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(54) **Title:** METHODS OF INTRODUCING MULTIPLE EXPRESSION CONSTRUCTS INTO A EUKARYOTIC CELL

(57) **Abstract:** The present invention relates to methods of introducing multiple expression constructs into a eukaryotic cell, methods of constructing a eukaryotic cell having multiple target loci for expressing multiple heterologous proteins of interest, eukaryotic cells for expressing multiple heterologous proteins of interest, and methods of production of multiple heterologous proteins of interest.



METHODS OF INTRODUCING MULTIPLE EXPRESSION CONSTRUCTS INTO A EUKARYOTIC CELL

Reference to a Sequence Listing

5 This application contains a Sequence Listing in computer readable form, which is incorporated herein by reference.

Background of the Invention

Field of the Invention

10 The present invention relates to methods of introducing multiple expression constructs into a eukaryotic cell, methods of constructing a eukaryotic cell having multiple target loci for expressing multiple heterologous proteins of interest, eukaryotic cells for expressing multiple heterologous proteins of interest, and methods of production of multiple heterologous proteins of interest.

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Description of the Related Art

 Recombinant production of a protein in a eukaryotic host cell, e.g., fungal cell, may provide for a more desirable vehicle for producing the protein in commercially relevant quantities. The recombinant production of a protein is generally accomplished by
20 constructing an expression cassette in which the DNA coding for the protein is placed under the expression control of a promoter from a regulated gene. The expression cassette is introduced into the host cell, usually by plasmid-mediated transformation. Production of the protein is then achieved by culturing the transformed host cell under inducing conditions necessary for the proper functioning of the promoter contained on the expression cassette.

25 Fungal cells may be transformed with a vector by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Transformation of a fungal host cell with two or more vectors, alone or together (co-transformation) is very inefficient and limited by the availability of useful selectable markers.

30 There is a need in the art for methods of constructing eukaryotic strains capable of expressing multiple recombinant proteins by targeting expression constructs to two or more (e.g., several) specific genomic loci to achieve desired expression levels of multiple heterologous proteins of interest.

 The present invention provides improved methods for producing multiple
35 recombinant proteins in a eukaryotic cell.

Summary of the Invention

The present invention relates to methods of introducing multiple expression constructs into two or more target loci of a eukaryotic cell, said method comprising.

(a) transforming a population of the eukaryotic cell with one or more first constructs and one or more second constructs,

wherein the eukaryotic cell comprises (1) one or more first target loci (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprising a pair of a first recombination recognition site and a second recombination recognition site, and (2) one or more second target loci (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprising a first fragment of a first selectable marker lacking a selectable function;

wherein the one or more first constructs (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprises one or more first expression cassettes each comprising a first polynucleotide encoding a first protein of interest, wherein in each of the first constructs the one or more first expression cassettes are flanked on one side by the first recombination recognition site and on the other side by the second recombination recognition site corresponding to the same recombination recognition sites of the first target loci; and

wherein the one or more second constructs (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprises one or more second expression cassettes each comprising a second polynucleotide encoding a second protein of interest, and wherein in each of the second constructs the one or more second expression cassettes are flanked on one side by a homologous region of the corresponding second target locus and on the other side by a second fragment of the first selectable marker that lacks the selectable function, wherein the second fragment comprises a sequence overlapping homologously the corresponding sequence of the first fragment of the first selectable marker of the second target loci; and

(b) selecting a transformant using each of the first selectable markers wherein the first fragment and the second fragment of each of the first selectable markers undergo recombination (e.g., homologous recombination or recombinase-mediated recombination) to become functional and the homologous region of the second constructs undergo homologous recombination with the same corresponding region of the second target loci, wherein each of the second expression cassettes are integrated at the corresponding second target loci, and wherein the first and second recombination recognition sites of the first constructs undergo recombination at the corresponding first and second recombination recognition sites of the first target loci driven by at least one recombinase integrating each of the first expression cassettes at the corresponding first target loci.

The present invention also relates to methods of constructing a eukaryotic cell having multiple target loci for expressing multiple heterologous proteins of interest, comprising:

(a) transforming a population of the eukaryotic cell with a first construct comprising (1) a 5' homologous region of a first target locus, (2) a first recombination recognition site, (3) a first selectable marker conferring a first selectable function, (4) a second recombination recognition site, and (5) a 3' homologous region of the first target locus;

(b) selecting a first transformant having the first construct integrated at the first target locus using the first selectable marker for selection of the first transformant, wherein the 5' homologous region and the 3' homologous region of the first construct undergo homologous recombination with the corresponding regions of the first target locus integrating the first construct at the first target locus, and wherein the first recombination recognition site and the second recombination recognition site are integrated at the first target locus;

(c) transforming a population of the first transformant with a second construct comprising (1) a 5' homologous region of a second target locus, (2) a first fragment of a second selectable marker that lacks a second selectable function, (3) a third selectable marker conferring a third selectable function, and (4) a 3' homologous region of the second target locus;

(d) selecting a second transformant having the second construct integrated at the second target locus using the third selectable marker for selection of the second transformant, wherein the 5' homologous region and the 3' homologous region of the second construct undergo homologous recombination with the corresponding regions of the second target locus integrating the second construct at the second target locus, and wherein the first fragment of the second selectable marker that lacks a second selectable function is integrated at the second target locus;

(e) co-transforming a population of the second transformant with (i) a third construct comprising (1) the first recombination recognition site, (2) one or more first expression cassettes each comprising a first polynucleotide encoding a first protein of interest, and (3) the second recombination recognition site, and (ii) a fourth construct comprising (1) the 5' homologous region of the second target locus, (2) one or more second expression cassettes each comprising a second polynucleotide encoding a second protein of interest, and (3) a second fragment of the second selectable marker that lacks the second selectable function wherein the second fragment comprises a sequence overlapping homologously the corresponding sequence of the first fragment of the first selectable marker; and

(f) selecting a third transformant using the second selectable marker wherein the first integrated fragment and the second fragment of the second selectable marker become functional upon recombination (e.g., homologous recombination or recombinase-mediated recombination), wherein the 5' homologous region of the fourth construct undergoes

homologous recombination with the same corresponding region of the second target locus, wherein the one or more second expression cassettes are integrated at the second target locus, and wherein the first recombination recognition site and the second recombination recognition site undergo recombination at the first target locus driven by at least one recombina-
5 recombina- integrating the one or more first expression cassettes at the first target locus.

The present invention also relates to eukaryotic cells for expressing multiple heterologous proteins of interest, comprising:

(a) one or more first target loci (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprising a pair of a first recombination recognition site and a second recombination recognition site;

10 and

(b) one or more second target loci (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprising a first fragment of a first selectable marker lacking a selectable function.

The present invention also relates to eukaryotic cells comprising (1) one or more first target loci each comprising one or more first expression cassettes each comprising a first polynucleotide encoding a first protein of interest, wherein the one or more first expression cassettes are each flanked 5' by a first recombination recognition site and 3' by a second recombination recognition site, and (2) one or more second target loci each comprising one or more second expression cassettes each comprising a second polynucleotide encoding a second protein of interest, wherein each of the one or more second expression cassettes are
15 flanked on one side by a region of the second target locus and on the other side by a first fragment of a first selectable marker that lacks selectable function,

wherein each of the pairs of the first and second recombination recognition sites at the first loci are able to undergo recombination with a first construct comprising one or more third expression cassettes each comprising a third polynucleotide encoding a third protein of interest, wherein each of the one or more third expression cassettes are flanked on one side
25 by the first recombination recognition site and on the other side by the second recombination recognition site of the corresponding first target locus, and

wherein each of the target loci regions and the first fragment of the first selectable marker that lacks selectable function at the second loci are able to undergo recombination (e.g., homologous recombination or recombinase-mediated recombination) with a second construct comprising one or more fourth expression cassettes each comprising a fourth polynucleotide encoding a fourth protein of interest, wherein each of the one or more fourth expression cassettes are flanked on one side by a homologous region of the corresponding second target locus and on the other side by a second fragment of the first selectable
30 marker that lacks the selectable function wherein the second fragment comprises a sequence overlapping homologously the corresponding sequence of the first fragment of the first selectable marker.

The present invention also relates to methods for producing proteins of interest, comprising cultivating a eukaryotic cell of the present invention under conditions conducive for production of the proteins.

The present invention also relates to nucleic acid constructs selected from the group consisting of:

(a) a first nucleic acid construct comprising (i) a 5' homologous region of a first target locus of a eukaryotic cell, (ii) a first recombination recognition site, (iii) a first repeat sequence, (iv) a first selectable marker conferring a first selectable function, (v) a second repeat sequence, (vi) a second recombination recognition site, and (vii) a 3' homologous region of the first target locus of the eukaryotic cell;

(b) a second nucleic acid construct comprising: (1) a 5' homologous region of a second target locus of a eukaryotic cell, (2) a first fragment of a second selectable marker that lacks a second selectable function, (3) a third repeat sequence, (4) a third selectable marker conferring a third selectable function, (5) a fourth repeat sequence, and (6) a 3' homologous region of the second target locus of the eukaryotic cell;

(c) a third nucleic acid construct comprising: (1) a first recombination recognition site, (2) one or more first expression cassettes each comprising a first polynucleotide encoding a first protein of interest, and (3) a second recombination recognition site; and

(d) a fourth nucleic acid construct comprising: (1) a 5' homologous region of the second target locus, (2) one or more second expression cassettes each comprising a second polynucleotide encoding a second protein of interest, and (3) a second fragment of a second selectable marker that lacks a second selectable function.

The present invention also relates to methods of introducing multiple expression constructs into a eukaryotic cell, said method comprising.

(a) transforming a population of the eukaryotic cell with one or more first constructs and one or more second constructs,

wherein the eukaryotic cell comprises (1) one or more first target loci (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprising a pair of a first recombination recognition site and a second recombination recognition site, wherein the first recombination recognition site and a second recombination recognition site are TP901-1 sites of the Xis-att system, and (2) one or more second target loci (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprising a pair of a third recombination recognition site and a fourth recombination recognition site, wherein the third recombination recognition site and the fourth recombination recognition site are flippase recognition sites of the FLP-FRT system;

wherein the one or more first constructs (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprises one or more first expression cassettes each comprising a first polynucleotide encoding a first protein of interest, wherein in each of the first constructs the one or more

first expression cassettes are flanked on one side by the first recombination recognition site and on the other side by the second recombination recognition site corresponding to the same recombination recognition sites of the first target loci;

wherein the one or more second constructs (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprises one or more second expression cassettes each comprising a second polynucleotide encoding a second protein of interest, and wherein in each of the second constructs the one or more second expression cassettes are flanked on one side by the third recombination recognition site and on the other side by the fourth recombination recognition site corresponding to the same recombination recognition sites of the second target loci; and

wherein one or more of the first constructs and second constructs comprise one or more first selectable markers;

(b) selecting a transformant using the one or more first selectable markers, wherein each of the second expression cassettes are integrated at the corresponding second target loci, and wherein the first and second recombination recognition sites of the first constructs undergo recombination at the corresponding first and second recombination recognition sites of the first target loci driven by at least one recombinase integrating each of the first expression cassettes at the corresponding first target loci.

Brief Description of the Figures

Figure 1 shows a restriction map of plasmid pJfyS147.

Figure 2 shows a restriction map of plasmid pJfyS156.

Figure 3 shows a restriction map of plasmid pQM41.

Figure 4 shows a restriction map of plasmid pJfyS145.

Figure 5 shows a restriction map of plasmid pECW2.

Figure 6 shows a restriction map of plasmid pECW7.

Figure 7 shows a restriction map of plasmid pGMEr193.

Figure 8 shows a restriction map of plasmid pCKle137.

Figure 9 shows a restriction map of plasmid pDM315.

Figure 10 shows a restriction map of plasmid pQM43.

Figure 11 shows a restriction map of plasmid pQM45.

Figure 12 shows a restriction map of plasmid pDM325.

Figure 13 shows a restriction map of plasmid pDM318.

Figure 14 shows a restriction map of plasmid pDM320.

Figure 15 shows a restriction map of plasmid pDM324.

Definitions

Aspartic protease: The term "aspartic protease" means a protease that uses an aspartate residue(s) for catalyzing the hydrolysis of peptide bonds in peptides, polypeptides, and proteins. Aspartic proteases are a family of protease enzymes that use an aspartate residue for catalytic hydrolysis of their peptide substrates. In general, they have two highly-conserved aspartates in the active site and are optimally active at acidic pH (Szecsi, 1992, *Scand. J. Clin. Lab. Invest. Suppl.* 210: 5–22). For purposes of the present invention, aspartic protease activity is determined according to the procedure described by Aikawa *et al.*, 2001, *J. Biochem.* 129: 791-794.

Auxiliary Activity 9 polypeptide: The term "Auxiliary Activity 9 polypeptide" or "AA9 polypeptide" means a polypeptide classified as a lytic polysaccharide monooxygenase (Quinlan *et al.*, 2011, *Proc. Natl. Acad. Sci. USA* 208: 15079-15084; Phillips *et al.*, 2011, *ACS Chem. Biol.* 6: 1399-1406; Lin *et al.*, 2012, *Structure* 20: 1051-1061). AA9 polypeptides were formerly classified into the glycoside hydrolase Family 61 (GH61) according to Henrissat, 1991, *Biochem. J.* 280: 309-316, and Henrissat and Bairoch, 1996, *Biochem. J.* 316: 695-696.

Beta-glucosidase: The term "beta-glucosidase" means a beta-D-glucoside glucohydrolase (E.C. 3.2.1.21) that catalyzes the hydrolysis of terminal non-reducing beta-D-glucose residues with the release of beta-D-glucose. For purposes of the present invention, beta-glucosidase activity is determined using *p*-nitrophenyl-beta-D-glucopyranoside as substrate according to the procedure of Venturi *et al.*, 2002, Extracellular beta-D-glucosidase from *Chaetomium thermophilum* var. *coprophilum*: production, purification and some biochemical properties, *J. Basic Microbiol.* 42: 55-66. One unit of beta-glucosidase is defined as 1.0 μ mole of *p*-nitrophenolate anion produced per minute at 25°C, pH 4.8 from 1 mM *p*-nitrophenyl-beta-D-glucopyranoside as substrate in 50 mM sodium citrate containing 0.01% TWEEN® 20.

cDNA: The term "cDNA" means a DNA molecule that can be prepared by reverse transcription from a mature, spliced, mRNA molecule obtained from a eukaryotic or prokaryotic cell. cDNA lacks intron sequences that may be present in the corresponding genomic DNA. The initial, primary RNA transcript is a precursor to mRNA that is processed through a series of steps, including splicing, before appearing as mature spliced mRNA.

Cellobiohydrolase: The term "cellobiohydrolase" means a 1,4-beta-D-glucan cellobiohydrolase (E.C. 3.2.1.91 and E.C. 3.2.1.176) that catalyzes the hydrolysis of 1,4-beta-D-glucosidic linkages in cellulose, cellooligosaccharides, or any beta-1,4-linked glucose containing polymer, releasing cellobiose from the reducing end (cellobiohydrolase I) or non-reducing end (cellobiohydrolase II) of the chain (Teeri, 1997, *Trends in Biotechnology* 15:

160-167; Teeri *et al.*, 1998, *Biochem. Soc. Trans.* 26: 173-178). Cellobiohydrolase activity is determined according to the procedures described by Lever *et al.*, 1972, *Anal. Biochem.* 47: 273-279; van Tilbeurgh *et al.*, 1982, *FEBS Letters*, 149: 152-156; van Tilbeurgh and Claeysens, 1985, *FEBS Letters*, 187: 283-288; and Tomme *et al.*, 1988, *Eur. J. Biochem.* 170: 575-581. In the present invention, the Tomme *et al.* method can be used to determine cellobiohydrolase activity.

Cellulolytic enzyme or cellulase: The term “cellulolytic enzyme” or “cellulase” means one or more (*e.g.*, several) enzymes that hydrolyze a cellulosic material. Such enzymes include endoglucanase(s), cellobiohydrolase(s), beta-glucosidase(s), or combinations thereof. The two basic approaches for measuring cellulolytic enzyme activity include: (1) measuring the total cellulolytic enzyme activity, and (2) measuring the individual cellulolytic enzyme activities (endoglucanases, cellobiohydrolases, and beta-glucosidases) as reviewed in Zhang *et al.*, Outlook for cellulase improvement: Screening and selection strategies, 2006, *Biotechnology Advances* 24: 452-481. Total cellulolytic enzyme activity can be measured using insoluble substrates, including Whatman №1 filter paper, microcrystalline cellulose, bacterial cellulose, algal cellulose, cotton, pretreated lignocellulose, *etc.* The most common total cellulolytic activity assay is the filter paper assay using Whatman №1 filter paper as the substrate. The assay was established by the International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987, Measurement of cellulase activities, *Pure Appl. Chem.* 59: 257-68).

For purposes of the present invention, cellulolytic enzyme activity is determined by measuring the increase in production/release of sugars during hydrolysis of a cellulosic material by cellulolytic enzyme(s) under the following conditions: 1-50 mg of cellulolytic enzyme protein/g of cellulose in pretreated corn stover (PCS) (or other pretreated cellulosic material) for 3-7 days at a suitable temperature such as 40°C-80°C, *e.g.*, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, or 80°C, and a suitable pH, such as 4-9, *e.g.*, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, or 9.0, compared to a control hydrolysis without addition of cellulolytic enzyme protein. Typical conditions are 1 ml reactions, washed or unwashed PCS, 5% insoluble solids (dry weight), 50 mM sodium acetate pH 5, 1 mM MnSO₄, 50°C, 55°C, or 60°C, 72 hours, sugar analysis by AMINEX® HPX-87H column chromatography (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Coding sequence: The term “coding sequence” means a polynucleotide, which directly specifies the amino acid sequence of a protein. The boundaries of the coding sequence are generally determined by an open reading frame, which begins with a start codon such as ATG, GTG, or TTG and ends with a stop codon such as TAA, TAG, or TGA. The coding sequence may be a genomic DNA, cDNA, synthetic DNA, or a combination thereof.

Control sequences: The term “control sequences” means nucleic acid sequences necessary for expression of a polynucleotide encoding a protein. Each control sequence may be native (*i.e.*, from the same gene) or foreign (*i.e.*, from a different gene) to the polynucleotide encoding the protein or native or foreign to each other. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the polynucleotide encoding a protein.

Ectopic integration: The term “ectopic integration” means the insertion of a nucleic acid into the genome of a microorganism at a non-targeted site or at a site other than its usual chromosomal locus, *i.e.*, random integration.

Endoglucanase: The term “endoglucanase” means a 4-(1,3;1,4)-beta-D-glucan 4-glucanohydrolase (E.C. 3.2.1.4) that catalyzes endohydrolysis of 1,4-beta-D-glycosidic linkages in cellulose, cellulose derivatives (such as carboxymethyl cellulose and hydroxyethyl cellulose), lichenin, beta-1,4 bonds in mixed beta-1,3-1,4 glucans such as cereal beta-D-glucans or xyloglucans, and other plant material containing cellulosic components. Endoglucanase activity can be determined by measuring reduction in substrate viscosity or increase in reducing ends determined by a reducing sugar assay (Zhang *et al.*, 2006, *Biotechnology Advances* 24: 452-481). For purposes of the present invention, endoglucanase activity is determined using carboxymethyl cellulose (CMC) as substrate according to the procedure of Ghose, 1987, *Pure and Appl. Chem.* 59: 257-268, at pH 5, 40°C.

Expression: The term “expression” includes any step involved in the production of a protein including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion.

Expression vector: The term “expression vector” means a linear or circular DNA molecule that comprises a polynucleotide encoding a protein and is operably linked to control sequences that provide for its expression.

Flanking sequence or region: The term “flanking sequence or region” means DNA sequences extending on either side of a specific DNA sequence, locus, or gene. The flanking DNA is immediately adjacent to another DNA sequence, locus, or gene that is to be integrated into the genome of a filamentous fungal cell.

Fragment: The term “fragment” means a polypeptide having one or more (*e.g.*, several) amino acids absent from the amino and/or carboxyl terminus of a mature polypeptide, wherein the fragment has enzyme activity. In one aspect, a fragment contains

at least 85%, e.g., at least 90% or at least 95% of the amino acid residues of the mature polypeptide of an enzyme.

Heterologous protein: The term “heterologous protein” means a protein which is not native to a eukaryotic cell, a native protein in which modifications have been made to alter the native sequence, or a native protein whose expression is quantitatively altered as a result of a manipulation of the eukaryotic cell by recombinant DNA techniques. For example, a native protein may be recombinantly produced by, e.g., placing a gene encoding the protein under the control of a promoter foreign to the gene.

Homolog: The term “homolog” means a polynucleotide related to a second polynucleotide by descent from a common ancestral DNA sequence. The term “homolog” may apply to the relationship between genes separated by the event of speciation or to the relationship between genes separated by the event of genetic duplication. In one aspect, a homolog of a polynucleotide of interest has a sequence identity of at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to the polynucleotide. In another aspect, a homolog of a polynucleotide of interest hybridizes under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with the polynucleotide.

Homologous 3' or 5' region: The term “homologous 3' region” means a fragment of DNA that is identical in sequence or has a sequence identity of at least 70%, e.g., at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% to a region in the genome and when combined with a homologous 5' region can target integration of a piece of DNA to a specific site in the genome by homologous recombination. The term “homologous 5' region” means a fragment of DNA that is identical in sequence or has a sequence identity of at least 70%, e.g., at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% to a region in the genome and when combined with a homologous 3' region can target integration of a piece of DNA to a specific site in the genome by homologous recombination. The homologous 5' and 3' regions must be linked in the genome which means they are on the same chromosome and within at least 200 kb of one another. The homologous regions contain a sufficient number of nucleic acids, such as 20 to 10,000 base pairs, 50 to 10,000 base pairs, 100 to 10,000 base pairs,

400 to 10,000 base pairs, and 800 to 10,000 base pairs.

Homologous flanking sequence or region: The term "homologous flanking sequence or region" means a fragment of DNA that is identical or has a sequence identity of at least 70%, e.g., at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% to a region in the genome and is located immediately upstream or downstream of a specific site in the genome into which extracellular DNA is targeted for integration.

Host cell: The term "host cell" means any cell type that is susceptible to transformation, transfection, transduction, or the like with a nucleic acid construct or expression vector comprising a polynucleotide encoding a protein. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication.

Isolated: The term "isolated" means a substance in a form or environment that does not occur in nature. Non-limiting examples of isolated substances include (1) any non-naturally occurring substance, (2) any substance including, but not limited to, any enzyme, variant, nucleic acid, protein, peptide or cofactor, that is at least partially removed from one or more or all of the naturally occurring constituents with which it is associated in nature; (3) any substance modified by the hand of man relative to that substance found in nature; or (4) any substance modified by increasing the amount of the substance relative to other components with which it is naturally associated (e.g., recombinant production in a host cell; multiple copies of a gene encoding the substance; and use of a stronger promoter than the promoter naturally associated with the gene encoding the substance).

Mature polypeptide: The term "mature polypeptide" means a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc. It is known in the art that a host cell may produce a mixture of two or more different mature polypeptides (*i.e.*, with a different C-terminal and/or N-terminal amino acid) expressed by the same polynucleotide.

Mature polypeptide coding sequence: The term "mature polypeptide coding sequence" means a polynucleotide that encodes a mature polypeptide having enzyme activity.

Nucleic acid construct: The term "nucleic acid construct" means a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or is modified to contain segments of nucleic acids in a manner that would not otherwise exist in nature or which is synthetic, which comprises one or more (e.g., several) control sequences.

Operably linked: The term “operably linked” means a configuration in which a control sequence is placed at an appropriate position relative to the coding sequence of a polynucleotide such that the control sequence directs expression of the coding sequence.

Recombinase: The term “recombinase” is an enzyme that catalyzes directionally sensitive exchange DNA reactions between short target site sequences that are specific to each recombinase.

Recombinase-mediated recombination: The term “recombinase-mediated recombination” means recombination between two recognition sites of identical sequences catalyzed by a recombinase.

Repeat: The term “repeat” means a fragment of DNA that is repeated at least twice in the recombinant DNA introduced into a host cell and which can facilitate the loss of the DNA, *i.e.*, selectable marker that is inserted between two repeats, by homologous recombination. A repeat is also known as a direct repeat or a homologous repeat. In a preferred aspect, the two repeats are identical in sequence. However, two repeats may have a sequence identity to each other of at least 70%, *e.g.*, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%.

Repeat sequences: The term “repeat sequences” means a set of two sequences, one of which is positioned 5' to a polynucleotide of interest and the other is positioned 3' to the polynucleotide sequence of interest, such that the repeat sequences undergo homologous recombination to remove the polynucleotide. A repeat sequence can be at least 20 nucleotides, at least 30 nucleotides, at least 40 nucleotides, at least 50 nucleotides, at least 100 nucleotides, at least 150 nucleotides, at least 200 nucleotides, at least 300 nucleotides, at least 400 nucleotides, or at least 500 nucleotides.

Sequence identity: The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter “sequence identity”.

For purposes of the present invention, the sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 5.0.0 or later. The parameters used are a gap open penalty of 10, a gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled “longest identity” (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

$$(\text{Identical Residues} \times 100) / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

For purposes of the present invention, the sequence identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *supra*), preferably version 5.0.0 or later. The parameters used are a gap open penalty of 10, a gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled "longest identity" (obtained using the -nobrief option) is used as the percent identity and is calculated as follows:

(Identical Deoxyribonucleotides x 100)/(Length of Alignment – Total Number of Gaps in Alignment)

Stringency conditions: The term "very low stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 25% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 45°C.

The term "low stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 25% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 50°C.

The term "medium stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 55°C.

The term "medium-high stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 60°C.

The term "high stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 50% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 65°C.

The term "very high stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 50% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 70°C.

Subsequence: The term "subsequence" means a polynucleotide having one or more (e.g., several) nucleotides absent from the 5' and/or 3' end of a mature polypeptide coding sequence, wherein the subsequence encodes a fragment having enzyme activity. In one aspect, a subsequence contains at least 85%, e.g., at least 90% or at least 95% of the nucleotides of the mature polypeptide coding sequence of an enzyme.

Subtilisin-like serine protease: The term "subtilisin-like serine protease" means a protease with a substrate specificity similar to subtilisin that uses a serine residue for catalyzing the hydrolysis of peptide bonds in peptides and proteins. Subtilisin-like proteases (subtilases) are serine proteases characterized by a catalytic triad of the three amino acids aspartate, histidine, and serine. The arrangement of these catalytic residues is shared with the prototypical subtilisin from *Bacillus licheniformis* (Siezen and Leunissen, 1997, *Protein Science* 6: 501-523). Subtilisin-like serine protease activity can be determined using a synthetic substrate, N-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide (AAPF) (Bachem AG, Bubendorf, Switzerland) in 100 mM NaCl-100 mM MOPS pH 7.0 at 50°C for 3 hours and then the absorbance at 405 nm is measured.

Targeted integration: The term "targeted integration" means the stable integration of extracellular DNA at a defined genomic locus.

Transformant: The term "transformant" means a cell which has taken up extracellular DNA (foreign, artificial or modified) and expresses the gene(s) contained therein.

Transformation: The term "transformation" means the introduction of extracellular DNA into a cell, i.e., the genetic alteration of a cell resulting from the direct uptake, incorporation and expression of exogenous genetic material (exogenous DNA) from its surroundings and taken up through the cell membrane(s).

Transformation efficiency: The term "transformation efficiency" means the efficiency by which cells can take up the extracellular DNA and express the gene(s) contained therein, which is calculated by dividing the number of positive transformants expressing the gene(s) by the amount of DNA used during a transformation procedure.

Trypsin-like serine protease: The term "trypsin-like serine protease" means a protease with a substrate specificity similar to trypsin that uses a serine residue for catalyzing the hydrolysis of peptide bonds in peptides and proteins. For purposes of the present invention, trypsin-like serine protease activity is determined according to the

procedure described by Dienes *et al.*, 2007, *Enzyme and Microbial Technology* 40: 1087-1094.

Variant: The term “variant” means a polypeptide having enzyme activity comprising an alteration, *i.e.*, a substitution, insertion, and/or deletion, at one or more (e.g., several) positions. A substitution means replacement of the amino acid occupying a position with a different amino acid; a deletion means removal of the amino acid occupying a position; and an insertion means adding an amino acid adjacent to and immediately following the amino acid occupying a position.

Xylanase: The term “xylanase” means a 1,4-beta-D-xylan-xylohydrolase (E.C. 3.2.1.8) that catalyzes the endohydrolysis of 1,4-beta-D-xylosidic linkages in xylans. For purposes of the present invention, xylanase activity is determined with 0.2% AZCL-arabinoxylan as substrate in 0.01% TRITON® X-100 and 200 mM sodium phosphate buffer pH 6 at 37°C. One unit of xylanase activity is defined as 1.0 μ mole of azurine produced per minute at 37°C, pH 6 from 0.2% AZCL-arabinoxylan as substrate in 200 mM sodium phosphate pH 6.

Detailed Description of the Invention

The present invention relates to methods of introducing multiple expression constructs into a eukaryotic cell, methods of constructing a eukaryotic cell having multiple target loci for expressing multiple heterologous proteins of interest, eukaryotic cells for expressing multiple heterologous proteins of interest, and methods of production of multiple heterologous proteins of interest.

The methods of introducing multiple expression constructs into a eukaryotic cell have several advantages. One advantage is the ability to introduce multiple protein expression constructs at two or more specific target loci in the genome of the eukaryotic cell to achieve desirable expression levels of all the proteins of interest. Another advantage is the efficiency of the method for constructing the eukaryotic cell by reducing the steps and time compared to methods known in the art. Another advantage is the flexibility of the method to easily delete one or more of the introduced expression constructs. Another advantage is the flexibility of the method to easily replace one or more of the introduced expression constructs with different expression constructs.

Methods of Constructing a Eukaryotic Cell Having Multiple Target Loci for Expressing Multiple Heterologous Proteins of Interest

In an embodiment, the present invention relates to methods of constructing a eukaryotic cell having multiple target loci for expressing multiple heterologous proteins of interest, comprising:

- 5 (a) transforming a population of the eukaryotic cell with a first construct comprising (1) a 5' homologous region of a first target locus, (2) a first recombination recognition site, (3) a first selectable marker conferring a first selectable function, (4) a second recombination recognition site, and (5) a 3' homologous region of the first target locus;
- 10 (b) selecting a first transformant having the first construct integrated at the first target locus using the first selectable marker for selection of the first transformant, wherein the 5' homologous region and the 3' homologous region of the first construct undergo homologous recombination with the corresponding regions of the first target locus integrating the first construct at the first target locus, and wherein the first recombination recognition site and the second recombination recognition site are integrated at the first target locus;
- 15 (c) transforming a population of the first transformant with a second construct comprising (1) a 5' homologous region of a second target locus, (2) a first fragment of a second selectable marker that lacks a second selectable function, (3) a third selectable marker conferring a third selectable function, and (4) a 3' homologous region of the second target locus;
- 20 (d) selecting a second transformant having the second construct integrated at the second target locus using the third selectable marker for selection of the second transformant, wherein the 5' homologous region and the 3' homologous region of the second construct undergo homologous recombination with the corresponding regions of the second target locus integrating the second construct at the second target locus, and wherein the first
25 fragment of the second selectable marker that lacks a second selectable function is integrated at the second target locus;
- 30 (e) co-transforming a population of the second transformant with (i) a third construct comprising (1) the first recombination recognition site, (2) one or more first expression cassettes each comprising a first polynucleotide encoding a first protein of interest, and (3) the second recombination recognition site, and (ii) a fourth construct
35 comprising (1) the 5' homologous region of the second target locus, (2) one or more second expression cassettes each comprising a second polynucleotide encoding a second protein of interest, and (3) a second fragment of the second selectable marker that lacks the second selectable function wherein the second fragment comprises a sequence overlapping homologously the corresponding sequence of the first fragment of the first selectable marker; and

(f) selecting a third transformant using the second selectable marker wherein the first integrated fragment and the second fragment of the second selectable marker become functional upon recombination (e.g., homologous recombination or recombinase-mediated recombination), wherein the 5' homologous region of the fourth construct undergoes homologous recombination with the same corresponding region of the second target locus, wherein the one or more second expression cassettes are integrated at the second target locus, and wherein the first recombination recognition site and the second recombination recognition site undergo recombination at the first target locus driven by at least one recombinase integrating the one or more first expression cassettes at the first target locus.

In step (a), a population of a eukaryotic cell is transformed with a first construct comprising (1) a 5' homologous region of a first target locus, (2) a first recombination recognition site, (3) a first selectable marker conferring a first selectable function, (4) a second recombination recognition site, and (5) a 3' homologous region of the first target locus. Step (a) introduces recombination recognition sites for integration of the third construct at the first locus. The 5' and 3' homologous regions direct the construct to integrate at the first locus. The first selectable marker is used for selection of the first construct integrated at the first locus.

In step (b), a first transformant is selected having the first construct integrated at the first target locus using the first selectable marker for selection. The 5' homologous region and the 3' homologous region of the first construct undergo homologous recombination with the corresponding regions of the first target locus integrating the first construct at the first target locus. Thus, the first recombination recognition site and the second recombination recognition site are integrated at the first target locus.

In step (c), a population of the first transformant is transformed with a second construct comprising (1) a 5' homologous region of a second target locus, (2) a first fragment of a second selectable marker that lacks a second selectable function, (3) a third selectable marker conferring a third selectable function, and (4) a 3' homologous region of the second target locus. Step (c) introduces a first fragment of a second selectable marker that lacks a second selectable function for integration at the second locus. The first fragment of the second selectable marker will recombine by recombination (e.g., homologous recombination or recombinase-mediated recombination) with a second fragment of the second selectable marker to become functional for use as a selectable marker in step (f) below. The 5' and 3' homologous regions direct the construct to integrate at the second locus. The third selectable marker is used for selection of the second construct integrated at the second locus.

In step (d), a second transformant is selected having the second construct integrated at the second target locus using the third selectable marker for selection. The 5' homologous

region and the 3' homologous region of the second construct undergo homologous recombination with the corresponding regions of the second target locus integrating the second construct at the second target locus. The first fragment of the second selectable marker that lacks a second selectable function is integrated at the second target locus.

5 In step (e), a population of the second transformant is co-transformed with (i) a third construct comprising (1) the first recombination recognition site, (2) one or more first expression cassettes each comprising a first polynucleotide encoding a first protein of interest, and (3) the second recombination recognition site, and (ii) a fourth construct comprising (1) the 5' homologous region of the second target locus, (2) one or more second
10 expression cassettes each comprising a second polynucleotide encoding a second protein of interest, and (3) a second fragment of the second selectable marker that lacks the second selectable function. The second fragment comprises a sequence overlapping homologously the corresponding sequence of the first fragment of the first selectable marker. The overlapping homologous sequence contains a sufficient number of nucleic acids, such as 20
15 to 10,000 base pairs, 50 to 10,000 base pairs, 100 to 10,000 base pairs, 400 to 10,000 base pairs, and 800 to 10,000 base pairs, which have a high degree of sequence identity to the corresponding target sequence to enhance recombination.

In step (f), a third transformant is selected using the second selectable marker where (1) the first integrated fragment and the second fragment of the second selectable marker
20 become functional upon recombination (e.g., homologous recombination or recombinase-mediated recombination), (2) the 5' homologous region of the fourth construct undergoes homologous recombination with the same corresponding region of the second target locus, (3) the one or more second expression cassettes are integrated at the second target locus, and (4) the first recombination recognition site and the second recombination recognition site
25 undergo recombination at the first target locus driven by at least one recombinase integrating the one or more first expression cassettes at the first target locus.

In one aspect, steps (a) and (b) are performed before steps (c) and (d). In another aspect, steps (c) and (d) are performed before steps (a) and (b).

The target loci can be any region of the genome of the eukaryotic cell. In one aspect,
30 the target loci are genes. In another aspect, the target loci are non-genes. In another aspect, the target loci are genes and non-genes. The loci can be targets for introducing a heterologous gene encoding a protein of interest. The loci can also be targets for replacing a native gene with a heterologous gene that encodes a protein with the same function but with superior properties, e.g., thermostability, thermal activity, specific activity, pH optimum,
35 substrate specificity, etc. In one preferred embodiment, the target locus is a *Trichoderma reesei* CBHI gene. In another preferred embodiment, the target locus is a *Trichoderma reesei* CBHII gene. In another preferred embodiment, the target locus is a *Trichoderma*

reesei EGI gene. In another preferred embodiment, the target locus is a *Trichoderma reesei* EGII gene. In another preferred embodiment, the target locus is a *Trichoderma reesei* EGIII gene. In another preferred embodiment, the target locus is a *Trichoderma reesei* beta-glucosidase gene. In another preferred embodiment, the target locus is a *Trichoderma reesei* AA9A (GH61A) polypeptide gene. In another preferred embodiment, the target locus is a *Trichoderma reesei* AA9B (GH61B) polypeptide gene. In another preferred embodiment, the target locus is a *Trichoderma reesei* aspartic protease gene. In another preferred embodiment, the target locus is a *Trichoderma reesei* subtilisin-like serine protease gene. In another preferred embodiment, the target locus is a *Trichoderma reesei* trypsin-like serine protease gene. In another preferred embodiment, the target locus is a *Trichoderma reesei* xylanase gene. In another preferred embodiment, the target locus is a *Trichoderma reesei* dihydroflavonol-4-reductase gene. In another preferred embodiment, the target locus is a *Trichoderma reesei* paracelsin synthetase gene.

The first and second recombination recognition sites at each of the first target loci can be the same recombination recognition sites, different recombination recognition sites, or a combination of the same and different recombination recognition sites. In one aspect, the first and second recombination recognition sites at each of the first target loci are the same recombination recognition site. In another aspect, the first and second recombination recognition sites at each of the first target loci are a different recombination recognition site. In another aspect, the first and second recombination recognition sites at each of the first target loci are a combination of the same and different recombination recognition sites. In a preferred embodiment, the first and second recombination recognition sites at each of the first target loci are a different recombination recognition site. In an alternative preferred embodiment, the first and second recombination recognition sites at each of the first target loci are the same recombination recognition site.

The recombination recognition sites can be any recombination recognition sites useful in the methods of the present invention. In one aspect, the recombination recognition sites are selected from the group consisting of a B2 system from *Zygosaccharomyces bailii*, B3 system from *Zygosaccharomyces bisporus*, beta-recombinase-six system from a *Bacillus subtilis* plasmid, Bxb1 from phage Bxb1, Cre-lox system of bacteriophage P1, Dre from Bacteriophage D6, FLP-FRT of *Saccharomyces cerevisiae*, Delta-gamma-es system from bacterial transposon Tn1000, Gin-gix system from bacteriophage Mu, HK022 from phage HK022, KD system from *Kluyveromyces drosophilum*, Mx9 phage transformation system, *Streptomyces* phage ϕ C31, R-RS system of *Zygosaccharomyces rouxii*, Tn3 from *E. coli*, Vika recombinase from *Vibrio coralliilyticus*, and Xis-att system of temperate lactococcal bacteriophage TP901-1; and combinations thereof. It is understood herein that the enumerated recombination recognition sites encompass homologs and variants thereof. In

one embodiment, one of the recombination recognition sites is a B2 system from *Zygosaccharomyces bailii*. In another embodiment, one of the recombination recognition sites is a B3 system from *Zygosaccharomyces bisporus*. In another embodiment, one of the recombination recognition sites is a beta-recombinase-six system from a 25 *Bacillus subtilis* plasmid. In another embodiment, one of the recombination recognition sites is a Bxb1 from phage Bxb1. In another embodiment, one of the recombination recognition sites is a Cre-lox system of bacteriophage P1. In another embodiment, one of the recombination recognition sites is a Dre from Bacteriophage D6. In another embodiment, one of the recombination recognition sites is a FLP-FRT of *Saccharomyces cerevisiae*. In another embodiment, one of the recombination recognition sites is a Delta-gamma-es system from bacterial transposon Tn1000. In another embodiment, one of the recombination recognition sites is a Gin-gix system from bacteriophage Mu. In another embodiment, one of the recombination recognition sites is a HK022 from phage HK022. In another embodiment, one of the recombination recognition sites is a KD system from *Kluyveromyces drosophilarum*. In another embodiment, one of the recombination recognition sites is a Mx9 phage transformation system. In another embodiment, one of the recombination recognition sites is a *Streptomyces* phage ϕ C31. In another embodiment, one of the recombination recognition sites is a R-RS system of *Zygosaccharomyces rouxii*. In another embodiment, one of the recombination recognition sites is a Tn3 from *E. coli*. In another embodiment, one of the recombination recognition sites is a Vika recombinase from *Vibrio coralliilyticus*. In another embodiment, one of the recombination recognition sites is a Xis-att system of temperate lactococcal bacteriophage TP901-1.

In one aspect, the flippase recognition sites of the FLP-FRT system are selected from the group consisting F, F3, F10, F13, F14, F15, Fa, and F3a, and combinations thereof. It is understood herein that the enumerated flippase recognition sites encompass homologs and variants thereof. In one embodiment, the flippase recognition site is F. In another embodiment, the flippase recognition site is F3. In another embodiment, the flippase recognition site is F10. In another embodiment, the flippase recognition site is F13. In another embodiment, the flippase recognition site is F14. In another embodiment, the flippase recognition site is F15. In another embodiment, the flippase recognition site is Fa. In another embodiment, the flippase recognition site is F3a. See Turan *et al.*, 2010, *J. Mol. Biol.* 402: 52-69, which is incorporated herein in its entirety.

In another aspect, the TP901-1 sites of the Xis-att system are selected from the group consisting of *attB* and *attP*, and combinations thereof. It is understood herein that the enumerated TP901-1 sites encompass homologs and variants thereof. In one embodiment, the TP901-1 site is *attB*. In another embodiment, the TP901-1 site is *attP*.

In another aspect, the Lox sites of the Cre-lox system are selected from the group consisting of LoxP, Lox71, Lox66, Lox511, Lox5171, Lox2272, M2, M3, M7, and M11, and combinations thereof. It is understood herein that the enumerated Lox sites encompass homologs and variants thereof. In one embodiment, the Lox site is LoxP. In another
5 embodiment, the Lox site is Lox71. In another embodiment, the Lox site is Lox66. In another embodiment, the Lox site is Lox511. In another embodiment, the Lox site is Lox5171. In another embodiment, the Lox site is M2. In another embodiment, the Lox site is M3. In another embodiment, the Lox site is M7. In another embodiment, the Lox site is M11.

In the methods of the present invention, the eukaryotic cell requires one or more
10 recombinases to catalyze the recombination between a specific set of recombination recognition sites. The one or more recombinases can be permanent or transient in the eukaryotic cell. The term "permanent" means the gene encoding the recombinase is already present in the genome of the eukaryotic cell. The term "transient" means the gene encoding the recombinase is introduced into the eukaryotic cell when the recombinase encoded by the
15 gene is needed to catalyze the recombination between a specific set of recombination recognition sites and is removed thereafter.

The one or more recombinases can be any recombinase useful in the methods of the present invention. In one aspect, the one or more recombinases are native to the eukaryotic cell. In another aspect, the one or more recombinases are heterologous to the eukaryotic
20 cell. In another aspect, the recombinases are a combination of native and heterologous recombinases. In another aspect, the one or more recombinases are selected from the group consisting of a Bxb1 recombinase, a Cre recombinase, a CinH recombinase, a Flp flippase, a HK022 integrase, a ParA recombinase, a Tn1721 recombinase, a Tn5053 recombinase, a TP901-1 integrase, an U153 recombinase, a λ integrase, and a ϕ C31
25 recombinase. It is understood herein that the enumerated recombinases encompass homologs and variants thereof. In one embodiment, one of the recombinases is a Bxb1 recombinase. In another embodiment, one of the recombinases is a Cre recombinase. In another embodiment, one of the recombinases is a CinH recombinase. In another embodiment, one of the recombinases is a Flp flippase. In another embodiment, one of the
30 recombinases is a HK022 integrase. In another embodiment, one of the recombinases is a ParA recombinase. In another embodiment, one of the recombinases is a Tn1721 recombinase. In another embodiment, one of the recombinases is a Tn5053 recombinase. In another embodiment, one of the recombinases is a TP901-1 integrase. In another embodiment, one of the recombinases is an U153 recombinase. In another embodiment,
35 one of the recombinases is a λ integrase. In another embodiment, one of the recombinases is a ϕ C31 recombinase.

The selectable markers can be any selectable markers useful in the methods of the present invention. In one aspect, the selectable markers are selected from the group consisting of ADE2, ARO4-OPF, FLD1, HIS3, LEU2, LYS2, MET3, TRP1, URA3, *adeA*, *adeB*, *amdS*, *argB*, *bar*, *bleR*, *bsd*, *fcy1*, *hpt*, *hpt-tk*, *nat1*, *niaD*, *ptr1*, *pyrG*, *sC*, *tk*, Tn903kan^r, *trpC*, and beta-tubulin.

The first selectable marker can be a dual selectable marker system. The dual selectable marker can be useful to facilitate the removal the first selectable marker and/or to improve screening strength for selecting the third transformant in step (f). The dual selectable marker system can be any useful combination of a negative screenable marker and a positive screenable marker. For example, the screenable marker can be a screenable morphological phenotype. In one aspect, the dual selectable marker system is *hpt-tk*. In another aspect, the dual selectable marker system is *hpt-fcy1*. In another aspect, the dual selectable marker system is *hpt-laccase*.

The first selectable marker can also be a counter selectable marker for negative selection.

The first selectable marker can further comprise a first repeat 5' of the first selectable marker and a second repeat 3' of the first selectable marker. The first and second repeat sequences of the integrated first construct can undergo homologous recombination to remove the first selectable marker.

The third selectable marker can further comprise a third repeat 5' of the third selectable marker and a fourth repeat 3' of the third selectable marker. The third and fourth repeat sequences of the second construct undergo homologous recombination to remove the third selectable marker.

The third selectable marker can be the same as the first selectable marker or different than the first selectable marker. In one aspect, the third selectable marker is the same as the first selectable marker when the third selectable marker is introduced after the first selectable marker is removed. In another aspect, the third selectable marker is different than the first selectable marker when the first selectable marker is present in the genome of the eukaryotic cell.

The second selectable marker can be the same as or different than the first and third selectable markers. In one aspect, the second selectable marker is the same as the first selectable marker when the first selectable marker is removed before step (c). In another aspect, the second selectable marker is the same as the third selectable marker when the third selectable marker is removed before step (e). In another aspect, the second selectable marker is the same as the third selectable marker when a portion of the third selectable marker is removed leaving a non-functional selectable marker in the genome. In another aspect, the second selectable marker is different than the first and third selectable markers.

The first and second non-functional fragments of the second selectable marker may each further comprise a recombination recognition site in an intron of the second selectable marker. In one aspect, each recombination recognition site in the first and second non-functional fragments is the same, which can undergo recombinase-mediated recombination.

5 In another aspect, each recombination recognition site in the first and second non-functional fragments are different but can undergo recombinase-mediated recombination.

The first construct can further comprise a third counter selectable marker for negative selection between the first and the second recombination recognition sites to improve screening strength for selecting the third transformant in step (f).

10 The second construct can further comprise a third counter selectable marker for negative selection before or after the non-functional first fragment of the second selectable marker. In one embodiment, the second construct further comprises a third counter selectable marker for negative selection before the non-functional first fragment of the second selectable marker. In another embodiment, the second construct further comprises a

15 third counter selectable marker for negative selection after the non-functional first fragment of the second selectable marker

In one aspect, the second transformant comprises a third counter selectable marker at both the first and the second target loci to improve screening strength for selecting the third transformant in step (f). In another aspect, the third transformant comprises no counter

20 selectable marker at both the first and second target loci and is selected using only the selection function of the second selectable marker.

The counter selectable marker for negative selection can be any useful counter selectable marker. In one aspect, the counter selectable marker is selected from the group consisting of URA3, *amdS*, *fcy1*, *pyrG*, *tk*, and beta-tubulin. In one embodiment, the counter

25 selectable marker is URA3. In another embodiment, the counter selectable marker is *amdS*. In another embodiment, the counter selectable marker is *fcy1*. In another embodiment, the counter selectable marker is *pyrG*. In another embodiment, the counter selectable marker is *tk*. In another embodiment, the counter selectable marker is beta-tubulin.

In one embodiment of the above method, steps (a)-(f) are repeated at two or more

30 additional target loci with different recombination recognition sites, different polynucleotides encoding proteins of interest, and the same or a different second selectable marker.

In another embodiment of the above method, steps (a)-(b) or (c)-(d) are repeated at one additional target locus with different recombination recognition sites, different polynucleotides encoding proteins of interest, and the same or a different selectable marker.

35 **Methods of Introducing Multiple Expression Constructs into a Eukaryotic Cell**

In one embodiment, the present invention relates to methods of introducing multiple

expression constructs into two or more target loci of a eukaryotic cell, comprising.

(a) transforming a population of the eukaryotic cell with one or more first constructs and one or more second constructs,

wherein the eukaryotic cell comprises (1) one or more first target loci (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprising a pair of a first recombination recognition site and a second recombination recognition site, and (2) one or more second target loci (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprising a first fragment of a first selectable marker lacking a selectable function;

wherein the one or more first constructs (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprises one or more first expression cassettes each comprising a first polynucleotide encoding a first protein of interest, wherein in each of the first constructs the one or more first expression cassettes are flanked on one side by the first recombination recognition site and on the other side by the second recombination recognition site corresponding to the same recombination recognition sites of the first target loci; and

wherein the one or more second constructs (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprises one or more second expression cassettes each comprising a second polynucleotide encoding a second protein of interest, and wherein in each of the second constructs the one or more second expression cassettes are flanked on one side by a homologous region of the corresponding second target locus and on the other side by a second fragment of the first selectable marker that lacks the selectable function, wherein the second fragment comprises a sequence overlapping homologously the corresponding sequence of the first fragment of the first selectable marker of the second target loci; and

(b) selecting a transformant using each of the first selectable markers wherein the first fragment and the second fragment of each of the first selectable markers undergo recombination (e.g., homologous recombination or recombinase-mediated recombination) to become functional and the homologous region of the second constructs undergo homologous recombination with the same corresponding region of the second target loci, wherein each of the second expression cassettes are integrated at the corresponding second target loci, and wherein the first and second recombination recognition sites of the first constructs undergo recombination at the corresponding first and second recombination recognition sites of the first target loci driven by at least one recombinase integrating each of the first expression cassettes at the corresponding first target loci.

The transformation of the population of the eukaryotic cell can be performed sequentially in any order with the one or more first constructs and the one or more second constructs. Alternatively, the transformation of the population of the eukaryotic cell can be performed as a co-transformation with the one or more first constructs and the one or more second constructs.

The selectable markers can be any selectable markers useful in the methods of the present invention. In one aspect, the selectable markers can be selected from the group consisting of ADE2, ARO4-OPF, FLD1, HIS3, LEU2, LYS2, MET3, TRP1, URA3, *adeA*, *adeB*, *amdS*, *argB*, *bar*, *bleR*, *bsd*, *fcy1*, *hpt*, *hpt-tk*, *nat1*, *niaD*, *ptr1*, *pyrG*, *sC*, *tk*, Tn903kan^r, *trpC*, and beta-tubulin.

The one or more first target loci can each further comprise a second selectable marker between the recombination recognition sites. In one aspect, the second selectable marker at each of the first target loci is a counter selectable marker for negative selection. The counter selectable marker for negative selection can be any useful counter selectable marker. In one aspect, the counter selectable marker is selected from the group consisting of URA3, *amdS*, *fcy1*, *pyrG*, *tk*, and beta-tubulin. In one embodiment, the counter selectable marker is URA3. In another embodiment, the counter selectable marker is *amdS*. In another embodiment, the counter selectable marker is *fcy1*. In another embodiment, the counter selectable marker is *pyrG*. In another embodiment, the counter selectable marker is *tk*. In another embodiment, the counter selectable marker is beta-tubulin.

The one or more second target loci can each further comprise a third selectable marker before or after the non-functional first fragment of the first selectable marker. In one aspect, the third selectable marker is before the non-functional first fragment of the first selectable marker. In another aspect, the third selectable marker is after the non-functional first fragment of the first selectable marker.

In one aspect, the third selectable marker at each of the second target loci is a counter selectable marker for negative selection. The counter selectable marker for negative selection can be any useful counter selectable marker. In one aspect, the counter selectable marker is selected from the group consisting of URA3, *amdS*, *fcy1*, *pyrG*, *tk*, and beta-tubulin. In one embodiment, the counter selectable marker is URA3. In another embodiment, the counter selectable marker is *amdS*. In another embodiment, the counter selectable marker is *fcy1*. In another embodiment, the counter selectable marker is *pyrG*. In another embodiment, the counter selectable marker is *tk*. In another embodiment, the counter selectable marker is beta-tubulin.

The first selectable marker can be the same selectable marker, a different selectable marker, or a combination of the same and different selectable markers at each of the second target loci. In one aspect, the first selectable marker is the same selectable marker at each of the second target loci. In another aspect, the first selectable marker is a different selectable marker at each of the second target loci. In another aspect, the first selectable marker is a combination of the same and different selectable markers at each of the second target loci.

The first and second fragments of the first selectable markers can each further comprise a repeat sequence 5' of the first fragment and a repeat sequence 3' of the second fragment. The repeat sequences undergo homologous recombination to remove the selectable markers.

5 The first and second fragments of the first selectable markers can each further comprise repeat sequences allowing removal of a portion of each of the selectable markers by homologous recombination resulting in selectable markers lacking a selectable function, which can undergo recombination (e.g., homologous recombination or recombinase-mediated recombination) with the second fragment of a first selectable marker lacking a
10 selectable function. This allows reuse of the eukaryotic cell for expressing different multiple heterologous proteins of interest.

In one aspect, one of the repeat sequences can be located within an intron of the first selectable markers and the other of the repeat sequences can be located 5' or 3' of the first selectable markers, wherein the repeat sequences undergo homologous recombination to
15 remove a portion of the first selectable markers.

In another aspect, one of the repeat sequences is located 5' or 3' of the first selectable markers and the other of the repeat sequences is a homologous region of the first selectable markers, wherein the repeat sequences undergo homologous recombination to remove a portion of the first selectable markers.

20 The one or more first constructs can each further comprise a fourth selectable marker between the recombination recognition sites.

Each of the fourth selectable markers can be the same selectable marker, a different selectable marker, or a combination of the same and different selectable markers. In one aspect, each of the fourth selectable markers is the same selectable marker. In another
25 aspect, each of the fourth selectable markers is a different selectable marker. In another aspect, each of the fourth selectable markers is a combination of the same and different selectable markers.

Each of the fourth selectable markers can be the same as the first selectable markers of the second constructs, different from the first selectable markers of the second
30 constructs, or a combination of the same and different selectable markers from the first selectable markers of the second constructs. In one aspect, each of the fourth selectable markers is the same as the first selectable markers of the second constructs. In another aspect, each of the fourth selectable markers is different from the first selectable markers of the second constructs. In another aspect, each of the fourth selectable markers is a
35 combination of the same and different selectable markers from the first selectable markers of the second constructs.

In one aspect, each of the fourth selectable markers of the one or more first constructs is a counter selectable marker for negative selection. The counter selectable marker for negative selection can be any useful counter selectable marker. In one aspect, the counter selectable marker is selected from the group consisting of URA3, *amdS*, *fcy1*, *pyrG*, *tk*, and beta-tubulin. In one embodiment, the counter selectable marker is URA3. In another embodiment, the counter selectable marker is *amdS*. In another embodiment, the counter selectable marker is *fcy1*. In another embodiment, the counter selectable marker is *pyrG*. In another embodiment, the counter selectable marker is *tk*. In another embodiment, the counter selectable marker is beta-tubulin.

Each of the fourth selectable markers can comprise a repeat sequence 5' and a repeat sequence 3' of each of the selectable markers, wherein the repeat sequences undergo homologous recombination to remove the selectable markers.

The first and second recombination recognition sites at each of the first target loci can be the same recombination recognition sites, different recombination recognition sites, or a combination of the same and different recombination recognition sites. In one embodiment, the first and second recombination recognition sites at each of the first target loci are the same recombination recognition site. In another embodiment, the first and second recombination recognition sites at each of the first target loci are a different recombination recognition site. In another embodiment, the first and second recombination recognition sites at each of the first target loci are a combination of the same and different recombination recognition sites.

The recombination recognition sites can be any recombination recognition sites useful in the methods of the present invention. In one aspect, the recombination recognition sites are selected from the group consisting of a B2 system from *Zygosaccharomyces bailii*, B3 system from *Zygosaccharomyces bisporus*, beta-recombinase-six system from a 25 *Bacillus subtilis* plasmid, Bxb1 from phage Bxb1, Cre-lox system of bacteriophage P1, Dre from Bacteriophage D6, FLP-FRT of *Saccharomyces cerevisiae*, Delta-gamma-es system from bacterial transposon Tn1000, Gin-gix system from bacteriophage Mu, HK022 from phage HK022, KD system from *Kluyveromyces drosophilum*, Mx9 phage transformation system, *Streptomyces* phage ϕ C31, R-RS system of *Zygosaccharomyces rouxii*, Tn3 from *E. coli*, Vika recombinase from *Vibrio coralliilyticus*, and Xis-att system of temperate lactococcal bacteriophage TP901-1; and combinations thereof. It is understood herein that the enumerated recombination recognition sites encompass homologs and variants thereof. In one embodiment, one of the recombination recognition sites is a B2 system from *Zygosaccharomyces bailii*. In another embodiment, one of the recombination recognition sites is a B3 system from *Zygosaccharomyces bisporus*. In another embodiment, one of the recombination recognition sites is a beta-recombinase-six system from a 25 *Bacillus subtilis*

plasmid. In another embodiment, one of the recombination recognition sites is a Bxb1 from phage Bxb1. In another embodiment, one of the recombination recognition sites is a Cre-lox system of bacteriophage P1. In another embodiment, one of the recombination recognition sites is a Dre from Bacteriophage D6. In another embodiment, one of the recombination recognition sites is a FLP-FRT of *Saccharomyces cerevisiae*. In another embodiment, one of the recombination recognition sites is a Delta-gamma-es system from bacterial transposon Tn1000. In another embodiment, one of the recombination recognition sites is a Gin-gix system from bacteriophage Mu. In another embodiment, one of the recombination recognition sites is a HK022 from phage HK022. In another embodiment, one of the recombination recognition sites is a KD system from *Kluyveromyces drosophilarum*. In another embodiment, one of the recombination recognition sites is a Mx9 phage transformation system. In another embodiment, one of the recombination recognition sites is a *Streptomyces* phage ϕ C31. In another embodiment, one of the recombination recognition sites is a R-RS system of *Zygosaccharomyces rouxii*. In another embodiment, one of the recombination recognition sites is a Tn3 from *E. coli*. In another embodiment, one of the recombination recognition sites is a Vika recombinase from *Vibrio coralliilyticus*. In another embodiment, one of the recombination recognition sites is a Xis-att system of temperate lactococcal bacteriophage TP901-1.

In one aspect, the flippase recognition sites of the FLP-FRT system are selected from the group consisting F, F3, F10, F13, F14, F15, Fa, and F3a; and combinations thereof. It is understood herein that the enumerated Lox sites encompass homologs and variants thereof. In one embodiment, the flippase recognition site is F. In another embodiment, the flippase recognition site is F3. In another embodiment, the flippase recognition site is F10. In another embodiment, the flippase recognition site is F13. In another embodiment, the flippase recognition site is F14. In another embodiment, the flippase recognition site is F15. In another embodiment, the flippase recognition site is Fa. In another embodiment, the flippase recognition site is F3a.

In another aspect, the TP901-1 sites of the Xis-att system are selected from the group consisting *attB* and *attP*, and combinations thereof. It is understood herein that the enumerated TP901-1 sites encompass homologs and variants thereof. In one embodiment, the TP901-1 site is *attB*. In another embodiment, the TP901-1 site is *attP*.

In another aspect, the Lox sites of the Cre-lox system are selected from the group consisting of LoxP, Lox71, Lox66, Lox511, Lox5171, Lox2272, M2, M3, M7, and M11, and combinations thereof. It is understood herein that the enumerated Lox sites encompass homologs and variants thereof. In one embodiment, the Lox site is LoxP. In another embodiment, the Lox site is Lox71. In another embodiment, the Lox site is Lox66. In another embodiment, the Lox site is Lox511. In another embodiment, the Lox site is Lox5171. In

another embodiment, the Lox site is M2. In another embodiment, the Lox site is M3. In another embodiment, the Lox site is M7. In another embodiment, the Lox site is M11.

In a preferred embodiment, the first and second recombination recognition sites at each of the first target loci are the same recombination recognition sites. In another preferred embodiment, the first and second recombination recognition sites at each of the first target loci are different recombination recognition sites. In another preferred embodiment, the first and second recombination recognition sites at each of the first target loci are a combination of the same and different recombination recognition sites.

In another preferred embodiment, the first and second recombination recognition sites at one of the first target loci are flippase recognition sites of the FLP-FRT system and at another of the first target loci are TP901-1 sites of the Xis-att system.

In the methods of the present invention, the eukaryotic cell requires one or more recombinases to catalyze the recombination between a specific set of recombination recognition sites. The one or more recombinases can be permanent or transient in the eukaryotic cell. The term "permanent" means the gene encoding the recombinase is already present in the genome of the eukaryotic cell. The term "transient" means the gene encoding the recombinase is introduced into the eukaryotic cell when the recombinase encoded by the gene is needed to catalyze the recombination between a specific set of recombination recognition sites and is removed thereafter.

The one or more recombinases can be any recombinase useful in the methods of the present invention. In one aspect, the one or more recombinases are native to the eukaryotic cell. In another aspect, the one or more recombinases are heterologous to the eukaryotic cell. In another aspect, the recombinases are a combination of native and heterologous recombinases. In one aspect, the one or more recombinases are selected from the group consisting of a Bxb1 recombinase, a Cre recombinase, a CinH recombinase, a Flp flippase, a HK022 integrase, a ParA recombinase, a Tn1721 recombinase, a Tn5053 recombinase, a TP901-1 integrase, an U153 recombinase, a λ integrase, and a ϕ C31 recombinase. It is understood herein that the enumerated recombinases encompass homologs and variants thereof. In one embodiment, one of the recombinases is a Bxb1 recombinase. In another embodiment, one of the recombinases is a Cre recombinase. In another embodiment, one of the recombinases is a CinH recombinase. In another embodiment, one of the recombinases is a Flp flippase. In another embodiment, one of the recombinases is a HK022 integrase. In another embodiment, one of the recombinases is a ParA recombinase. In another embodiment, one of the recombinases is a Tn1721 recombinase. In another embodiment, one of the recombinases is a Tn5053 recombinase. In another embodiment, one of the recombinases is a TP901-1 integrase. In another embodiment, one of the recombinases is

an U153 recombinase. In another embodiment, one of the recombinases is a λ integrase. In another embodiment, one of the recombinases is a ϕ C31 recombinase.

Each of the first polynucleotides can be the same polynucleotide, a different polynucleotide, or a combination of the same and different polynucleotides. In one aspect, each of the first polynucleotides is the same polynucleotide. In another aspect, each of the first polynucleotides is a different polynucleotide. In another aspect, each of the first polynucleotides is a combination of the same and different polynucleotides.

Each of the second polynucleotides can be the same polynucleotide, a different polynucleotide, or a combination of the same and different polynucleotides. In one aspect, each of the second polynucleotides is the same polynucleotide. In another aspect, each of the second polynucleotides is a different polynucleotide. In another aspect, each of the second polynucleotides is a combination of the same and different polynucleotides.

Each of the first polynucleotides and second polynucleotides can be the same polynucleotide, a different polynucleotide, or a combination of the same and different polynucleotides. In one aspect, each of the first polynucleotides and second polynucleotides is the same polynucleotide. In another aspect, each of the first polynucleotides and second polynucleotides is a different polynucleotide. In another aspect, each of the first polynucleotides and second polynucleotides is a combination of the same and different polynucleotides.

In the above methods of the present invention, the eukaryotic cell is reusable by repeating steps (a) and (b) with one or more different first constructs, second constructs, or first and second constructs each comprising an expression cassette comprising a polynucleotide encoding a different protein of interest. The repeat sequences described above can undergo homologous recombination to remove a selectable marker or a portion thereof.

The present invention also relates to methods of introducing multiple expression constructs into a eukaryotic cell, said method comprising.

(a) transforming a population of the eukaryotic cell with one or more first constructs and one or more second constructs,

wherein the eukaryotic cell comprises (1) one or more first target loci (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprising a pair of a first recombination recognition site and a second recombination recognition site, wherein the first recombination recognition site and a second recombination recognition site are TP901-1 sites of the Xis-att system, and (2) one or more second target loci (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprising a pair of a third recombination recognition site and a fourth recombination recognition site, wherein the third recombination recognition site and a fourth recombination recognition site are flippase

recognition sites of the FLP-FRT system;

wherein the one or more first constructs (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprises one or more first expression cassettes each comprising a first polynucleotide encoding a first protein of interest, wherein in each of the first constructs the one or more first expression cassettes are flanked on one side by the first recombination recognition site and on the other side by the second recombination recognition site corresponding to the same recombination recognition sites of the first target loci;

wherein the one or more second constructs (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprises one or more second expression cassettes each comprising a second polynucleotide encoding a second protein of interest, and wherein in each of the second constructs the one or more second expression cassettes are flanked on one side by the third recombination recognition site and on the other side by the fourth recombination recognition site corresponding to the same recombination recognition sites of the second target loci; and

wherein one or more of the first constructs and second constructs comprise one or more first selectable markers;

(b) selecting a transformant using the one or more first selectable markers, wherein each of the second expression cassettes are integrated at the corresponding second target loci, and wherein the first and second recombination recognition sites of the first constructs undergo recombination at the corresponding first and second recombination recognition sites of the first target loci driven by at least one recombinase integrating each of the first expression cassettes at the corresponding first target loci.

In one aspect, the TP901-1 sites of the Xis-att system are selected from the group consisting of *attB* and *attP*, and combinations thereof.

In another aspect, the flippase recognition sites of the FLP-FRT system are selected from the group consisting of F, F3, F10, F13, F14, F15, Fa, and F3a; and combinations thereof.

The selectable markers can be any selectable markers useful in the methods of the present invention. In one aspect, the selectable markers can be selected from the group consisting of ADE2, ARO4-OPF, FLD1, HIS3, LEU2, LYS2, MET3, TRP1, URA3, *adeA*, *adeB*, *amdS*, *argB*, *bar*, *bleR*, *bsd*, *fcy1*, *hpt*, *hpt-tk*, *nat1*, *niaD*, *ptr1*, *pyrG*, *sC*, *tk*, Tn903kan^r, *trpC*, and beta-tubulin.

In one aspect, the one or more first selectable markers are the same selectable markers.

In another aspect, the one or more first selectable markers are different selectable markers.

The one or more first target loci can each further comprise a second selectable marker between the recombination recognition sites. In one aspect, the second selectable marker at each of the first target loci is a counter selectable marker for negative selection.

The counter selectable marker for negative selection can be any useful counter selectable marker. In one aspect, the counter selectable marker is selected from the group consisting of URA3, *amdS*, *fcy1*, *pyrG*, *tk*, and beta-tubulin. In one embodiment, the counter selectable marker is URA3. In another embodiment, the counter selectable marker is *amdS*. In another embodiment, the counter selectable marker is *fcy1*. In another embodiment, the counter selectable marker is *pyrG*. In another embodiment, the counter selectable marker is *tk*. In another embodiment, the counter selectable marker is beta-tubulin.

The one or more second target loci can each further comprise a third selectable marker between the recombination recognition sites.

In one aspect, the third selectable marker at each of the second target loci is a counter selectable marker for negative selection. The counter selectable marker for negative selection can be any useful counter selectable marker. In one aspect, the counter selectable marker is selected from the group consisting of URA3, *amdS*, *fcy1*, *pyrG*, *tk*, and beta-tubulin. In one embodiment, the counter selectable marker is URA3. In another embodiment, the counter selectable marker is *amdS*. In another embodiment, the counter selectable marker is *fcy1*. In another embodiment, the counter selectable marker is *pyrG*. In another embodiment, the counter selectable marker is *tk*. In another embodiment, the counter selectable marker is beta-tubulin.

The first selectable marker can be the same selectable marker, a different selectable marker, or a combination of the same and different selectable markers at each of the second target loci. In one aspect, the first selectable marker is the same selectable marker at each of the second target loci. In another aspect, the first selectable marker is a different selectable marker at each of the second target loci. In another aspect, the first selectable marker is a combination of the same and different selectable markers at each of the second target loci.

The one or more first constructs, the one or more second constructs, or the one or more first constructs and the second constructs can each further comprise a fourth selectable marker between the recombination recognition sites.

Each of the fourth selectable markers can be the same selectable marker, a different selectable marker, or a combination of the same and different selectable markers. In one aspect, each of the fourth selectable markers is the same selectable marker. In another aspect, each of the fourth selectable markers is a different selectable marker. In another aspect, each of the fourth selectable markers is a combination of the same and different selectable markers.

Each of the fourth selectable markers can be the same as the first selectable markers of the second constructs, different from the first selectable markers of the second constructs, or a combination of the same and different selectable markers from the first

selectable markers of the second constructs. In one aspect, each of the fourth selectable markers is the same as the first selectable markers of the second constructs. In another aspect, each of the fourth selectable markers is different from the first selectable markers of the second constructs. In another aspect, each of the fourth selectable markers is a combination of the same and different selectable markers from the first selectable markers of the second constructs.

In one aspect, each of the fourth selectable markers of the one or more first constructs is a counter selectable marker for negative selection. The counter selectable marker for negative selection can be any useful counter selectable marker. In one aspect, the counter selectable marker is selected from the group consisting of URA3, *amdS*, *fcy1*, *pyrG*, *tk*, and beta-tubulin. In one embodiment, the counter selectable marker is URA3. In another embodiment, the counter selectable marker is *amdS*. In another embodiment, the counter selectable marker is *fcy1*. In another embodiment, the counter selectable marker is *pyrG*. In another embodiment, the counter selectable marker is *tk*. In another embodiment, the counter selectable marker is beta-tubulin.

Each of the fourth selectable markers can comprise a repeat sequence 5' and a repeat sequence 3' of each of the selectable markers, wherein the repeat sequences undergo homologous recombination to remove the selectable markers.

All other aspects for this method are described above.

Expression Constructs

The present invention also relates to expression constructs comprising a polynucleotide encoding a protein of interest, wherein the polynucleotide is operably linked to one or more control sequences that direct the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences.

The polynucleotide may be manipulated in a variety of ways to provide for expression of the protein. Manipulation of the polynucleotide prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotides utilizing recombinant DNA methods are well known in the art.

The control sequence may be a promoter, a polynucleotide that is recognized by a host cell for expression of a polynucleotide encoding a protein. The promoter contains transcriptional control sequences that mediate the expression of the protein. The promoter may be any polynucleotide that shows transcriptional activity in the host cell including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular proteins either homologous or heterologous to the host cell.

Examples of suitable promoters for directing transcription of the nucleic acid constructs of the present invention in a filamentous fungal host cell are promoters obtained

from the genes for *Aspergillus nidulans* acetamidase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Aspergillus oryzae* TAKA amylase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Fusarium oxysporum* trypsin-like protease (WO 96/00787), *Fusarium venenatum* amyloglucosidase (WO 00/56900), *Fusarium venenatum* Daria (WO 00/56900), *Fusarium venenatum* Quinn (WO 00/56900), *Rhizomucor miehei* lipase, *Rhizomucor miehei* aspartic proteinase, *Trichoderma reesei* beta-glucosidase, *Trichoderma reesei* cellobiohydrolase I, *Trichoderma reesei* cellobiohydrolase II, *Trichoderma reesei* endoglucanase I, *Trichoderma reesei* endoglucanase II, *Trichoderma reesei* endoglucanase III, *Trichoderma reesei* endoglucanase V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma reesei* xylanase III, *Trichoderma reesei* beta-xylosidase, and *Trichoderma reesei* translation elongation factor, as well as the NA2-tpi promoter (a modified promoter from an *Aspergillus* neutral alpha-amylase gene in which the untranslated leader has been replaced by an untranslated leader from an *Aspergillus* triose phosphate isomerase gene; non-limiting examples include modified promoters from an *Aspergillus niger* neutral alpha-amylase gene in which the untranslated leader has been replaced by an untranslated leader from an *Aspergillus nidulans* or *Aspergillus oryzae* triose phosphate isomerase gene); and mutant, truncated, and hybrid promoters thereof. Other promoters are described in U.S. Patent No. 6,011,147.

In a yeast host, useful promoters are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* galactokinase (GAL1), *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH1, ADH2/GAP), *Saccharomyces cerevisiae* triose phosphate isomerase (TPI), *Saccharomyces cerevisiae* metallothionein (CUP1), and *Saccharomyces cerevisiae* 3-phosphoglycerate kinase. Other useful promoters for yeast host cells are described by Romanos *et al.*, 1992, *Yeast* 8: 423-488.

The control sequence may also be a transcription terminator, which is recognized by a host cell to terminate transcription. The terminator is operably linked to the 3'-terminus of the polynucleotide encoding the protein. Any terminator that is functional in the host cell may be used in the present invention.

Preferred terminators for filamentous fungal host cells are obtained from the genes for *Aspergillus nidulans* acetamidase, *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* glucoamylase, *Aspergillus niger* alpha-glucosidase, *Aspergillus oryzae* TAKA amylase, *Fusarium oxysporum* trypsin-like protease, *Trichoderma reesei* beta-glucosidase, *Trichoderma reesei* cellobiohydrolase I, *Trichoderma reesei* cellobiohydrolase II, *Trichoderma reesei* endoglucanase I, *Trichoderma reesei* endoglucanase II, *Trichoderma*

reesei endoglucanase III, *Trichoderma reesei* endoglucanase V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma reesei* xylanase III, *Trichoderma reesei* beta-xylosidase, and *Trichoderma reesei* translation elongation factor.

Preferred terminators for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1), and *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase. Other useful terminators for yeast host cells are described by Romanos *et al.*, 1992, *supra*.

The control sequence may also be a leader, a nontranslated region of an mRNA that is important for translation by the host cell. The leader is operably linked to the 5'-terminus of the polynucleotide encoding the protein. Any leader that is functional in the host cell may be used.

Preferred leaders for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TKA amylase and *Aspergillus nidulans* triose phosphate isomerase.

Suitable leaders for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* 3-phosphoglycerate kinase, *Saccharomyces cerevisiae* alpha-factor, and *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP).

The control sequence may also be a polyadenylation sequence, a sequence operably linked to the 3'-terminus of the polynucleotide and, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence that is functional in the host cell may be used.

Preferred polyadenylation sequences for filamentous fungal host cells are obtained from the genes for *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* glucoamylase, *Aspergillus niger* alpha-glucosidase *Aspergillus oryzae* TKA amylase, and *Fusarium oxysporum* trypsin-like protease.

Useful polyadenylation sequences for yeast host cells are described by Guo and Sherman, 1995, *Mol. Cellular Biol.* 15: 5983-5990.

The control sequence may also be a signal peptide coding region that encodes a signal peptide linked to the N-terminus of a polypeptide and directs the polypeptide into the cell's secretory pathway. The 5'-end of the coding sequence of the polynucleotide may inherently contain a signal peptide coding sequence naturally linked in translation reading frame with the segment of the coding sequence that encodes the protein. Alternatively, the 5'-end of the coding sequence may contain a signal peptide coding sequence that is foreign to the coding sequence. A foreign signal peptide coding sequence may be required where the coding sequence does not naturally contain a signal peptide coding sequence. Alternatively, a foreign signal peptide coding sequence may simply replace the natural signal peptide coding sequence in order to enhance secretion of the polypeptide. However, any

signal peptide coding sequence that directs the expressed polypeptide into the secretory pathway of a host cell may be used.

Effective signal peptide coding sequences for filamentous fungal host cells are the signal peptide coding sequences obtained from the genes for *Aspergillus niger* neutral amylase, *Aspergillus niger* glucoamylase, *Aspergillus oryzae* TAKA amylase, *Humicola insolens* cellulase, *Humicola insolens* endoglucanase V, *Humicola lanuginosa* lipase, and *Rhizomucor miehei* aspartic proteinase.

Useful signal peptides for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* alpha-factor and *Saccharomyces cerevisiae* invertase. Other useful signal peptide coding sequences are described by Romanos *et al.*, 1992, *supra*.

The control sequence may also be a propeptide coding sequence that encodes a propeptide positioned at the N-terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to an active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding sequence may be obtained from the genes for *Bacillus subtilis* alkaline protease (*aprE*), *Bacillus subtilis* neutral protease (*nprT*), *Myceliophthora thermophila* laccase (WO 95/33836), *Rhizomucor miehei* aspartic proteinase, and *Saccharomyces cerevisiae* alpha-factor.

Where both signal peptide and propeptide sequences are present, the propeptide sequence is positioned next to the N-terminus of a polypeptide and the signal peptide sequence is positioned next to the N-terminus of the propeptide sequence.

It may also be desirable to add regulatory sequences that regulate expression of the polypeptide relative to the growth of the host cell. Examples of regulatory sequences are those that cause expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. In filamentous fungi, the *Aspergillus niger* glucoamylase promoter, *Aspergillus oryzae* TAKA alpha-amylase promoter, and *Aspergillus oryzae* glucoamylase promoter, *Trichoderma reesei* cellobiohydrolase I promoter, and *Trichoderma reesei* cellobiohydrolase II promoter may be used. Other examples of regulatory sequences are those that allow for gene amplification. In eukaryotic systems, these regulatory sequences include the dihydrofolate reductase gene that is amplified in the presence of methotrexate, and the metallothionein genes that are amplified with heavy metals. In these cases, the polynucleotide encoding the polypeptide would be operably linked to the regulatory sequence.

In one embodiment, a first nucleic acid construct comprises (i) a 5' homologous region of a first target locus of a eukaryotic cell, (ii) a first recombination recognition site, (iii) a first repeat sequence, (iv) a first selectable marker conferring a first selectable function, (v) a second repeat sequence, (vi) a second recombination recognition site, and (vii) a 3'

homologous region of the first target locus of the eukaryotic cell. In a preferred embodiment, the second recombination recognition site is before the first repeat sequence. In another preferred embodiment, the second recombination recognition site is after the second repeat sequence.

5 In another embodiment, a second nucleic acid construct comprises (1) a 5' homologous region of a second target locus of a eukaryotic cell, (2) a first fragment of a second selectable marker that lacks a second selectable function, (3) a third repeat sequence, (4) a third selectable marker conferring a third selectable function, (5) a fourth repeat sequence, and (6) a 3' homologous region of the second target locus of the
10 eukaryotic cell. In a preferred embodiment, the first fragment of the second selectable marker is before the third repeat sequence. In another preferred embodiment, the first fragment of the second selectable marker is after the fourth repeat sequence.

In another embodiment, a third nucleic acid construct comprises (1) a first recombination recognition site, (2) one or more first expression cassettes each comprising a
15 first polynucleotide encoding a first protein of interest, and (3) a second recombination recognition site.

In another embodiment, a fourth nucleic acid construct comprises (1) a 5' homologous region of the second target locus, (2) one or more second expression cassettes each comprising a second polynucleotide encoding a second protein of interest, and (3) a
20 second fragment of a second selectable marker that lacks a second selectable function.

Proteins of Interest

The proteins of interest may be any protein having a biological activity of interest. The term "protein" is not meant herein to refer to a specific length of the encoded product
25 and, therefore, encompasses peptides, oligopeptides, and polypeptides. The term "protein" also encompasses two or more (e.g., several) proteins combined to form the encoded product. The proteins also include fusion proteins, which comprise a combination of partial or complete polypeptide sequences obtained from at least two different proteins wherein one or more (e.g., several) may be heterologous to the eukaryotic cell. The proteins can further
30 include naturally occurring allelic and engineered variations of the below-mentioned proteins and hybrid proteins.

The techniques used to isolate or clone a polynucleotide encoding a protein of interest are known in the art and include isolation from genomic DNA or cDNA, or a combination thereof. The cloning of the polynucleotides from genomic DNA can be effected,
35 e.g., by using the well-known polymerase chain reaction (PCR) or antibody screening of expression libraries to detect cloned DNA fragments with shared structural features. See, e.g., Innis *et al.*, 1990, *PCR: A Guide to Methods and Application*, Academic Press, New

York. Other nucleic acid amplification procedures such as ligase chain reaction (LCR), ligation activated transcription (LAT) and polynucleotide-based amplification (NASBA) may be used. The polynucleotides may be cloned from any suitable source.

In one aspect, the proteins are selected from the group consisting of an antibody, an antigen, an antimicrobial peptide, an enzyme, a growth factor, a hormone, an immunodilator, a neurotransmitter, a receptor, a reporter protein, a structural protein, or a transcription factor.

In one embodiment, the enzyme is selected from the group consisting of an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase, and a ligase. In another embodiment, the enzyme is selected from the group consisting of an acetylmannan esterase, acetyxylan esterase, aminopeptidase, alpha-amylase, alpha-galactosidase, alpha-glucosidase, alpha-1,6-transglucosidase, arabinanase, arabinofuranosidase, beta-galactosidase, beta-glucosidase, beta-xylosidase, carbohydrase, carboxypeptidase, catalase, cellobiohydrolase, cellulase, chitinase, coumaric acid esterase, cyclodextrin glycosyltransferase, cutinase, deoxyribonuclease, endoglucanase, esterase, feruloyl esterase, AA9 polypeptide having cellulolytic enhancing activity, glucocerebrosidase, glucose oxidase, glucuronidase, glucuronoyl esterase, haloperoxidase, hemicellulase, invertase, isomerase, laccase, ligase, lipase, mannanase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phospholipase, phytase, phenoloxidase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, urokinase, and xylanase.

In another aspect, the proteins are selected from the group consisting of an albumin, a collagen, a tropoelastin, an elastin, and a gelatin.

In another aspect, the proteins having biological activity may be different proteins. In another aspect, two or more (e.g., several) of the proteins having biological activity are the same protein.

In another aspect, the proteins comprise one or more enzymes selected from the group consisting of a cellulase, a cip1 protein, a AA9 polypeptide having cellulolytic enhancing activity, a hemicellulase, an esterase, an expansin, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin. In another aspect, the cellulase is one or more enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase. In another aspect, the hemicellulase is one or more enzymes selected from the group consisting of a xylanase, an acetylxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, and a glucuronidase.

In one embodiment, one of the proteins is a cellulase. In another embodiment, one of the proteins is an endoglucanase. In another embodiment, one of the proteins is a cellobiohydrolase. In another embodiment, one of the proteins is a beta-glucosidase. In

another embodiment, one of the proteins is a AA9 protein having cellulolytic enhancing activity. In another embodiment, one of the proteins is a swollenin protein. In another embodiment, one of the proteins is a cip1 protein. In another embodiment, one of the proteins is an esterase. In another embodiment, one of the proteins is an expansin. In another embodiment, one of the proteins is a laccase. In another embodiment, one of the proteins is a ligninolytic enzyme. In another embodiment, one of the proteins is a pectinase. In another embodiment, one of the proteins is a peroxidase. In another embodiment, one of the proteins is a protease. In another embodiment, one of the proteins is a swollenin.

In another embodiment, one of the proteins is a hemicellulase. In another embodiment, one of the proteins is a xylanase. In another embodiment, one of the proteins is a beta-xylosidase. In another embodiment, one of the proteins is an acetyxylan esterase. In another embodiment, one of the proteins is a feruloyl esterase. In another embodiment, one of the proteins is an arabinofuranosidase. In another embodiment, one of the proteins is a glucuronidase. In another embodiment, one of the proteins is an acetylmannan esterase. In another embodiment, one of the proteins is an arabinanase. In another embodiment, one of the proteins is a coumaric acid esterase. In another embodiment, one of the proteins is a galactosidase. In another embodiment, one of the proteins is a glucuronoyl esterase. In another embodiment, one of the proteins is a mannanase. In another embodiment, one of the proteins is a mannosidase.

Eukaryotic Cells

The present invention also relates to a eukaryotic cell obtained by the methods of the present invention.

In one embodiment, the eukaryotic cell for expressing multiple heterologous proteins of interest, comprises:

(a) one or more first target loci (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprising a pair of a first recombination recognition site and a second recombination recognition site; and

(b) one or more second target loci (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprising a first fragment of a first selectable marker lacking a selectable function.

In one aspect, the eukaryotic cell further comprises one or more first expression cassettes each comprising a first polynucleotide encoding a first protein of interest at each of the first target loci, wherein each of the one or more first expression cassettes are flanked on one side by the first recombination recognition site and on the other side by the second recombination recognition site of the corresponding first target locus.

In another aspect, the eukaryotic cell further or even further comprises one or more second expression cassettes each comprising a second polynucleotide encoding a second

protein of interest at each of the second target loci, wherein each of the one or more second expression cassettes are flanked on one side by the first fragment of the selectable marker lacking a selectable function.

In another embodiment, the eukaryotic cell comprises (1) one or more first target loci each comprising one or more first expression cassettes each comprising a first polynucleotide encoding a first protein of interest, wherein the one or more first expression cassettes are each flanked 5' by a first recombination recognition site and 3' by a second recombination recognition site, and (2) one or more second target loci each comprising one or more second expression cassettes each comprising a second polynucleotide encoding a second protein of interest, wherein each of the one or more second expression cassettes are flanked on one side by a region of the second target locus and on the other side by a first fragment of a first selectable marker that lacks selectable function,

wherein each of the pairs of the first and second recombination recognition sites at the first loci are able to undergo recombination with a first construct comprising one or more third expression cassettes each comprising a third polynucleotide encoding a third protein of interest, wherein each of the one or more third expression cassettes are flanked on one side by the first recombination recognition site and on the other side by the second recombination recognition site of the corresponding first target locus, and

wherein each of the target loci regions and the first fragment of the first selectable marker that lacks selectable function at the second loci are able to undergo recombination (e.g., homologous recombination or recombinase-mediated recombination) with a second construct comprising one or more fourth expression cassettes each comprising a fourth polynucleotide encoding a fourth protein of interest, wherein each of the one or more fourth expression cassettes are flanked on one side by a homologous region of the corresponding second target locus and on the other side by a second fragment of the first selectable marker that lacks the selectable function wherein the second fragment comprises a sequence overlapping homologously the corresponding sequence of the first fragment of the first selectable marker.

The eukaryotic cell can be any eukaryotic cell useful in the methods of the present invention. The eukaryotic cell can be a mammalian, insect, plant, or fungal cell.

The eukaryotic cell may be a fungal cell. "Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota as well as the Oomycota and all mitosporic fungi (as defined by Hawksworth *et al.*, *In, Ainsworth and Bisby's Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK).

The fungal cell may be a yeast cell. "Yeast" as used herein includes ascosporogenous yeast (Endomycetales), basidiosporogenous yeast, and yeast belonging

to the Fungi Imperfecti (Blastomycetes). Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in *Biology and Activities of Yeast* (Skinner, Passmore, and Davenport, editors, Soc. App. Bacteriol. Symposium Series No. 9, 1980).

The yeast cell may be a *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* cell, such as a *Kluyveromyces lactis*, *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, *Saccharomyces oviformis*, or *Yarrowia lipolytica* cell.

The fungal cell may be a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth *et al.*, 1995, *supra*). The filamentous fungi are generally characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative.

The filamentous fungal cell may be an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes*, or *Trichoderma* cell.

For example, the filamentous fungal cell may be an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Bjerkandera adusta*, *Ceriporiopsis aneirina*, *Ceriporiopsis caregiea*, *Ceriporiopsis gilvescens*, *Ceriporiopsis pannocinta*, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*, *Ceriporiopsis subvermispora*, *Chrysosporium inops*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium merdarium*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium tropicum*, *Chrysosporium zonatum*, *Coprinus cinereus*, *Coriolus hirsutus*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Phlebia radiata*, *Pleurotus eryngii*, *Talaromyces emersonii*, *Talaromyces leycettanus*, *Thielavia terrestris*, *Trametes villosa*, *Trametes versicolor*, *Trichoderma*

harzianum, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell.

In one aspect, the filamentous fungal cell is an *Aspergillus* cell. In another aspect, the filamentous fungal cell is *Aspergillus nidulans*. In another aspect, the filamentous fungal cell is *Aspergillus niger*. In another aspect, the filamentous fungal cell is *Aspergillus oryzae*.

In another aspect, the filamentous fungal cell is a *Fusarium* cell. In another aspect, the filamentous fungal cell is *Fusarium venenatum*.

In another aspect, the filamentous fungal cell is a *Myceliophthora* cell. In another aspect, the filamentous fungal cell is *Myceliophthora thermophila*.

In another aspect, the filamentous fungal cell is a *Talaromyces* cell. In another aspect, the filamentous fungal cell is *Talaromyces emersonii*.

In another aspect, the filamentous fungal cell is a *Trichoderma* cell. In another aspect, the filamentous fungal cell is *Trichoderma reesei*.

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* and *Trichoderma* host cells are described in EP 238023, Yelton *et al.*, 1984, *Proc. Natl. Acad. Sci. USA* 81: 1470-1474, and Christensen *et al.*, 1988, *Bio/Technology* 6: 1419-1422. Suitable methods for transforming *Fusarium* species are described by Malardier *et al.*, 1989, *Gene* 78: 147-156, and WO 96/00787. Yeast may be transformed using the procedures described by Becker and Guarente, *In* Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, *J. Bacteriol.* 153: 163; and Hinnen *et al.*, 1978, *Proc. Natl. Acad. Sci. USA* 75: 1920.

In one aspect, the filamentous fungal cell is an *Aspergillus* cell. In another aspect, the filamentous fungal cell is *Aspergillus nidulans*. In another aspect, the filamentous fungal cell is *Aspergillus niger*. In another aspect, the filamentous fungal cell is *Aspergillus oryzae*.

In another aspect, the filamentous fungal cell is a *Fusarium* cell. In another aspect, the filamentous fungal cell is *Fusarium venenatum*.

In another aspect, the filamentous fungal cell is a *Myceliophthora* cell. In another aspect, the filamentous fungal cell is *Myceliophthora thermophila*.

In another aspect, the filamentous fungal cell is a *Talaromyces* cell. In another aspect, the filamentous fungal cell is *Talaromyces emersonii*.

In another aspect, the filamentous fungal cell is a *Trichoderma* cell. In another aspect, the filamentous fungal cell is *Trichoderma reesei*.

Methods of Production

The present invention also relates to methods of producing a protein of interest, comprising (a) cultivating a eukaryotic cell of the present invention under conditions conducive for production of the protein; and optionally, (b) recovering the protein.

The eukaryotic cell is cultivated in a nutrient medium suitable for production of the protein using methods known in the art. For example, the eukaryotic cell may be cultivated by shake flask cultivation, or small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors in a suitable medium and under conditions allowing the protein to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). If the protein is secreted into the nutrient medium, the protein can be recovered directly from the medium. If the protein is not secreted, it can be recovered from cell lysates.

The protein may be detected using methods known in the art that are specific for the protein. These detection methods include, but are not limited to, use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate. For example, an enzyme assay may be used to determine the activity of the protein.

The protein may be recovered using methods known in the art. For example, the protein may be recovered from the nutrient medium by conventional procedures including, but not limited to, collection, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation. In one aspect, a whole fermentation broth comprising the protein is recovered.

The protein may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), SDS-PAGE, or extraction (see, e.g., *Protein Purification*, Janson and Ryden, editors, VCH Publishers, New York, 1989) to obtain substantially pure proteins.

Examples

Strains

T. reesei RutC30 (ATCC 56765) is described by Montenecourt and Eveleigh, 1979, *Adv. Chem. Ser.* 181: 289-301.

Trichoderma reesei strain 981-O-8 (D4) is a mutagenized strain of *T. reesei* RutC30.

Trichoderma reesei strain AgJg115-104-7B1 (WO 2011/075677) is a ku70- derivative of *T. reesei* strain 981-O-8 (D4).

Media and Buffer Solutions

Cellulase-inducing medium (CIM) was composed of 20 g of cellulose, 10 g of corn steep solids, 1.45 g of $(\text{NH}_4)_2\text{SO}_4$, 2.08 g of KH_2PO_4 , 0.28 g of CaCl_2 , 0.42 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.42 ml of *Trichoderma* trace metals solution, 1-2 drops of antifoam, and deionized water to 1 liter; pH adjusted to 6.0.

COVE plates were composed of 342.3 g of sucrose, 20 ml of COVE salt solution, 10 ml of 1 M acetamide, 10 ml of 1.5 M CsCl, 25 g of Noble agar (Difco), and deionized water to 1 liter.

COVE + 2% beta-lactose plates were composed of 342.3 g of sucrose, 20 g of beta-lactose, 20 ml of COVE salt solution, 10 ml of 1 M acetamide, 10 ml of 1.5 M CsCl, 25 g of Noble agar (Difco), and deionized water to 1 liter.

COVE + 2% beta-lactose overlay was composed of 20 g of beta-lactose, 20 ml of COVE salt solution, 10 ml of 1 M acetamide, 10 ml of 1.5 M CsCl, 25 g of Noble agar (Difco), and deionized water to 1 liter. The autoclaved medium was melted in a microwave and then tempered to 55°C before use.

COVE + 2% glucose transformation plates were composed of 342 g of sucrose, 20 ml of COVE salts solution, 10 ml of 1 M acetamide, 10 ml of 1.5 M CsCl, 20 g of glucose, 25 g of Noble agar, and deionized water to 1 liter.

COVE + 2% glucose overlay was composed of 20 ml of COVE salts solution, 10 ml of 1 M acetamide, 10 ml of 1.5 M CsCl, 20 g of glucose, 25 g of Noble agar, and deionized water to 1 liter. The autoclaved medium was melted in a microwave and then tempered to 55°C before use.

COVE + 2% glucose plates were composed of 20 ml of COVE salts solution, 10 ml of 1 M acetamide, 20 g of glucose, 25 g of Noble agar, and deionized water to 1 liter.

COVE glycerol plates were composed of 40 ml of 75% glycerol, 20 ml of COVE salt solution, 10 ml of 1 M acetamide, 25 g of Noble agar (Difco), and deionized water to 1 liter.

COVE 2 plates were composed of 30 g of sucrose, 20 ml of COVE salt solution, 10 ml of 1 M acetamide, 25 g of Noble agar (Difco), and deionized water to 1 liter.

COVE salt solution was composed of 26 g of KCl, 26 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 76 g of KH_2PO_4 , 50 ml of COVE trace metals solution, and deionized water to 1 liter.

COVE trace metals solution was composed of 0.04 g of $\text{NaB}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 0.4 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1.2 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.7 g of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.8 g of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 10 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and deionized water to 1 liter.

Denaturing Solution was composed of 0.5 M NaOH and 1.5 M NaCl.

FdU plates were composed of 20 ml of COVE salts solution, 0.6 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 25 g of Noble agar, and deionized water to 967 ml. After the solution was autoclaved and cooled

to 55°C, 20 ml filter sterilized 50% glucose, 12.5 ml filter sterilized 1 M urea, and 12.4 µl 40.6 µM FdU (5-fluoro-2'-deoxyuridine) were added.

LB plus ampicillin medium was composed of 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and deionized water to 1 liter. After autoclaving 1 ml of a 100 mg/ml solution of ampicillin in water was added.

LB plates were composed of 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 15 g of Bacto agar, and deionized water to 1 liter.

Neutralization Solution was composed of 1 M Tris pH 8.0 and 1.5 M NaCl.

NZY+ medium was composed of 5 g of NaCl, 3 g of MgSO₄·7H₂O, 5 g of yeast extract, 10 g of NZ amine, 1.2 g of MgCl₂, 4 g of glucose, and deionized water to 1 liter.

PDA plates were composed of 39 g of Potato Dextrose Agar (Difco) and deionized water to 1 liter.

PDA overlay medium was composed of 39 g of Potato Dextrose Agar (Difco), 2.44 g uridine, and deionized water to 1 liter. The autoclaved medium was melted in a microwave and then tempered to 55°C before use.

PEG buffer was composed of 500 g of polyethylene glycol 4000 (PEG 4000), 10 mM CaCl₂, 10 mM Tris-HCl pH 7.5, and deionized water to 1 liter; filter sterilized.

SOC medium was composed of 0.5 g of NaCl, 5 g of yeast extract, 20 g of tryptone, 10 ml of 250 mM KCl, and deionized water to 1 liter.

20X SSC was composed of 175.3 g of NaCl, 88.2 g of sodium citrate, and deionized water to 1 liter.

STC was composed of 1 M sorbitol, 10 mM CaCl₂, and 10 mM Tris-HCl, pH 7.5; filter sterilized.

TAE buffer was composed of 4.84 g of Tris Base, 1.14 ml of Glacial acetic acid, 2 ml of 0.5 M EDTA pH 8, and deionized water to 1 liter.

TBE buffer was composed of 10.8 g of Tris Base, 5 g of boric acid, 4 ml of 0.5 M EDTA pH 8, and deionized water to 1 liter.

TE Buffer was composed of 1 M Tris pH 8.0 and 0.5 M EDTA pH 8.0.

Trichoderma Minimal Medium (TrMM) plates (for transformation) were composed of 342.3 g of sucrose, 20 ml of COVE salt solution, 0.6 g CaCl₂·2H₂O, 6 g (NH₄)₂SO₄, 25 g Noble agar, and deionized water to 1 liter.

Trichoderma Minimal Medium (TrMM) plates (for sub-culturing) were composed of 30 g sucrose, 20 ml COVE salt solution, 0.6 g of CaCl₂·2H₂O, 6 g of (NH₄)₂SO₄, 25 g of Noble agar, and deionized water to 1 liter.

TrMM medium was composed of 20 ml of COVE salt solution, 6 g of (NH₄)₂SO₄, 0.6 g of CaCl₂, 25 g of Noble agar (Difco), 30 g of sucrose, and deionized water to 1 liter.

TrMM-G medium was composed of 20 ml of COVE salt solution, 6 g of (NH₄)₂SO₄,

0.6 g of CaCl₂, 25 g of Nobel agar (Difco), 20 g of glucose, and deionized water to 1 liter.

Trichoderma trace metals solution was composed of 216 g of FeCl₃·6H₂O, 58 g of ZnSO₄·7H₂O, 27 g of MnSO₄·H₂O, 10 g of CuSO₄·5H₂O, 2.4 g of H₃BO₃, 336 g of citric acid, and deionized water to 1 liter.

5 YP medium was composed of 10 g of yeast extract, 20 g of Bacto peptone, and deionized water to 1 liter.

YPG medium was composed of 4 g of yeast extract, 1 g of K₂HPO₄, 0.5 g of MgSO₄, 15.0 g of glucose, and deionized water to 1 liter (pH 6.0).

10 2XYT plus ampicillin plates were composed of 16 g of tryptone, 10 g of yeast extract, 5 g of sodium chloride, 15 g of Bacto agar, and deionized water to 1 liter. One ml of a 100 mg/ml solution of ampicillin was added after the autoclaved medium was tempered to 55°C.

DNA sequencing

15 DNA sequencing was performed with a Model 377 XL Automated DNA Sequencer (Applied Biosystems Inc.) using dye-terminator chemistry (Giesecke *et al.*, 1992, *J. Virol. Methods* 38: 47-60).

Example 1: *Trichoderma reesei* protoplast generation and transformation

20 Protoplast preparation and transformation were performed using the following protocol based on Penttilä *et al.*, 1987, *Gene* 61: 155-164. A *Trichoderma reesei* strain was cultivated in 25 ml of YP medium supplemented with 2% (w/v) glucose and 10 mM uridine at 27°C for 17 hours with gentle agitation at 90 rpm. Mycelia were collected by filtration using a Vacuum Driven Disposable Filtration System (Millipore) and washed twice with deionized water and twice with 1.2 M sorbitol. Protoplasts were generated by suspending the washed
25 mycelia in 20 ml of 1.2 M sorbitol containing 15 mg of GLUCANEX® 200 G (Novozymes A/S, Bagsvaerd, Denmark) per ml and 0.36 units of chitinase (Sigma Chemical Co.) per ml for 15-25 minutes at 34°C with gentle shaking at 90 rpm. Protoplasts were collected by centrifuging for 7 minutes at 400 x g and washed twice with cold 1.2 M sorbitol. The protoplasts were counted using a haemocytometer and resuspended to a final concentration
30 of 1 x 10⁸ protoplasts/ml in STC.

Approximately 1-10 µg of DNA were added to 100 µl of the protoplast solution and mixed gently. PEG buffer (250 µl) was then added, and the transformation reaction was mixed and incubated at 34°C for 30 minutes. STC (3 ml) was then added to the transformation reaction and mixed. The transformation reaction was then spread onto COVE
35 plates for *amdS* selection. The plates were incubated at 28°C for 6-11 days.

For transformation requiring hygromycin selection, the reaction mix in STC was

spread onto PDA plates supplemented with 1 M sucrose. After incubation at 28°C for 16 hours, 20 ml of overlay PDA medium supplemented with 35 µg of hygromycin B per ml were added to each plate. The plates were incubated at 28°C for 4-7 days.

5 **Example 2: Genomic DNA extraction from *Trichoderma reesei* strains**

A *Trichoderma reesei* strain was grown in 50 ml of YP medium supplemented with 2% glucose (w/v) in a 250 ml baffled shake flask at 28°C for 2 days with agitation at 200 rpm. Mycelia from each cultivation were collected using a MIRACLOTH® (EMD Chemicals Inc.) lined funnel, squeeze-dried, and frozen under liquid nitrogen. The frozen mycelia were transferred to a pre-chilled mortar and pestle. Each mycelia preparation was ground into a fine powder and kept frozen with liquid nitrogen. A total of 1-2 g of powder was transferred to a 50 ml tube and genomic DNA was extracted from the ground mycelial powder using a DNEASY® Plant Maxi Kit (QIAGEN Inc.). Five ml of Buffer AP1 (QIAGEN Inc.) pre-heated to 65°C were added to the 50 ml tube followed by 10 µl of a RNase A 100 mg/ml stock solution (QIAGEN Inc.), and incubated for 2-3 hours at 65°C. A total of 1.8 ml of AP2 Buffer (QIAGEN Inc.) was added and the tube was incubated on ice for 5 minutes followed by centrifugation at 3000-5000 x g for 5 minutes in a LEGEND™ RT swinging bucket centrifuge (Thermo Fisher Scientific Inc.). The supernatant was transferred to a QIA shredder™ Maxi Spin Column (QIAGEN Inc.) placed in a 50 ml collection tube, and centrifuged at 3000-5000 x g at room temperature for 5 minutes (15-25°C) in a swing-out rotor. The flow-through in the collection tube was transferred, without disturbing the pellet, into a new 50 ml tube. A 1.5 ml volume of Buffer AP3/E (QIAGEN Inc.) was added to the cleared lysate, and mixed immediately by vortexing. The sample (maximum 15 ml), including any precipitate that may have formed, was pipetted into a DNEASY® Maxi Spin Column (QIAGEN Inc.) placed in a 50 ml collection tube and centrifuged at 3000-5000 x g for 5 minutes at room temperature (15-20°C) in a swing-out rotor. The flow-through was discarded. Twelve ml of Buffer AW (QIAGEN Inc.) were added to the DNEASY® Maxi Spin Column, and centrifuged at 3000-5000 x g for 10 minutes to dry the membrane. The flow-through and collection tube were discarded. The DNEASY® Maxi Spin Column was transferred to a new 50 ml tube. The DNA was eluted by adding 1-1.5 ml of Kit-supplied buffer AE, pre-heated to 65°C, directly onto the DNEASY® Maxi Spin Column membrane, incubating at room temperature for 5 minutes (15-25°C), and then centrifuging at 3000-5000 x g for 5 minutes. The concentration and purity of the genomic DNA was determined by measuring the absorbance at 260 nm and 280 nm.

35 **Example 3: Southern blot analysis of transformants**

Two µg of genomic DNA from each transformant were digested with selected restriction enzyme(s). The digestions were submitted to 0.7-0.8% agarose gel

electrophoresis in TAE buffer and blotted onto a HYBOND® N+ blotting membrane (GE Healthcare Life Sciences) or a NYTRAN® SuperCharge membrane (Schleicher & Schuell BioScience) using a TURBOBLOTTER® (GE Healthcare Life Sciences) for approximately 1-2 hours. The membrane was hybridized with a digoxigenin-labeled gene-specific or site-specific probe, which was synthesized by PCR using a PCR DIG Probe Synthesis Kit (Roche Applied Science Corp.).

The probe was boiled for 5 minutes, chilled on ice for 2 minutes, and added to 10 ml of DIG Easy Hyb to produce the hybridization solution. Hybridization was performed in DIG Easy Hyb buffer at 42°C for 15-17 hours. The membrane was then washed in 2X SSC plus 0.1% SDS at room temperature for 5 minutes followed by two washes in 0.5X SSC plus 0.1% SDS each at 65°C for 15 minutes. After one wash in 1X Blocking Solution (Roche Applied Science Corp.) for a minimum of one hour, the membrane was incubated with 50 ml of 1X Blocking Solution containing 3.75 U of anti-digoxigenin-AP Fab fragments (Roche Applied Science Corp.) for 10 minutes, followed by two washes in 1X Washing Solution (Roche Applied Science Corp.). The CDP-STAR® ready-to-use reagent (disodium 2-chloro-5-(4-methoxyspiro (1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)-1-phenyl phosphate; Roche Applied Science Corp.) was then applied to the membrane and the probe-target hybrids were detected by autoradiography.

Example 4: Construction of plasmid pJfyS147 to insert FRT-F and FRT-F3 sites at the *cbh1* locus of *Trichoderma reesei*

A FRT site integration plasmid pJfyS147 was constructed so that the *T. reesei cbh1* gene and 1 kb of the upstream region thereof is deleted when the plasmid integrates. The promoter region and desired gene could then be introduced back with a FLP/FRT expression construct. To construct the FRT site integration plasmid the FRT-F site (SEQ ID NO: 1) was inserted downstream of the 5' *cbh1* flanking region using splicing by overlap extension (SOE) PCR. First the FRT-F site and 5' *cbh1* flanking region were amplified separately and then combined by overlapping PCR.

The 5' *cbh1* flanking region was amplified from *T. reesei* RutC30 genomic DNA using the primers shown below. *T. reesei* RutC30 genomic DNA was prepared as described in Example 1.

Forward primer:

5'-TCACATGGTTTAAACGGCGCGCCGGTGAAACACCGCCCCCTTC-3' (SEQ ID NO: 2)

Reverse primer:

5'-TTCGAACAGCCCCAGTCGGT-3' (SEQ ID NO: 3)

The PCR was composed of 150 ng of *T. reesei* RutC30 genomic DNA, 200 µM dNTPs, 0.4 µM primers, 1X PHUSION® Reaction Buffer (Thermo Fisher Scientific, Inc.), and

2 units of PHUSION® High Fidelity DNA polymerase (Thermo Fisher Scientific, Inc.) in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 25 seconds, 57°C for 25 seconds, and 72°C for 1.5 minutes; and 1 cycle at 72°C for 7 minutes.

5 The FRT-F site was amplified from plasmid pRika147 (WO 2012/120093) using the primers shown below.

Forward primer:

5'-ACCGACTGGGGCTGTTCTGAACGGCCGCGAATTCATCTTGA-3' (SEQ ID NO: 4)

Reverse primer:

10 5'-AGCCTTGTTTTGTCGGGCGCGCCGCTGCTCTCGGCTAGCGAAG-3' (SEQ ID NO: 5)

The PCR was composed of 20 ng of pRika147, 200 µM dNTPs, 0.4 µM primers, 1X PHUSION® Reaction Buffer, and 2 units of PHUSION® High Fidelity DNA polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 25 seconds, 51°C for 25 seconds, and 72°C for 40 seconds; and 1 cycle at 72°C for 7 minutes.

The completed PCRs were submitted to either 2% agarose (FRT-F PCR) or 1% agarose (5' *cbh1* flanking region) gel electrophoresis in TAE buffer where a 0.1 kb band (FRT-F PCR) and 1.5 kb band (5' *cbh1* flanking region), respectively, were excised from the gels and agarose was extracted using a MINELUTE® Gel Extraction Kit (QIAGEN Inc.). Briefly, 3 volumes of Kit-supplied buffer QG were added to the gel slice and dissolved at 50°C for approximately 10 minutes. The dissolved gel slice was applied to a Kit-supplied spin column and centrifuging at 13,000 rpm for 1 minute. The column was washed with 750 µl of Kit-supplied buffer PE and then re-centrifuged. DNA was eluted with 10 µl of Kit-supplied buffer EB.

25 A single fragment was generated from the above individual PCR products by SOE PCR using PHUSION® High Fidelity DNA polymerase and the fragment-specific forward and reverse primers shown below. The region in italics corresponds to sequence homologous to the site of insertion into target vector pJfyS1579-41-11(WO 2011/075677).

Forward primer:

30 5'-*tcacatgggttaaacggcgcgcc*GGTGAAACACCGCCCCCTTC-3' (SEQ ID NO: 6)

Reverse primer:

5'-*agccttggtttgtcgggcgcgcc*GCTGCTCTCGGCTAGCGAAG-3' (SEQ ID NO: 7)

The SOE PCR was composed of 1 µl of each gel-purified PCR product above, 200 µM dNTPs, 1X PHUSION® Reaction Buffer, and 2 units of PHUSION® High Fidelity DNA polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; and 5 cycles each at 95°C for 25 seconds, 51°C for 25 seconds, and 72°C for 1.5 minutes. Following the above cycling parameters,

primers were added to a final concentration of 0.4 μ M and subjected to 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 25 seconds, 51°C for 25 seconds, and 72°C for 40 seconds; and 1 cycle at 72°C for 7 minutes. The completed PCR was submitted to 1% agarose gel electrophoresis in TAE buffer where a 1.5 kb band containing both sequences was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit as above.

To generate more PCR product the above gel purified fragment was re-amplified using the forward and reverse primers listed above. The PCR was composed of 1 μ l of the gel purified fragment, 200 μ M dNTPs, 0.4 μ M of primers, 1X PHUSION® Reaction Buffer, and 2 units of PHUSION® High Fidelity DNA polymerase in a final volume of 50 μ l. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; 30 cycles each at 95°C for 25 seconds, 58°C for 25 seconds, and 72°C for 1.5 minutes; and 1 cycle at 72°C for 7 minutes. The completed PCR was submitted to 1% agarose gel electrophoresis in TAE buffer where the 1.5 kb band was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit as above.

The 1.5 kb fragment was inserted into Asc I-digested pJfyS1579-41-11 (WO 2011/075677) using an IN-FUSION® Advantage PCR Cloning Kit (Clontech Laboratories, Inc.). The reaction was composed of 1X IN-FUSION® Reaction Buffer (Clontech Laboratories, Inc.), 120 ng of pJfyS1579-41-11, 120 ng of the SOE PCR product, and 1 μ l of IN-FUSION® Enzyme (Clontech Laboratories, Inc.) in a 10 μ l reaction volume. The reaction was incubated at 50°C for 15 minutes. Then 40 μ l of TE were added to the reaction and 2 μ l were transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells (Invitrogen Corp.) by addition to a single use tube containing the competent cells and incubating the cells on ice for 5 minutes. The tube was incubated at 42°C for 30 seconds after which 250 μ l of SOC medium were added. The tube was then incubated at 37°C with agitation at 200 rpm for 1 hour and 250 μ l were transferred to a 150 mm 2XYT plus ampicillin plate and incubated overnight at 37°C. *E. coli* transformants were inoculated into 3 ml of LB plus ampicillin medium in 14 ml tubes and incubated overnight at 37°C with agitation at 200 rpm. Plasmid DNA was isolated using a BIOROBOT® 9600 (QIAGEN Inc.). The insert was confirmed by DNA sequencing. One transformant was identified as containing the insert with no PCR errors and the plasmid was designated pJfyS147A.

The FRT-F3 (SEQ ID NO: 8) site was inserted upstream of the 3' *cbh1* flanking region using SOE PCR. First the FRT-F3 site and 3' *cbh1* flanking region were amplified separately and then combined by SOE PCR.

The 3' *cbh1* flanking region was amplified from *T. reesei* RutC30 genomic DNA using the primers shown below.

Forward primer:

5'-GATAACGGAATAGAAGAAAGAG-3' (SEQ ID NO: 9)

Reverse primer:

5'-GCCATATTTAAATCCGTTTAAACAGTCAACACGTCTCCTATGTCT-3' (SEQ ID NO: 10)

The PCR was composed of 150 ng of *T. reesei* RutC30 genomic DNA, 200 µM dNTPs, 0.4 µM primers, 1X PHUSION® Reaction Buffer, and 2 units of PHUSION® High Fidelity DNA polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 25 seconds, 54°C for 25 seconds, and 72°C for 1.5 minutes; and 1 cycle at 72°C for 7 minutes.

The FRT site was amplified from pRika147 using the primers shown below.

Forward primer:

5'-CCTAGTTGGAGTATTCCTGCAGGTCAACTCTCTCCTCTAGGTTGAAGTTCCTATTCCGAGTTC-3' (SEQ ID NO: 11)

Reverse primer:

5'-CTCTTTCTTCTATTCCGTTATCGCATGCACTAGCTAGTTGAAGTTCCTATAC-3' (SEQ ID NO: 12)

The PCR was composed of 20 ng of pRika147, 200 µM dNTPs, 0.4 µM primers, 1X PHUSION® Reaction Buffer, and 2 units of PHUSION® High Fidelity DNA polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 25 seconds, 50°C for 25 seconds, and 72°C for 40 seconds; and 1 cycle at 72°C for 7 minutes.

The completed PCRs were submitted to either 2% agarose (FRT-F3 PCR) or 1% agarose (3' *cbh1* flank) gel electrophoresis in TAE buffer where a 0.1 kb band (FRT-F3 PCR) and 1.5 kb band (3' *cbh1* flanking region), respectively, were excised from the gels and agarose was extracted using a MINELUTE® Gel Extraction Kit as above.

A single fragment was generated from the above individual PCRs by SOE PCR using the primers shown below. The region in italics corresponds to sequence homologous to the desired site of insertion into pJfyS147A (described above).

Forward primer:

5'-CCTAGTTGGAGTATTCCTGCAGGTCAACTCTCTCCTCTAGGTTGAAGTTCCTATTCCGAGTTC-3' (SEQ ID NO: 13)

Reverse primer:

5'-GCCATATTTAAATCCGTTTAAACAGTCAACACGTCTCCTATGTCT-3' (SEQ ID NO: 14)

The PCR was composed of 1 µl of each gel-purified PCR product above, 200 µM dNTPs, 1X PHUSION® Reaction Buffer, and 2 units of PHUSION® High Fidelity DNA polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; and 5 cycles each at 95°C for 25 seconds, 51°C for 25 seconds, and 72°C for 1.5 minutes. Following the above cycling parameters,

primers were added to a final concentration of 0.4 μ M and subjected to 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 25 seconds, 51°C for 25 seconds, and 72°C for 40 seconds; and 1 cycle at 72°C for 7 minutes. The completed PCR was submitted to 1% agarose gel electrophoresis in TAE buffer where a 1.5 kb band containing both sequences was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit as above.

To generate more PCR product the above gel-purified fragment was re-amplified using the forward and reverse primers used in the preceding step. The PCR was composed of 1 μ l of the gel purified 1.5 kb fragment, 200 μ M dNTPs, 0.4 μ M of primers, 1X PHUSION® Reaction Buffer, and 2 units of PHUSION® High Fidelity DNA polymerase in a final volume of 50 μ l. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; 30 cycles each at 95°C for 25 seconds, 58°C for 25 seconds, and 72°C for 1.5 minutes; and 1 cycle at 72°C for 7 minutes. The completed PCR was submitted to 1% agarose gel electrophoresis in TAE buffer where the 1.5 kb band was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit as above.

The 1.5 kb fragment was inserted into *Sbf* I-digested pJfyS147A using an IN-FUSION® Advantage PCR Cloning Kit. The reaction was composed of 1X IN-FUSION® Reaction Buffer, 120 ng of pJfyS147A, 60 ng of the 1.5 kb PCR product, and 1 μ l of IN-FUSION® Enzyme in a 10 μ l reaction volume. The reaction was incubated at 50°C for 15 minutes. Then 40 μ l of TE were added to the reaction and 2 μ l were transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells as described above. Transformants were screened by sequencing. *E. coli* transformants were inoculated into 3 ml of LB plus ampicillin medium in 14 ml tubes and incubated overnight at 37°C with agitation at 200 rpm. Plasmid DNA was isolated using a BIOROBOT® 9600. The insert was confirmed by DNA sequencing. One transformant was identified as containing the insert with no PCR errors and the plasmid was designated pJfyS147 (Figure 1). Plasmid pJfyS147 was used to integrate the FRT-F and FRT-F3 sites at the *cbh1* locus of *T. reesei* 981-O-8.

Example 5: Insertion of FRT-F and FRT-F3 sequences at the *cbh1* locus of *Trichoderma reesei* 981-O-8 (D4)

Protoplast preparation and transformation of *Trichoderma reesei* 981-O-8 were performed according to Example 1. In order to insert the FRT-F and FRT-F3 sequences at the *cbh1* locus of *T. reesei* 981-O-8, the strain was transformed with 6 x 2.5 μ g of *Pme* I-linearized pJfyS147 (Example 4). One hundred and thirty-one transformants were obtained and each one was picked and transferred to a 25 mm PDA plate and incubated for 7 days at 28°C. Transformants were each analyzed by transferring a small amount of spores with a sterile 10 μ l inoculation loop into 25 ml of CIM in a 125 ml polycarbonate shake flask and

incubating at 28°C for 5 days with agitation at 200 rpm. Supernatant from each culture was subjected to SDS-PAGE using a CRITERION® 8-16% SDS-PAGE gel (Bio-Rad Laboratories, Inc.) and PRECISION PLUS® Protein Standards (Bio-Rad Laboratories, Inc.). Since successful targeted integration of the FRT-F/FRT-F3 construct at the *cbh1* locus effectively removes the *cbh1* gene, SDS-PAGE gels were visually analyzed for loss of the CBH1 protein from the proteome. Two transformants, *T. reesei* JfyS147-20 and *T. reesei* JfyS147-74, were identified, which produced no CBH1 protein. *T. reesei* JfyS147-20 and *T. reesei* JfyS147-74 were grown on PDA plates at 28°C for 7 days. Spores were collected from the plates using 0.01% TWEEN® 20 and diluted to 10⁶ spores/ml using a hemocytometer. One ml and 0.1 ml of each spore suspension were spread onto TrMM-G plates supplemented with 1 µM 5-fluorodeoxyuridine (FDU) and incubated at 28°C for 6 days. Eight spore isolates were picked for *T. reesei* JfyS147-20 and 1 spore isolate for *T. reesei* JfyS147-74, which were each transferred to a new 75 mm PDA plate. The plates were incubated at 28°C for 7 days. Genomic DNA was isolated from *T. reesei* JfyS147-20 and *T. reesei* JfyS147-74 as described in Example 1 and analyzed by Southern blotting to insure correct integration of the original FRT integration construct in the primary transformant as well as to insure correct excision of the markers in the resulting FdU-resistant spore progeny.

A probe hybridizing to the 3' flanking region of the *cbh1* gene was generated using a PCR DIG Probe Synthesis Kit and the forward and reverse primers shown below. The PCR was composed of 1X HERCULASE® Reaction Buffer (Stratagene Corp.), 400 nM each primer, 200 µM DIG-labeled dUTP-containing dNTPs, 20 ng of pJfyS139 (WO 2013/028927), and 1.5 units of HERCULASE® DNA polymerase (Stratagene Corp.). The reaction was incubated in a thermocycler programmed for 1 cycle at 95°C for 2 minute; 25 cycles each at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 40 seconds; and 1 cycle at 72°C for 7 minutes.

Forward primer:

5'-AAAAAACAACATCCCGTTCATAAC-3' (SEQ ID NO: 15)

Reverse primer:

5'-AACAAGGTTTACCGGTTTCGAAAAG-3' (SEQ ID NO: 16)

The probe was purified by 1% agarose gel electrophoresis in TAE buffer where a band corresponding to the probe was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

For Southern blot analysis, 2 µg of each genomic DNA was digested with *Nde* I in a 50 µl reaction volume. The digested DNA was subjected to 1% agarose gel electrophoresis in TAE buffer and transferred to a NYTRAN® SuperCharge membrane as described to Example 3 using the probe hybridizing to the 3' flanking region of the *cbh1* gene. Southern

blot analysis identified primary transformant *T. reesei* JfyS147-20 as having correctly integrated the FRT site construct at the *cbh1* locus and the resulting *hpt/tk*- spore isolate JfyS147-20B as having correctly excised the markers leaving the FRT-F and FRT-F3 sites at the *cbh1* locus, with a single copy of the 262 bp repeat DNA used for marker excision, separating the FRT-F and FRT-F3 sites.

Example 6: Construction of FLP/FRT integration plasmid pJfyS150

Expression plasmid pJfyS150 was constructed for integrating a desired gene at the *T. reesei cbh1* locus using the *Saccharomyces cerevisiae* flippase (FLP) and flippase recognition sequences FRT-F and FRT-F3. The FRT-F3 site was first inserted into plasmid pSMai155 (WO 05/074647) using a QUICKCHANGE® II XL Site-Directed Mutagenesis Kit (Agilent Technologies) with the mutagenic insertion primers shown below.

Forward primer:

5'-CGAATTCTGCATTGAAGTTCCTATTCCGAGTTCCTATTCTTCAAATAGTATAGGAACTT
CA
GATATCCATCACACTGGCG-3' (SEQ ID NO: 17)

Reverse primer:

5'-GCCAGTGTGATGGATATCTGAAGTTCCTATACTATTTGAAGAATAGGAACTCGGAATA
GGAACCTTCAATGCAGAATTCGC-3' (SEQ ID NO: 18)

The mutagenic PCR contained 10 ng of pSMai155, 200 µM dNTPs, 125 ng of each primer, 1X QUICKCHANGE® Reaction Buffer, 3 µl of QUIKSOLUTION® reagent (Agilent Technologies), and 2.5 units of *Pfu* Ultra High Fidelity DNA polymerase (Agilent Technologies) in a final volume of 50 µl. The PCR was performed in a thermocycler programmed for 1 cycle at 95°C for 1 minute; 18 cycles each at 95°C for 50 seconds, 60°C for 50 seconds, and 68°C for 40 seconds; and 1 cycle at 68°C for 7 minutes. One µl of Kit-supplied *Dpn* I was added and the reaction was incubated at 37°C for 1 hour. Two µl of the *Dpn* I-treated reaction were added to 45 µl of Kit-supplied XL10-Gold Ultracompetent *E. coli* cells (Agilent Technologies) in a 14 ml tube and incubated on ice for 30 minutes. The tube was incubated at 42°C for 30 seconds after which 0.5 ml of SOC medium was added. The tube was then incubated at 37°C with agitation at 200 rpm for 1 hour after which 250 µl each were plated onto 2 X 150 mm 2XYT plus ampicillin plates and incubated at 37°C overnight. *E. coli* transformants were inoculated into 3 ml of LB plus ampicillin medium in 14 ml tubes and incubated overnight at 37°C with agitation at 200 rpm. Plasmid DNA was isolated using a BIOROBOT® 9600. The insert was confirmed by DNA sequencing. One transformant was identified as containing the desired sequence insertion corresponding to the FRT-F3 site and the plasmid was designated pJfyS148A.

The FRT-F sequence was then inserted into plasmid pJfyS148A using an IN-

FUSION® Advantage PCR Cloning Kit. The FRT-F site was first amplified by PCR from plasmid pRika147 using the primers shown below.

Forward primer:

5'-ATATCCATCACACTGGCGGCCGCTCAACTCTCTCCTCTAGGTTGAAGTTCCTATTCCG
5 AG

TTC-3' (SEQ ID NO: 19)

Reverse primer:

5'-AGGATGCATGCTCGAGCATGCACTAGCTAGTTGAAGTTCCTATAC-3' (SEQ ID NO:
20)

10 The PCR was composed of 20 ng of pRika147, 200 µM dNTPs, 0.4 µM primers, 1X PHUSION® Reaction Buffer, and 2 units of PHUSION® High Fidelity DNA polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 25 seconds, 50°C for 25 seconds, and 72°C for 40 seconds; and 1 cycle at 72°C for 7 minutes. The completed PCR was submitted
15 to 2% agarose gel electrophoresis in TAE buffer where a 0.1 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The 0.1 kb PCR product was inserted into *Sa*I-digested pJfyS148A using an IN-FUSION® Advantage PCR Cloning Kit. The reaction was composed of 1X IN-FUSION® Reaction Buffer, 125 ng of pJfyS147A, 20 ng of FRT-F PCR product, and 1 µl of IN-
20 FUSION® Enzyme in a 10 µl reaction volume. The reaction was incubated at 50°C for 15 minutes. Then 40 µl of TE were added to the reaction and 2 µl were transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Plasmid DNA from *E. coli* transformants was isolated as described in Example 4. The insert was confirmed by DNA sequencing. One transformant was identified as containing the insert with no PCR
25 errors and the plasmid was designated pJfyS148B. Plasmid pJfyS148B was used to insert the *Saccharomyces cerevisiae* flippase (FLP) gene.

A *S. cerevisiae* flippase cassette (WO 2012/120093) was amplified from plasmid pRika147 using the primers shown below.

Forward primer:

30 5'-ATCTACGCGTACTAGTTAATTAAGGCTTTCGTGACCGGGCTTCAAACA-3' (SEQ ID NO: 21)

Reverse primer:

5'-GCGGCCGTTACTAGTGGATCCACTCGGAGTTGTTATACGCTACTCG-3' (SEQ ID NO:
22)

35 The PCR was composed of 20 ng of pRika147, 200 µM dNTPs, 0.4 µM primers, 1X PHUSION® Reaction Buffer, and 2 units of PHUSION® High Fidelity DNA polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle

at 95°C for 2 minutes; 25 cycles each at 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 2 minutes; and 1 cycle at 72°C for 7 minutes. The completed PCR was submitted to 1% agarose gel electrophoresis in TAE buffer where a 2.4 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

5 The 2.4 kb PCR product was inserted into *Bam* HI-digested pJfyS148B using an IN-FUSION® Advantage PCR Cloning Kit. The reaction was composed of 1X IN-FUSION® Reaction Buffer, 150 ng of pJfyS142-A, 150 ng of the 2.4 kb PCR product, and 1 µl of IN-FUSION® Enzyme in a 10 µl reaction volume. The reaction was incubated at 50°C for 15 minutes. Then 40 µl of TE were added to the reaction and 2 µl were transformed into ONE
10 SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Plasmid DNA from *E. coli* transformants was isolated as described in Example 4. The insert was confirmed by DNA sequencing. One transformant was identified as containing the insert with no PCR errors and the plasmid was designated pJfyS148. Plasmid pJfyS148 was used to insert an *Aspergillus fumigatus* beta-glucosidase gene.

15 The *A. fumigatus* beta-glucosidase gene was amplified by PCR from pEJG107 (WO 05/047499) using the primers shown below.

Forward primer:

5'-ACCGCGGACTGCGCACCATGAGATTCGGTTGGCTCGAGG-3' (SEQ ID NO: 23)

Reverse primer:

20 5'-TTCGCCACGGAGCTTACTAGTAGACACGGGGCAGAGGC-3' (SEQ ID NO: 24)

The PCR was composed of 20 ng of pEJG107, 200 µM dNTPs, 0.4 µM primers, 1X PHUSION® Reaction Buffer, and 2 units of PHUSION® High Fidelity DNA polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 25 cycles each at 95°C for 30 seconds, 57°C for 30 seconds, and
25 72°C for 2 minutes; and 1 cycle at 72°C for 7 minutes. The completed PCR was submitted to 1% agarose gel electrophoresis in TAE buffer where a 3 kb band was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The *A. fumigatus* beta-glucosidase gene was inserted into *Nco* I/*Pac* I-linearized pJfyS148 using an IN-FUSION® Advantage PCR Cloning Kit. The reaction was composed
30 of 1X IN-FUSION® Reaction Buffer, 150 ng of *Nco* I/*Pac* I-linearized pJfyS148, 80 ng of the 3 kb PCR product, and 1 µl of IN-FUSION® Enzyme in a 10 µl reaction volume. The reaction was incubated at 50°C for 15 minutes. Then 40 µl of TE were added to the reaction and 2 µl were transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Plasmid DNA from *E. coli* transformants was isolated as described in Example 4.
35 The insert was confirmed by DNA sequencing. One transformant was identified as containing the insert with no PCR errors and the plasmid was designated pJfyS150.

Example 7: Integration of FLP/FRT construct pJfyS150 at the *cbh1* locus of *Trichoderma reesei* strain JfyS147-20B

In order to integrate the FLP/FRT expression construct at the *cbh1* locus using the FLP/FRT system, protoplasts of *Trichoderma reesei* strain JfyS147-20B were generated and transformed according to Example 1 except that xylose was added to a final concentration of 1% to STC. Xylose was included to properly induce transcription of the *Saccharomyces cerevisiae* flippase gene as its expression in pJfyS150 is controlled by the *Aspergillus nidulans xlnA* promoter which is induced by xylose (WO 2012/120093). Protoplasts were transferred to nine 14 ml round-bottom polypropylene tubes and transformed with 2 µg of the *Pme* I-linearized and gel purified pJfyS150 (Example 6). The transformations were divided into 3 parts and spread onto 150 mm PDA plates supplemented with 1 M sucrose and 1% xylose and incubated overnight at 28°C. After approximately 16 hours of incubation 20 ml of PDA medium supplemented with 10 mM uridine, 1% xylose, and 35 µg of hygromycin B per ml were added to each plate and the plates incubated for 6 days at 28°C. Nineteen transformants were obtained and each was transferred to a 25 mm PDA plate and incubated for 7 days at 28°C. A fungal spore PCR method using a modified protocol by Suzuki *et al.*, 2006, *J. Bioscience and Bioengineering* 102: 572-574 was used to screen for transformants bearing site-specific integration.

The spore PCR was accomplished by collecting spores with a sterile 1 µl inoculation loop and transferring them to 25 µl of TE buffer in a 0.6 ml EPPENDORF® tube. Spores were microwaved on high for 1 minute and 1 µl of the microwaved spore solution was immediately added to ADVANTAGE® GC Genomic LA Polymerase Mix (Clontech Laboratories Inc.) containing the following components: 1X Reaction Buffer (Clontech Laboratories Inc.), 200 µM dNTPs, 400 nM each primer (3 primers for each PCR), and 1.25 units of ADVANTAGE® GC Genomic LA Polymerase (Clontech Laboratories Inc.) in a 25 µl volume. The PCR products were amplified using the forward and reverse primers shown below for either 5' recombination or 3' recombination.

5' Recombination:

Forward primer:

5'-TTCCCTTCCTCTAGTGTGAAT-3' (SEQ ID NO: 25)

Reverse No integration:

5'-TCGTCTGAATACTAACATCTTGC-3' (SEQ ID NO: 26)

Reverse Integration:

5'-CACGGACCTCGAACCTTTATAT-3' (SEQ ID NO: 27)

3' Recombination:

Forward primer:

5'-CAGCGAGAGCCTGACCTATTGCATC-3' (SEQ ID NO: 28)

Reverse No integration:

5'-AACAAAGGTTTACCGGTTTCGAAAAG-3' (SEQ ID NO: 29)

Reverse Integration:

5'-GTGGCTGCCGAGGTGTGTATACCA-3' (SEQ ID NO: 30)

5 The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 10 minutes; 35 cycles each at 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute and 40 seconds; 1 cycle at 72°C for 7 minutes; and a 4°C hold. The completed PCRs were analyzed by 1% agarose gel electrophoresis in TAE buffer.

10 The primers were designed such that depending on whether or not the cassette had targeted to the locus two different sized PCR amplicons would be produced. A successful targeted integration at the 5' end produces a 1.8 kb band while an ectopic integration produces a 1 kb band. At the 3' end a successful integration produces a 1.8 kb band while integration elsewhere results in a 0.7 kb band. The results of the spore PCR indicated that three of the nineteen transformants had undergone a successful integration at both 5' and 3' ends corresponding to the FRT-F and FRT-F3 sites, respectively.

Example 8: Construction of FLP integration plasmid pJfyS156 containing the *Saccharomyces cerevisiae* flippase controlled by the *Trichoderma reesei cbh2* promoter

20 In an attempt to increase the frequency of flippase-mediated targeted integration a new construct was constructed in which the promoter driving the *Saccharomyces cerevisiae* flippase gene was replaced with the *Trichoderma reesei cbh2* promoter. To construct the plasmid the *cbh2* gene promoter and flippase gene were PCR amplified using gene-specific primers shown below and inserted into plasmid pJfyS148B (Example 6).

25 Flippase

Forward primer:

5'-CACCTCTGTGTATTGCACCATGCCCCAGTTCGATATCCTCTGCA-3' (SEQ ID NO: 31)

Reverse primer:

5'-AAACTCTAGGATGCATGCAAGTGAGGCTATTGCCTATCAGCTC-3' (SEQ ID NO: 32)

30 *cbh2* gene promoter

Forward primer:

5'-CATCACACTGGCGGCCGCGAATTCTAGGCTAGGTATGC-3' (SEQ ID NO: 33)

Reverse primer:

5'-GGTGCAATACACAGAGGGTG-3' (SEQ ID NO: 34)

35 The PCR was composed of 20 ng of template pRiKa147 for the flippase PCR or 150 ng of *T. reesei* 981-O-8 genomic DNA for the *cbh2* promoter PCR, 200 µM dNTPs, 0.4 µM primers, 1X PHUSION® Reaction Buffer, and 2 units of PHUSION® High Fidelity DNA

polymerase in a final volume of 50 µl. The reactions were performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 2 minutes; and 1 cycle at 72°C for 7 minutes. The completed PCRs were submitted to 1% agarose gel electrophoresis in TAE buffer where 1.2 and 0.6 kb bands, corresponding to the coding region for the *S. cerevisiae* flippase gene and the *niaD* terminator and *cbh2* gene promoter, respectively, were excised from the gels and agarose was extracted using a Nucleospin® Extract II Kit (Macherey Nagel, Bethlehem, PA, USA). Three volumes of Kit-supplied NT buffer were added to the gel slice and the sample was heated at 50°C for 10 minutes. The entire solution was transferred to a Kit-supplied centrifugal column. The column was centrifuged at 13,000 rpm for 1 minute, and washed with Kit-supplied wash buffer NT3 and re-centrifuged. DNA was eluted with 30 µl of Kit-supplied elution buffer NE and centrifuged at 13,000 rpm for 1 minute.

The *cbh2* gene promoter and flippase coding sequence were inserted in a single step into *Xho* I-linearized pJfyS148B using an IN-FUSION® Advantage PCR Cloning Kit. The reaction was composed of 1X IN-FUSION® Reaction Buffer, 180 ng of *Xho* I-linearized pJfyS148B, 100 ng of the 0.6 kb *cbh2* promoter PCR product, 240 ng of the 1.2 kb flippase PCR product, and 1 µl of IN-FUSION® Enzyme in a 10 µl reaction volume. The reaction was incubated for 15 minutes at 37°C and then 15 minutes at 50°C. Then 40 µl of TE were added to the reaction and 2 µl were transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. *E. coli* transformants were inoculated into 3 ml of LB plus ampicillin medium in 14 ml tubes and incubated overnight at 37°C with agitation at 200 rpm. Plasmid DNA was isolated using a BIOROBOT® 9600. The insert was confirmed by DNA sequencing. One transformant was identified as containing the inserts with no PCR errors and the plasmid was designated pJfyS155.

The *A. fumigatus* beta-glucosidase PCR product (Example 6) was inserted into *Nco* I/*Pac* I-digested pJfyS155 using an IN-FUSION® Advantage PCR Cloning Kit. The reaction was composed of 150 ng of *Nco* I/*Pac* I-digested pJfyS155, 100 ng of the *A. fumigatus* beta-glucosidase PCR product (Example 6), 1X IN-FUSION® Advantage Buffer, and 1 µl of IN-FUSION® Enzyme in a 10 µl reaction volume. The reaction was incubated for 15 minutes at 37°C and then 15 minutes at 50°C. Then 40 µl of TE were added to the reaction and 2 µl were transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Plasmid DNA from *E. coli* transformants was isolated as described in Example 4. The insert was confirmed by DNA sequencing. One transformant was identified as containing the inserts with no PCR errors and the plasmid was designated pJfyS156 (Figure 2).

Example 9: Protoplast generation and transformation of *Trichoderma reesei* strain

AgJg115-104-7B1 to delete the *Trichoderma reesei* 42 kDa aspartic protease to create *Trichoderma reesei* AgJg115-118-1H1

Protoplast preparation and transformation of *Trichoderma reesei* strain AgJg115-104-7B1 were performed as described in Example 1.

5 Ninety-six µg of the transforming plasmid pAgJg118 (WO 2011/075677) was digested with *Pme* I and purified by 1% agarose gel electrophoresis in TAE buffer where a DNA band was excised from the gel and extracted using a QIAQUICK® Gel Extraction Kit (QIAGEN Inc.). Briefly 3 volumes of Kit-supplied Buffer QG were added to the gel slice and dissolved at 50°C for approximately 10 minutes. The dissolved gel slice was transferred to a
10 spin column and centrifuged at 13,000 rpm for 1 minute. The column was washed with 750 µl of Kit-supplied Buffer PE and then the centrifugation was repeated. DNA was eluted with 25 µl of Kit-supplied Buffer EB. Approximately 1 µg of the resulting purified DNA fragment was added to 100 µl of the protoplast solution for hygromycin selection transformation as described in Example 1. Seven transformants were sub-cultured onto new PDA plates to
15 generate spores.

The transformants of *T. reesei* strain AgJg115-104-7B1 were screened by Fungal Spore PCR for the presence of the pAgJg118 deletion vector at the 42 kDa aspartic protease locus. A small amount of spores from each transformant was suspended in 20 µl of Dilution buffer (PHIRE® Plant Direct PCR Kit, Thermo Fisher Scientific Inc.). The spore
20 suspensions were used as templates in the PCRs to screen for the aspartic protease gene deletion. Each reaction was composed of 0.5 µl of the spore suspension, 50 pmol of primer 069134 (shown below), 50 pmol of primer 067947 (shown below), 10 µl of 2X PHIRE® Plant PCR Buffer (PHIRE® Plant Direct PCR Kit), and 0.4 µl of PHIRE® Hot Start II DNA Polymerase (PHIRE® Plant Direct PCR Kit) in a 20 µl reaction. The reactions were
25 performed in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 5 seconds, 58°C for 5 seconds, and 72°C for 2 minutes and 20 seconds; 1 cycle at 72°C for 2 minutes; and a 10°C hold. Primer 069134 is located upstream of the 5' flanking region and primer 067947 is located at the beginning of the *E. coli* hygromycin phosphotransferase (*hpt*) gene coding region. If the deletion vector integrates into the
30 aspartic protease locus, the amplified PCR fragment will be 2.4 kb in length. One transformant designated *T. reesei* AgJg115-118-1 was identified as having the aspartic protease gene deleted.

Primer 069134 (forward):

5'-CGCAATCTATCGAATAGCAG-3' (SEQ ID NO: 35)

35 Primer 067947 (reverse):

5'-CTACATCGAAGCTGAAAGCACGAGA-3' (SEQ ID NO: 36)

The deletion construct pAgJg118 contains the *E. coli* hygromycin

phosphotransferase (*hpt*) gene and the Herpes simplex virus thymidine kinase (*tk*) gene flanked by direct repeats. The direct repeats were inserted to facilitate the curing out of the *hpt* and *tk* selectable markers and generate a clean deletion of the 42 kDa aspartic protease.

Spores from *T. reesei* AgJg115-118-1 were spread onto *Trichoderma* Minimal medium plates containing 1 μ M 5-fluoro-2'-deoxyuridine (FdU) and incubated at 28°C. Nine isolates were sub-cultured onto PDA plates and incubated at 28°C. The isolates were then screened for the absence of the *hpt* and *tk* markers by Fungal Spore PCR in a similar manner described above. The PCR screen was composed of 0.5 μ l of the spore suspension, 50 pmol of primer 069134, 50 pmol of primer 1200593, 10 μ l of 2X PHIRE® Plant PCR Buffer, and 0.4 μ l of PHIRE® Hot Start II DNA Polymerase in a 20 μ l reaction. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 5 seconds, 58°C for 5 seconds, and 72°C for 1 minute and 45 seconds; 1 cycle at 72°C for 1 minute; and a 10°C hold. Primer 069134 is located upstream of the 5' flanking region and primer 067947 is located at the downstream of the 3' flanking region. If the aspartic protease coding sequence is deleted and the *hpt* and *tk* markers are looped out, the amplified PCR fragment will be 3.6 kb in length.

Genomic DNA of the *T. reesei* AgJg115-118-1 isolates was prepared as described in Example 2 and analyzed by Southern blot analysis as described in Example 3 to confirm the deletion of the 42 kDa aspartic protease.

For Southern blot analysis, 2 μ g of each genomic DNA was digested with 10 units of *Nco* I in a 30 μ l reaction volume and subjected to 0.7% agarose gel electrophoresis in TAE buffer and transferred to a NYTRAN® SuperCharge membrane as described in Example 3.

The membrane was hybridized with a 500 bp digoxigenin-labeled *T. reesei* 42 kDa aspartic protease probe, which was synthesized by incorporation of digoxigenin-11-dUTP by PCR using the primers shown below.

Primer 069860 (sense):

5'-CTTCTATCTTGGGATGCTTCACGATACGTGA-3' (SEQ ID NO: 37)

Primer 069861 (antisense):

5'-CGCGCCCTTGAATATCGGAGAAGGT-3' (SEQ ID NO: 38)

The PCR was composed of 5 μ l of 10X *Taq* Buffer (New England Biolabs, Inc.), 2.5 μ l of PCR DIG Labeling Mix (Roche Applied Science Corp.), 5 ng of pAgJg118, 10 pmol of each primer, 2.5 μ l of 10 mM dNTPs, 5 units of *Taq* DNA polymerase (New England Biolabs, Inc.), and 36.5 μ l of water. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 40 seconds; 1 cycle at 72°C for 15 minutes; and a 4°C hold. The probe was purified by 1% agarose gel electrophoresis in TAE buffer, excised from the gel, and extracted using a QIAQUICK® Gel Extraction Kit as above.

Southern blot analysis identified primary transformant *T. reesei* AgJg115-118-1H1 as containing the replacement and being void of the *hpt/tk* markers.

Example 10: Protoplast generation and transformation of *Trichoderma reesei* strain AgJg115-118-1H1 to insert the FRT-F and FRT-F3 sequences at the *cbh1* locus

Protoplast preparation and transformation of *Trichoderma reesei* strain AgJg115-118-1H1 were performed as described in Example 1.

Eighty-five µg of the transforming plasmid pJfyS147 (Example 4) was digested with *Pme* I. The digestion reaction was purified by 1% agarose gel electrophoresis in TAE buffer where a DNA band of approximately 8.1 kb was excised from the gel, and extracted using a QIAQUICK® Gel Extraction Kit (Example 9). Approximately 3 µg of the resulting purified DNA fragment was used for each transformation according to the procedure described in Example 1. Seven transformants were sub-cultured onto PDA plates to generate spores.

The transformants of *T. reesei* strain AgJg115-104-7B1 were screened by Fungal Spore PCR according to Example 9 for the presence of the pJfyS147 FRT site integration vector at the *cbh1* locus, thereby deleting the promoter region and coding sequence of *cbh1*. The spore suspensions were used as templates in the PCRs to screen for the *cbh1* deletion. Each reaction was composed of 1 µl of spore suspension, 25 pmol of primer 1205412, 25 pmol of primer 1204415, 25 pmol of primer 1201430, 10 µl of 2X PHIRE® Plant PCR Buffer, and 0.4 µl of PHIRE® Hot Start II DNA Polymerase in a 20 µl reaction. The reactions were performed in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 5 seconds, 55°C for 5 seconds, and 72°C for 1 minute and 20 seconds; 1 cycle at 72°C for 1 minute; and a 10°C hold. Primer 1205412 is located within the *cbh1* coding sequence, primer 12054415 is located upstream of the *cbh1* 5' flanking region, and primer 1201430 is located at the beginning of the *E. coli* hygromycin phosphotransferase (*hpt*) gene coding region. If the integration vector integrates at the *cbh1* locus, the amplified PCR fragment will be 2.7 kb in length. But if integration of the FRT sites did not occur and the *cbh1* locus is intact, a 3.8 kb fragment will be produced. *T. reesei* AgJg-FRT1-2 was identified as a strain in which the FRT sites had been successfully integrated at the *cbh1* locus of *T. reesei* AgJg115-118-1H1.

Reverse primer 1205412:

5'-ACTGAGTCAGGCCGCCCTTGTCTGA-3' (SEQ ID NO: 39)

Forward primer 1204415:

5'-GTACAAACAACCTACCTGGTG-3' (SEQ ID NO: 40)

Reverse primer 1201430:

5'-GTTTCAGGCAGGTCTTGCAACG-3' (SEQ ID NO: 41)

The integration construct pJfyS147 contains the *E. coli* hygromycin

phosphotransferase (*hpt*) gene and the Herpes simplex virus thymidine kinase (*tk*) gene flanked by direct repeats. The direct repeats were inserted to facilitate the curing out of the *hpt* and *tk* selectable markers to generate a clean integration of the FRT sites.

Spores from *T. reesei* AgJg-FRT1-2 were spread onto *Trichoderma* Minimal medium plates containing 1 μ M 5-fluoro-2'-deoxyuridine (FdU) and incubated at 28°C. Nine isolates were sub-cultured onto PDA plates and incubated at 28°C for 6 days. The isolates were then screened for the absence of the *hpt* and *tk* markers by Fungal Spore PCR according to Example 9. Each reaction was composed of 1 μ l of spore suspension, 25 pmol of primer 1205412, 25 pmol of primer 1205537, 10 μ l of 2X PHIRE® Plant PCR Buffer, 0.4 μ l of PHIRE® Hot Start II DNA Polymerase in a 20 μ l reaction. The reactions were performed in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 5 seconds, 65°C for 5 seconds, and 72°C for 2 minute and 50 seconds; 1 cycle at 72°C for 1 minute; and a 10°C hold. If the *cbh1* promoter region and coding sequence are deleted, and the *hpt* and *tk* markers are looped out, the amplified PCR fragment will be 3.6 kb in length.

Genomic DNA was prepared according to Example 2 and analyzed by Southern blot analysis according to Example 3 to confirm replacement of the *cbh1* promoter region and coding sequence with the FRT sites.

For Southern blot analysis, 2 μ g of each genomic DNA was digested with 20 units of *Nde* I in a 20 μ l reaction volume. The digested DNA was subjected to 0.7% agarose gel electrophoresis in TAE buffer as described in Example 3.

The membrane was hybridized with a 500 bp digoxigenin-labeled *T. reesei* *cbh1* probe (SEQ ID NO: 42), which was synthesized by incorporation of digoxigenin-11-dUTP by PCR.

Southern blot analysis identified transformant *T. reesei* AgJg-FRT1-2B1A as a strain in which the *hpt/tk* markers were deleted and the *cbh1* coding sequence was deleted and replaced with FRT sites.

Example 11: Construction of plasmid pAgJg137 to insert FRT-F and FRT-F3 sites at the *cbh2* locus of *Trichoderma reesei*

A FRT site integration plasmid pAgJg137 was constructed so that the *T. reesei* *cbh2* gene and 1 kb of the upstream region thereof is deleted when the fragment integrates incorporating the FRT-F site (SEQ ID NO: 1) and FRT-F3 site (SEQ ID NO: 8).

The promoter region and a gene of interest could then be introduced with a FLP/FRT expression construct. To construct the FRT site integration plasmid the FRT-F site was inserted downstream of the 5' *cbh2* flanking region by SOE PCR. First the FRT-F site and 5' *cbh2* flanking region were amplified separately and then combined by overlapping PCR.

The 5' *cbh2* flanking region was amplified from *T. reesei* RutC30 genomic DNA using

the primers shown below. *T. reesei* RutC30 genomic DNA was prepared as described in Example 2. Bold letters represent sequence from the *cbh2* locus and the remaining sequence is homologous to the cloning sites of pJfyS1579-49-11 (WO 2011/075677).

Forward primer:

5 5'-TCACATGGTTTAAACGGCGCGCCACACTTACTCTTCTACACAG-3' (SEQ ID NO: 43)

Reverse primer:

5'-TTCGAACAGCCCCAGTCGGT-3' (SEQ ID NO: 44)

The PCR was composed of 180 ng of *T. reesei* RutC30 genomic DNA, 1 µl of 10 mM dNTPs, 50 pmol of each forward and reverse primer, 1X PHUSION® GC Buffer (Thermo Scientific), and 2 units of PHUSION® Hot Start DNA polymerase (Thermo Fisher Scientific, Inc.) in a final volume of 50 µl. The PCR was performed in a thermocycler programmed for 1 cycle at 98°C for 30 seconds; 30 cycles each at 98°C for 10 seconds, 55°C for 10 seconds, and 72°C for 1.5 minutes; and 1 cycle at 72°C for 10 minutes. The PCR products were separated by 1% agarose gel electrophoresis in TAE buffer where a 1.5 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The FRT-F site was amplified from plasmid pJfyS147 using the primers shown below. The sequence in italics denotes sequence that is homologous to the vector pJfyS1579-49-11. The sequence in bold is sequence homologous to the *cbh2* locus.

20 Forward primer:

5'-GGAAATACAGGATAGACACTCGGCCGCGAATTCATCTTGA-3' (SEQ ID NO: 45)

Reverse primer:

5'-AGCCTTGTTTTGTCGGGCGCGCCGCTGCTCTCGGCTAGCGAAG-3' (SEQ ID NO: 46)

The PCR was composed of 84 ng of pJfyS147 DNA, 1 µl of 10 mM dNTPs, 50 pmol of each forward and reverse primer, 1X PHUSION® GC Buffer, and 2 units of PHUSION® Hot Start DNA polymerase in a final volume of 50 µl. The PCR was performed in a thermocycler programmed for 1 cycle at 98°C for 30 seconds; 30 cycles each at 98°C for 10 seconds, 68°C for 10 seconds, and 72°C for 30 seconds; and 1 cycle at 72°C for 10 minutes. The PCR products were separated by 3% agarose gel electrophoresis in TAE buffer where a 0.1 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

A single fragment was generated from the above individual PCR products by SOE PCR using the primers shown below. The region in italics corresponds to sequence homologous to the site of insertion into vector pJfyS1579-41-11(WO 2011/075677).

35 Forward primer:

5'-TCACATGGTTTAAACGGCGCGCCACACTTACTCTTCTACACAG-3' (SEQ ID NO: 47)

Reverse primer:

5'-AGCCTTGTGTTTTGTCGGGCGCGCCGCTGCTCTCGGCTAGCGAAG-3' (SEQ ID NO: 48)

The PCR was composed of 1 µl of each gel-purified PCR product above, 1 µl of 10 mM dNTPs, 50 pmol of each forward and reverse primer, 1X PHUSION® GC Buffer, and 2 units of PHUSION® Hot Start DNA polymerase in a final volume of 50 µl. The PCR was performed in a thermocycler programmed for 1 cycle at 98°C for 30 seconds; 30 cycles each at 98°C for 10 seconds, 55°C for 10 seconds, and 72°C for 1 minute; and 1 cycle at 72°C for 10 minutes. The PCR products were separated by 1% agarose gel electrophoresis in TAE buffer where a 1.5 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The 1.5 kb fragment was inserted into Asc I-digested pJfyS1579-41-11 (WO 2011/075677) using an IN-FUSION® Advantage PCR Cloning Kit. The reaction was composed of 1X IN-FUSION® Reaction Buffer, 190 ng of pJfyS1579-41-11, 94 ng of the 1.5 kb fragment, and 2 µl of IN-FUSION® HD Premix Enzyme (Clontech Laboratories, Inc.) in a 10 µl reaction volume. The reaction was incubated at 50°C for 15 minutes. Then 2.5 µl of the reaction was transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Plasmid DNA was isolated using a Mini-prep Kit (QIAGEN Inc.). The plasmid was confirmed by DNA sequencing. The resulting plasmid was designated pAgJg137A.

The FRT-F3 site was inserted upstream of the 3' *cbh2* flanking region using SOE PCR. First the FRT-F3 site and 3' *cbh2* flanking region were amplified separately and then combined by SOE PCR.

The 3' *cbh2* flanking region was amplified from *T. reesei* RutC30 genomic DNA using the primers shown below. Bold letters represent sequence from the *cbh2* locus and the remaining sequence is homologous to the cloning sites of pJfyS1579-49-11

Forward primer:

5'-**CACAATGTCGAGTGTCTATT**-3' (SEQ ID NO: 49)

Reverse primer:

5'-GCCATATTTAAATCCGTTTAAACGTTTATAAAATGTTCTGCC-3' (SEQ ID NO: 50)

The PCR was composed of 180 ng of *T. reesei* RutC30 genomic DNA, 1 µl of 10 mM dNTPs, 50 pmol of each forward and reverse primer, 1X PHUSION® GC Buffer, and 2 units of PHUSION® Hot Start DNA polymerase in a final volume of 50 µl. The PCR was performed in a thermocycler programmed for 1 cycle at 98°C for 30 seconds; 30 cycles each at 98°C for 10 seconds, 55°C for 10 seconds, and 72°C for 1.5 minutes; and 1 cycle at 72°C for 10 minutes. The PCR products were separated by 1% agarose gel electrophoresis in TAE buffer where a 1.5 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The FRT-F3 site was amplified from pJfyS147 using the primers shown below. The

sequence in italics denotes sequence that is homologous to the vector pJfyS1579-49-11. The sequence in bold is sequence homologous to the *cbh2* locus.

Forward primer:

5'-*cctagttggagtattcctgcagg*TCAACTCTCTCCTCTAGGTTGAAGTTCCTATTCCGAGTTC-3'

5 (SEQ ID NO: 51)

Reverse primer:

5'-**aatagacactc****gacattgtg**GCATGCACTAGCTAGTTGAAGTTCCTATAC-3' (SEQ ID NO: 52)

The PCR was composed of 84 ng of pJfyS147, 1 µl of 10 mM dNTPs, 50 pmol of each forward and reverse primer, 1X PHUSION® GC Buffer, and 2 units of PHUSION® Hot Start DNA polymerase in a final volume of 50 µl. The PCR was performed in a thermocycler programmed for 1 cycle at 98°C for 30 seconds; 30 cycles each at 98°C for 10 seconds, 68°C for 10 seconds, and 72°C for 30 seconds; and 1 cycle at 72°C for 10 minutes. The PCR products were separated by 3% agarose gel electrophoresis in TAE buffer where a 0.1 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

A single fragment was generated from the above individual PCR products by SOE PCR using the primers shown below. The region in italics corresponds to sequence homologous to the desired site of insertion into pAgJg137A (described above).

Forward primer:

20 5'-*cctagttggagtattcctgcagg*TCAACTCTCTCCTCTAGGTTGAAGTTCCTATTCCGAGTTC-3' (SEQ ID NO: 53)

Reverse primer:

5'-*gccatatttaa***atccgtttaa**cGTTTATAAAATGTTCTGCC-3' (SEQ ID NO: 54)

The PCR was composed of 1 µl of the *cbh2* 3' flanking PCR product, 3 µl of the FRT-F3 PCR product, 1 µl of 10 mM dNTPs, 50 pmol of each forward and reverse primer, 1X PHUSION® GC Buffer, and 2 units of PHUSION® Hot Start DNA polymerase in a final volume of 50 µl. The PCR was performed in a thermocycler programmed for 1 cycle at 98°C for 30 seconds; 30 cycles each at 98°C for 10 seconds, 55°C for 10 seconds, and 72°C for 1 minute; and 1 cycle at 72°C for 10 minutes. The PCR products were separated by 1% agarose gel electrophoresis in TAE buffer where a 1.5 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The 1.5 kb fragment was inserted into *Sbf* I-digested pAgJg137A using an IN-FUSION® Advantage PCR Cloning Kit. The reaction was composed of 1X IN-FUSION® Reaction Buffer, 242 ng of the *Sbf* I-digested pAgJg137A, 140 ng of the 1.5 kb fragment, and 2 µl of IN-FUSION® HD Premix Enzyme in a 10 µl reaction volume. The reaction was incubated at 50°C for 15 minutes. Then 3.5 µl of the reaction were transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. One

transformant was identified as containing the insert with no PCR errors and the plasmid was designated pAgJg137. Plasmid pAgJg137 was used to integrate the FRT-F and FRT-F3 sites at the *cbh2* locus of *T. reesei*.

5 Example 12: Protoplast generation and transformation of *Trichoderma reesei* strain AgJg-FRT1-2B1A to insert the FRT-F and FRT-F3 sequences at the *cbh2* locus

Protoplast preparation and transformation of *T. reesei* AgJg-FRT1-2B1A were performed according to Example 1.

10 A total of 140 µg of the transforming plasmid pAgJg137 (Example 11) was digested with *Pme* I. The digestion reaction was purified by 1% agarose gel electrophoresis in TAE buffer where a DNA band was excised from the gel, and extracted using a MINELUTE® Gel Extraction Kit (Example 4). Approximately 3 µg of the resulting purified DNA fragment was added to 100 µl of the protoplast solution for hygromycin selection transformation as described in Example 1. Seven transformants were sub-cultured onto PDA plates to
15 generate spores.

The transformants of *Trichoderma reesei* AgJg-FRT1-2B1 were screened by Fungal Spore PCR according to Example 9 for the presence of the pAgJg137 FRT site integration vector at the *cbh2* locus, thereby deleting the promoter region and coding sequence of *cbh2*. Each spore suspension was used as a template in a PCR to screen for the *cbh2* deletion.
20 Each reaction was composed of 1 µl of the spore suspension, 25 pmol of primer 1201430, 25 pmol of primer 1206098, 25 pmol of primer 1206099, 10 µl of 2X PHIRE® Plant PCR Buffer, and 0.4 µl of PHIRE® Hot Start II DNA Polymerase in a 20 µl reaction. The reactions were performed in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minute and 10 seconds; 1
25 cycle at 72°C for 1 minute; and a 10°C hold. Primer 1206099 is located within the *cbh2* coding sequence, primer 1206098 is located upstream of the *cbh2* 5' flanking region, and primer 1201430 is located at the beginning of the *E. coli* hygromycin phosphotransferase (*hpt*) gene coding region. If the integration vector integrates at the *cbh2* locus thereby deleting the coding sequence, the amplified PCR fragment produced will be 2.6 kb in length.
30 But if integration of the FRT sites does not occur and the *cbh2* locus is intact, a 3.5 kb fragment will be produced. *T. reesei* AgJg-FRT2-4 was identified with the FRT sites successfully integrated at the *cbh2* locus of *T. reesei* AgJg-FRT1-2B1A.

Reverse primer (1201430):

5'-GTTTCAGGCAGGTCTTGCAACG-3' (SEQ ID NO: 55)

35 Forward primer (1206098):

5'-GCTGATCGAGAAGATTAGCATG-3' (SEQ ID NO: 56)

Reverse primer (1206099):

5'-GATCAGTGATGAAGAAGGCG-3' (SEQ ID NO: 57)

The integration construct pAgJg137 contains the *E. coli* hygromycin phosphotransferase (*hpt*) gene and the Herpes simplex virus thymidine kinase (*tk*) gene flanked by direct repeats. The direct repeats were inserted to facilitate the curing out of the *hpt* and *tk* selectable markers to generate clean integration of the FRT sites.

Spores from *T. reesei* AgJg-FRT2-4 were spread onto *Trichoderma* Minimal medium plates containing 1 μ M 5-fluoro-2'-deoxyuridine (FdU) and incubated at 28°C for 5 days. Seventeen isolates were sub-cultured onto PDA plates and incubated at 28°C for 7 days. The isolates were then screened for the absence of the *hpt* and *tk* markers by Fungal Spore PCR according to Example 9. The PCR was composed of 1 μ l of each spore suspension, 25 pmol of primer 1205457 shown below, 25 pmol of primer 1206335 shown below, 10 μ l of 2X PHIRE® Plant PCR Buffer, and 0.4 μ l of PHIRE® Hot Start II DNA Polymerase in a 20 μ l reaction. The reactions were performed in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 10 seconds, 55°C for 10 seconds, and 72°C for 2 minute and 15 seconds; 1 cycle at 72°C for 1 minute; and a 10°C hold. If the *cbh2* promoter region and coding sequence is deleted, and the *hpt* and *tk* markers are looped out, the amplified PCR fragment will be 2 kb in length.

Forward primer (1205457):

5'-CTCAGGCCATCGTAGGAAAT-3' (SEQ ID NO: 58)

Reverse primer (1206335):

5'-CTAGGTAGGTAGGTAGTATA-3' (SEQ ID NO: 59)

Genomic DNA of the isolates was prepared according to Example 2 and analyzed by Southern blot analysis according to Example 3 to confirm the replacement of *cbh1* with FRT sites.

For Southern blot analysis, 2 μ g of each genomic DNA was digested with 20 units of *Hind* III and 20 units of *Bam* HI in a 40 μ l reaction volume. The digested DNA was subjected to 0.7% agarose gel electrophoresis in TAE buffer and transferred to a NYTRAN® SuperCharge membrane according to Example 3.

The membrane was hybridized with a 500 bp digoxigenin-labeled *T. reesei cbh2* probe, which was synthesized by incorporation of digoxigenin-11-dUTP by PCR using the primers shown below.

Primer 1206366 (sense):

5'-CCAGTAACAACCTTTGCTTGGCC-3' (SEQ ID NO: 60)

Primer 1206367 (antisense):

5'-CACCATGTTACTTTACCCAAATACA-3' (SEQ ID NO: 61)

The PCR was composed of 10 μ l of 5X HF Buffer, 5 μ l of 10X PCR DIG Labeling Mix, 700 ng of pAgJg137, 50 pmol of primer 1206366, 10 pmol of primer 1206367, 1 μ l of 10

mM dNTPs, 1 unit of PHUSION® Hot Start II DNA Polymerase (Thermo Fisher Scientific, Inc.), and 31 µl of water. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 30 seconds; 30 cycles each at 98°C for 10 seconds, 55°C for 10 seconds, and 72°C for 30 seconds; 1 cycle at 72°C for 10 minutes; and a 4°C hold. The probe was
 5 purified by 1% agarose gel electrophoresis in TAE buffer, excised from the gel, and extracted using a QIAQUICK® Gel Extraction Kit.

Southern blot analysis identified transformant *T. reesei* AgJg-FRT2-4B17 as a strain in which the *hpt/tk* markers were deleted and the *cbh2* coding sequence was deleted and replaced with FRT sites.

Example 13: Construction of plasmid pAgJg136

In order to construct expression plasmid pAgJg136 containing the *amdS* gene as a selectable marker, the *amdS* gene was PCR amplified from pMJ09 (U.S. Patent 8,318,458) using the primers shown below.

15 Forward primer:

5'-CTTGGTACCGAGCTCTGGAAACGCAACCCTGAAGGGA-3' (SEQ ID NO: 62)

Reverse primer:

5'-CAGAATTCGCCCTTGTCTACGCCAGGACCGAGCAAGC-3' (SEQ ID NO: 63)

The PCR was composed of 10 ng of pMJ09, 200 µM dNTPs, 0.4 µM primers, 1X
 20 PHUSION® Reaction Buffer, and 2 units of PHUSION® High Fidelity DNA polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 25 seconds, 57°C for 25 seconds, and 72°C for 1.5 minutes; and 1 cycle at 72°C for 7 minutes.

The completed PCR and *Bam* HI/*Afe* I-digested pJfyS148B (Example 6) were
 25 submitted to 1% agarose gel electrophoresis in TAE buffer where a 4.1 kb band corresponding to the digested pJfyS148B and a 2.7 kb band corresponding to the *amdS* gene were both excised from the gels and agarose was extracted using a Nucleospin® Extract II Kit (Example 8).

The *amdS* gene was inserted into the digested pJfyS148B using an IN-FUSION® HD
 30 Cloning Kit (Clontech Laboratories, Inc.). The reaction was composed of 1X IN-FUSION® HD Premix, 120 ng of pJfyS148, and 150 ng of the *amdS* PCR product in a 10 µl reaction buffer. The reaction was incubated at 50°C for 15 minutes. Then 40 µl of TE were added to the reaction and 2 µl were transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Plasmid DNA was isolated from the transformants
 35 according to Example 4. The insert was confirmed by DNA sequencing. One transformant was identified as containing the inserts with no PCR errors and the plasmid was designated pJfyS164. Plasmid pJfyS164 was used to insert the *S. cerevisiae* flippase coding sequence

under transcriptional control of the *T. reesei cbh2* promoter.

The *cbh2* promoter and *S. cerevisiae* flippase coding sequence with terminator were amplified using the forward and reverse primers shown below.

cbh2 promoter Forward primer:

5 5'-CATCACACTGGCGGCCGCGAATTCTAGGCTAGGTATGC-3' (SEQ ID NO: 64)

Reverse primer:

5'-GGTGCAATACACAGAGGGTG-3' (SEQ ID NO: 65)

Flippase

Forward primer:

10 5'-CACCTCTGTGTATTGCACCATGCCCCAGTTCGATATCCTCTGCA-3' (SEQ ID NO: 66)

Reverse primer:

5'-AAACTCTAGGATGCATGCAAGTGAGGCTATTGCCTATCAGCTC-3' (SEQ ID NO: 67)

The PCR was composed of 150 ng *T. reesei* RutC30 genomic DNA for the *cbh2* promoter PCR or 10 ng of pRika147 for the flippase PCR, 200 µM dNTPs, 0.4 µM primers, 15 1X PHUSION® Reaction Buffer, and 2 units of PHUSION® High Fidelity DNA polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 30 seconds, 53°C (59°C for flippase PCR) for 30 seconds, and 72°C for 2 minutes; and 1 cycle at 72°C for 7 minutes.

The completed PCRs were submitted to 1% agarose gel electrophoresis in TAE 20 buffer where a 0.6 kb band corresponding to the *cbh2* promoter and a 2.0 kb band corresponding to the flippase gene were excised from the gels and agarose was extracted using a Nucleospin® Extract II Kit (Example 8).

The *cbh2* promoter and flippase coding sequence were inserted into *Xho* I- digested pJfyS164 using an IN-FUSION® HD Cloning Kit. The reaction was composed of 1X IN- 25 FUSION® HD Premix, 120 ng of the digested pJfyS164, 100 ng of the flippase PCR product, and 50 ng of the *cbh2* promoter PCR product in a 10 µl reaction buffer. The reaction was incubated at 50°C for 15 minutes. Then 40 µl of TE were added to the reaction and 2 µl were transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Plasmid DNA was isolated from the transformants according to Example 4. The 30 insert was confirmed by DNA sequencing. One transformant was identified as containing the inserts with no PCR errors and the plasmid was designated pJfyS165. Plasmid pJfyS165 was used to insert an *A. fumigatus* beta-glucosidase 4M variant coding sequence.

Two synthetic oligonucleotide primers shown below were designed to amplify by PCR the *A. fumigatus* beta-glucosidase variant gene from plasmid pDFng133-3 (WO 35 2013/028912) and introduce flanking regions for insertion into expression vector pJfyS165. Bold letters represent coding sequence and the remaining sequence is homologous to insertion sites of pJfyS165.

Forward primer 1205483:

5'-CGCGGACTGCGCACCATGAGATTCGGTTGGCTCGAG-3' (SEQ ID NO: 68)

Reverse primer 1205484:

5'-TCGCCACGGAGCTTACTAGTAGACACGGGGCAGAGGCG-3' (SEQ ID NO: 69)

5 The PCR was composed of 200 ng of plasmid pDFng133-3, 10 µl of 10 mM dNTPs, 50 pmol of primer 1205483, 50 pmol of primer 1205484, 1X PHUSION® HF buffer (Thermo Fisher Scientific, Inc.), and 2 units of PHUSION® Hot Start DNA polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 30 seconds; 30 cycles each at 98°C for 10 seconds, 62°C for 10 seconds, and 72°C for 1.5 minutes; and 1 cycle at 72°C for 10 minutes. The PCR products were separated by 1% agarose gel electrophoresis in TAE buffer where a 3 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

15 The 3 kb fragment was then cloned into pJfyS165 using an IN-FUSION™ HD Cloning Kit (Clontech Laboratories, Inc.). The vector was digested with *Nco* I and *Pac* I and isolated by 1% agarose gel electrophoresis in TAE buffer where a 9.1 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4). The 3 kb gene fragment of the *A. fumigatus* beta-glucosidase variant coding sequence and the digested vector were ligated together in a reaction resulting in expression plasmid pAgJg136 composed of the *A. fumigatus* beta-glucosidase variant coding sequence under transcriptional control of the *T. reesei cbh1* promoter, FRT recognition sites for efficient targeting, and *S. cerevisiae* flippase coding sequence under transcriptional control of the *T. reesei cbh2* promoter. The ligation reaction (10 µl) was composed of 1X IN-FUSION™ HD enzyme mix, 151 ng of pJfyS165 digested with *Nco* I and *Pac* I, and 201 ng of the *A. fumigatus* beta-glucosidase variant purified PCR product. The reaction was incubated at 50°C for 15 minutes. A 2.5 µl volume of the cloning reaction was transformed into ONE SHOT® TOP10 competent cells according to Example 4. Plasmid DNA was isolated using a Mini-prep Kit (QIAGEN Inc.). The insert was confirmed by DNA sequencing. The resulting plasmid was designated pAgJg136.

30 **Example 14: Construction of plasmid pDM313**

A 0.38 kb PCR fragment containing a portion of the *hpt* marker, the FRT-F3 site, and a portion of the *T. reesei gpdA* promoter was amplified from pJfyS156 (Example 8) using the primers shown below.

Forward primer:

35 5'-CGTGTTCCTTCCCATTCGCATGCGACCTCGTGGTCATTGAC-3' (SEQ ID NO: 70)

Reverse primer:

5'-GCTTTGACGTTACATTGACGTACTTATAAGCGGCCCGCCAGTGTGATGGA-3' (SEQ ID

NO: 71)

The PCR was composed of 50 picomoles of each of the primers, 100 ng of pJfyS156 DNA, 1X PHUSION™ High-Fidelity Hot Start DNA Polymerase buffer (Thermo Fisher Scientific, Inc.), 1 µl of a 10 mM blend of dNTPs, and 1 unit of PHUSION™ High-Fidelity Hot Start DNA Polymerase (Thermo Fisher Scientific, Inc.) in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; 34 cycles each at 98°C for 15 seconds, 59°C for 30 seconds, and 72°C for 1 minute; and 1 cycle at 72°C for 10 minutes.

A 1.0 kb PCR fragment containing the *T. reesei gpdA* promoter was amplified from *T. reesei* RutC30 genomic DNA using the primers shown below.

Forward primer:

5'-TCCATCACACTGGCGGCCGCTTATAAGTACGTCAATGTAACGTCAAAGC-3' (SEQ ID NO: 72)

Reverse primer:

5'-TGCAGAGGATATCGAACTGGGGCATTGTTGTATCTGCGAATTGAGCTTG-3' (SEQ ID NO: 73)

The PCR was composed of 50 picomoles of each of the primers, 100 ng of *T. reesei* RutC30 genomic DNA, 1X PHUSION™ High-Fidelity Hot Start DNA Polymerase buffer, 1 µl of a 10 mM blend of dNTPs, and 1 unit of PHUSION™ High-Fidelity Hot Start DNA Polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; 34 cycles each at 98°C for 15 seconds, 59°C for 30 seconds, and 72°C for 1 minute; and 1 cycle at 72°C for 10 minutes.

A 1.8 kb PCR fragment containing the coding region for the *S. cerevisiae* flippase gene and the *niaD* terminator was amplified from plasmid pJfyS156 (Example 8) using the primers shown below.

Forward primer:

5'-CAAGCTCAATTCGCAGATACAAAATGCCCCAGTTCGATATCCTCTGCA-3' (SEQ ID NO: 74)

Reverse primer:

5'-GCTGTTTAACTCTAGGATGCATGCAAGTGAGGCTATTGCC-3' (SEQ ID NO: 75)

The PCR was composed of 50 picomoles of each of the primers, 100 ng of pJfyS156 DNA, 1X PHUSION™ High-Fidelity Hot Start DNA Polymerase buffer, 1 µl of a 10 mM blend of dNTPs, and 1 unit of PHUSION™ High-Fidelity Hot Start DNA Polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; 34 cycles each at 98°C for 15 seconds, 59°C for 30 seconds, and 72°C for 1 minute; and 1 cycle at 72°C for 10 minutes.

The three completed PCRs described above were analysed by 0.8% agarose gel

electrophoresis in TAE buffer where fragments of 0.38 kb, 1.0 kb, and 1.8 kb were confirmed. The PCR fragments in the original reactions were used as template for a SOE PCR described below.

The 0.38 kb and 1.0 kb PCR fragments were joined by SOE PCR using the primers shown below.

Forward primer:

5'-CGTGTTCCTTCCCATTCGCATGCGACCTCGTGGTCATTGAC-3' (SEQ ID NO: 76)

Reverse primer:

5'-TGCAGAGGATATCGAACTGGGGCATTCTGTATCTGCGAATTGAGCTTG-3' (SEQ ID NO: 77)

The PCR was composed of 50 picomoles of each of the primers, 0.3 µl of the 0.38 kb fragment PCR, 0.6 µl of the 1.0 kb fragment PCR, 1X PHUSION™ High-Fidelity Hot Start DNA Polymerase buffer, 1 µl of a 10 mM blend of dNTPs, and 1 unit of PHUSION™ High-Fidelity Hot Start DNA Polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; 34 cycles each at 98°C for 15 seconds, 60°C for 30 seconds, and 72°C for 1.5 minutes, and 1 cycle at 72°C for 10 minutes. Five SOE PCRs were performed and the reactions were combined. The 1.36 kb SOE PCR fragment and the 1.8 kb PCR fragment containing the flippase coding sequence and *niaD* terminator were separated by 0.8% agarose gel electrophoresis in TAE buffer where a 1.36 kb fragment and a 1.8 kb fragment were excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

Seventeen µg of pJfyS156 DNA were digested with *Sph* I and purified by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 8.7 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

The 1.36 kb SOE PCR fragment and the 1.8 kb PCR fragment, containing the flippase coding sequence and *niaD* terminator, were inserted into *Sph* I digested pJfyS156 using an IN-FUSION® HD Cloning Kit. The reaction was composed of 116 ng of *Sph* I digested pJfyS156, 57 ng of the 1.36 kb SOE PCR fragment, 69 ng of the 1.8 kb PCR fragment, and 2 µl of IN-FUSION® buffer with Enzyme in a 10 µl reaction volume. The reaction was incubated at 50°C for 15 minutes and then chilled on ice. A 40 µl aliquot of TE was added. Two µl of the reaction transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Plasmid DNA was isolated from the transformants according to Example 4. Transformants were screened by restriction digestion with *Nsi* I which produced 2.1 kb, 2.6 kb, and 7 kb fragments. DNA sequencing of one clone verified that the construct contained the correct inserts with no PCR errors. The construct was designated pDM313.

Example 15: Multiple site specific integrations at *Trichoderma reesei* *cbh1* and *cbh2* loci with pJfyS156 using FLP/FRT system

T. reesei strain AgJg-FRT2-4B17 containing FRT sites inserted at the *cbh1* and *cbh2* loci (Example 12) was transformed with plasmid pJfyS156 (Example 8) to achieve targeting of one construct to the FRT sites at two different loci. Plasmid pJfyS156 contains an *A. fumigatus* beta-glucosidase expression cassette and the *hpt* marker for hygromycin resistance flanked by FRT sites, and the *S. cerevisiae* flippase gene under transcriptional control of the *T. reesei* *cbh2* promoter. Approximately 50 µg of plasmid pJfyS156 were digested with *Pme* I. The digested pJfyS156 DNA was recovered using a Nucleospin® Extract II Kit (Example 8).

Protoplasts of *T. reesei* strain AgJg-FRT2-4B17 were prepared as described in Example 1 except that media and solutions were supplemented with 2% (w/v) beta-lactose to induce the *T. reesei* *cbh2* promoter driving the flippase gene. The strain was cultivated in 25 ml of YP medium supplemented with 2% (w/v) beta-lactose at 27°C for 17 hours with gentle agitation at 90 rpm. Approximately 3 µg of *Pme* I digested pJfyS156 DNA were added to 100 µl of the protoplast solution and mixed gently. PEG buffer + 2% beta-lactose (250 µl) was added, and the transformation reaction was mixed and incubated at 34°C for 30 minutes. STC + 2% beta-lactose (3 ml) was then added, and the transformation reaction was mixed and then spread onto two PDA plates supplemented with 1 M sucrose and 2% beta-lactose. Eight transformation reactions were prepared and plated. After incubation at 30°C for 16 hours, 20 ml of overlay PDA medium supplemented with 2% beta-lactose and 35 µg of hygromycin B per ml were added to each plate.

The transformation plates were incubated at 30°C for 4 days and at room temperature for 5 days. Forty-four transformants were transferred to PDA plates and incubated for 4 days at 30°C. Fungal Spore PCR was utilized according to Example 9 to identify transformants that had integrated the *A. fumigatus* beta-glucosidase expression cassette and *hpt* marker at both the *cbh1* and *cbh2* loci. The spore PCR was composed of 1X PHIRE® Plant Direct PCR buffer (contains dNTPs and Mg), 10 pmol each of the forward and reverse primers shown below, 0.4 µl of PHIRE® II Hot Start DNA Polymerase, and 1 µl of supernatant from the spore suspension in a final volume of 20 µl.

PCR screen for pJfyS156 targeting: 5' end of *cbh1* and *cbh2* loci

Forward primer (homology to 5' flanking region of *cbh1*, upstream of FRT-F site):

5'-TTCCCTTCCTCTAGTGTGAAT-3' (SEQ ID NO: 78)

Forward primer (homology to 5' flanking region of *cbh2*, upstream of FRT-F site):

5'-GTTGGTATAGAGCAGCGTTC-3' (SEQ ID NO: 79)

Reverse primer (homology to *A. fumigatus* beta-glucosidase gene):

5'-CTATATCCGAAACAATGACG-3' (SEQ ID NO: 80)

The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 15 seconds, 61°C for 15 seconds, and 72°C for 1 minute and 10 seconds; and 1 cycle at 72°C for 15 minutes.

The completed PCRs were subjected to 0.8% agarose gel electrophoresis in TBE buffer. Transformants having correct targeting of pJfyS156 DNA to the *cbh1* locus produced a 2.0 kb fragment. Transformants having correct targeting of pJfyS156 DNA to the *cbh2* locus produced a 1.3 kb fragment. Transformants having correct targeting to both the *cbh1* and *cbh2* loci produced both the 2.0 kb and 1.3 kb fragments.

PCR screen for pJfyS156 targeting: 3' end of *cbh1* and *cbh2* loci

Forward primer (homology to *hpt* marker):

5'-CGTGTTCCTTCCCATTGCGATGCGACCTCGTGGTCATTGAC-3' (SEQ ID NO: 81)

Reverse primer (homology to 3' flanking region of *cbh1*, downstream of FRT-F3 site):

5'-AAAGACAGGCCAGCGACGAAG-3' (SEQ ID NO: 82)

Reverse primer (homology to 3' flanking region of *cbh2*, downstream of FRT-F3 site):

5'-GCATTGCAACCGCGGCTTTC-3' (SEQ ID NO: 83)

The PCRs were performed as described above. Transformants having correct targeting of pJfyS156 DNA to the *cbh1* locus produced a 1.4 kb fragment. Transformants having correct targeting of pJfyS156 DNA to the *cbh2* locus produced a 0.75 kb fragment. Transformants having correct targeting to both the *cbh1* and *cbh2* loci produced both the 1.4 kb and 0.75 kb fragments.

Transformants that produced PCR fragments indicating correct targeting to both the *cbh1* and *cbh2* loci were chosen for spore isolation. Spores from a 6 day old PDA plate were collected in 4 ml of 0.01% TWEEN® 20 and the spore concentration was determined using a hemocytometer. Spores were diluted appropriately to a concentration of 10³ spores per ml using sterile water and 100 spores were spread onto PDA plates supplemented with 10 µg of hygromycin per ml. The plates were incubated for 4 days at room temperature. Isolated colonies from each transformant were transferred with a sterile 10 µl inoculation loop to PDA plates and incubated at 30°C. Fungal Spore PCR was utilized according to Example 9 to identify spore isolates with correct targeting at both loci.

Genomic DNA from the spore isolates was prepared as described below and subjected to Southern blot analysis. *T. reesei* strains were grown in 25 ml of YP medium supplemented with 2% glucose (w/v) in a 125 ml baffled shake flask at 28°C for 2 days with agitation at 200 rpm. Mycelia were harvested by vacuum filtration through Whatman 1 filter paper in a Buchner funnel. The mycelia were washed twice in deionized water, dried under vacuum, and then transferred to 2 ml microfuge tubes. The mycelia were then dried approximately 16 hours in a Savant ISS110 SpeedVac concentrator (Thermo Scientific). The dried mycelia were ground to fine powders and total DNA was isolated using a MasterPure™

Yeast DNA Purification Kit (Epicentre). Ground mycelia equivalent to approximately a 50 µl volume were transferred to 2 ml microfuge tubes. Yeast Cell Lysis Solution (300 µl) was added to each mycelia sample and vortexed. The samples were incubated at 65°C for 20 minutes and then placed on ice for 5 minutes. MPC Protein Precipitation Reagent (150 µl) was added to each sample and briefly vortexed. The samples were centrifuged in a microcentrifuge at ≥10,000 rpm for 10 minutes. The supernatants were transferred to 1.7 ml microcentrifuge tubes and 500 µl of isopropanol were added to each tube. The samples were mixed thoroughly by inversion. The DNA was pelleted by centrifugation in a microcentrifuge for 10 minutes at ≥10,000 rpm. The supernatants were discarded. The pellets containing the DNA were washed with 0.5 ml of 70% ethanol. The samples were centrifuged in a microcentrifuge for 4 minutes at ≥10,000 rpm. The ethanol was removed with a pipette and the pellets were air dried for 7 minutes at room temperature. The DNA pellets were resuspended in 60 µl of TE. A 1.5 µl aliquot of 5 µg/µl RNase A was added to each tube and the samples were incubated at 37°C for 30 minutes.

For Southern blot analysis approximately 1 µg of each genomic DNA was digested with 20 units of *Nsi* I and *Sac* I. The digested DNA was subjected to 0.8% agarose gel electrophoresis in TAE buffer and transferred to a NYTRAN® SuperCharge membrane as described in Example 3.

A probe hybridizing to the *A. fumigatus* beta-glucosidase coding sequence was generated using a PCR DIG Probe Synthesis Kit with the primers shown below.

Primer 0615057:

5'-ATGAGATTCGGTTGGCTCGAG-3' (SEQ ID NO: 84)

Primer 068911:

5'-CCGTGATGTTGTAACCATAT-3' (SEQ ID NO: 85)

The PCR was composed of 100 ng of pDM313, 1 µl of 10 mM dNTPs, 1 µM primers, 1X PHUSION® High-Fidelity Reaction Buffer, and 1 unit of PHUSION® Hot Start High-Fidelity DNA Polymerase in a final volume of 50 µl. The PCR was performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; and 35 cycles each at 98°C for 15 seconds, 60°C for 30 seconds, and 72°C for 40 seconds; and 1 cycle at 72°C for 10 minutes. The PCR product was separated by 0.7% agarose gel electrophoresis in TAE buffer where an approximately 800 bp fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

The DIG Probe Synthesis PCR was composed of approximately 10 ng of the purified PCR fragment described above as template, 1 µM primers, 5 µl of PCR DIG Synthesis Mix (Roche Applied Science Corp.), 1X PCR buffer with MgCl₂ (Roche Applied Science Corp.), and 0.75 µl of Enzyme Mix (Roche Applied Science Corp.) in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 10

cycles each at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 40 seconds; 20 cycles each at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 40 seconds plus an additional 20 seconds for each successive cycle; and 1 cycle at 72°C for 7 minutes. The PCR product (the *A. fumigatus* BG probe) was purified using a Nucleospin® Extract II Kit (Example 8).

Southern blot analysis verified that the predicted 6.0 kb fragment characteristic of the correct FLP/FRT recombination at the *cbh1* locus and the predicted 4.4 kb fragment characteristic of the correct FLP/FRT recombination at the *cbh2* locus were both produced from the same transformant.

Example 16: Multiple site specific integrations at *Trichoderma reesei* *cbh1* and *cbh2* loci with pDM313 using FLP/FRT system

T. reesei strain AgJg-FRT2-4B17 with FRT sites inserted at the *cbh1* and *cbh2* loci (Example 12) was transformed with plasmid pDM313 (Example 14) to achieve targeting of one construct to the FRT sites at two different loci. Plasmid pDM313 contains an *A. fumigatus* beta-glucosidase expression cassette and the *hpt* marker for hygromycin resistance flanked by FRT sites, and the *S. cerevisiae* flippase gene under transcriptional control of the *T. reesei* *gpdA* promoter. Approximately 50 µg of plasmid pDM313 were digested with *Pme* I and recovered using a Nucleospin® Extract II Kit (Example 8). Protoplasts of *T. reesei* strain AgJg-FRT2-4B17 were prepared as described in Example 1. Approximately 3 µg of the *Pme* I digested pDM313 DNA were added to 100 µl of the protoplast solution and mixed gently. PEG buffer (250 µl) was added, and the reaction was mixed and incubated at 34°C for 30 minutes. STC (3 ml) was then added, and the reaction was mixed and then spread onto two PDA plates supplemented with 1 M sucrose. Eight transformation reactions were prepared and plated. After incubation at 30°C for 16 hours, 20 ml of overlay PDA medium supplemented with 35 µg of hygromycin B per ml were added to each plate.

The transformation plates were incubated at 30°C for 3 days and at room temperature for 5 days. Forty-eight transformants were transferred to PDA plates and incubated for 4 days at 30°C. Fungal Spore PCR was utilized according to Example 9 to identify transformants with the *A. fumigatus* beta-glucosidase expression cassette and *hpt* marker integrated at the *cbh1* and *cbh2* loci. The Spore PCR was composed of 1X PHIRE® Plant Direct PCR buffer (contains dNTPs and Mg), 10 pmol each of the primers shown below, 0.4 µl of PHIRE® II Hot Start DNA Polymerase, and 1 µl of supernatant from each spore suspension in a final volume of 20 µl.

PCR screen for pDM313 targeting: 5' end of *cbh1* and *cbh2* loci

Forward primer (homology to 5' flanking region of *cbh1*, upstream of FRT-F site):

5'-TTCCCTTCCTCTAGTGTGAAT-3' (SEQ ID NO: 86)

Forward primer (homology to 5' flanking region of *cbh2*, upstream of FRT-F site):

5'-GTTGGTATAGAGCAGCGTTC-3' (SEQ ID NO: 87)

Reverse primer (homology to *A. fumigatus* beta-glucosidase gene):

5 5'-CTATATCCGAAACAATGACG-3' (SEQ ID NO: 88)

The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 15 seconds, 61°C for 15 seconds, and 72°C for 1 minute and 10 seconds; and 1 cycle at 72°C for 15 minutes.

10 The completed PCRs were subjected to 0.8% agarose gel electrophoresis in TBE buffer. Transformants with correct targeting of pDM313 DNA to the *cbh1* locus produced a 2.0 kb fragment. Transformants with correct targeting of pDM313 DNA to the *cbh2* locus produced a 1.3 kb fragment. Transformants with correct targeting to both the *cbh1* and *cbh2* loci produced both the 2.0 kb and 1.3 kb fragments.

PCR screen for pDM313 targeting: 3' end of *cbh1* and *cbh2* loci

15 Forward primer (homology to *hpt* marker):

5'-CGTGTTCCTTCCCATTGCGATGCGACCTCGTGGTCATTGAC-3' (SEQ ID NO: 89)

Reverse primer (homology to 3' flanking region of *cbh1*, downstream of FRT-F3 site):

5'-AAAGACAGGCCAGCGACGAAG-3' (SEQ ID NO: 90)

Reverse primer (homology to 3' flanking region of *cbh2*, downstream of FRT-F3 site):

20 5'-GCATTGCAACCGCGGCTTTC-3' (SEQ ID NO: 91)

The PCRs were performed as described above. Transformants with correct targeting of pDM313 DNA to the *cbh1* locus produced a 1.4 kb fragment. Transformants with correct targeting of pDM313 DNA to the *cbh2* locus produced a 0.75 kb fragment. Transformants with correct targeting to both the *cbh1* and *cbh2* loci produced both the 1.4 kb and 0.75 kb
25 fragments.

Transformants that produced PCR fragments indicating correct targeting to both the *cbh1* and *cbh2* loci were chosen for spore isolation as described in Example 15. Fungal Spore PCR was utilized according to Example 9 to identify spore isolates with correct targeting at both loci.

30 Genomic DNA was isolated from the spore isolates according to Example 15 and subjected to Southern blot analysis according to Example 15 using the *A. fumigatus* beta-glucosidase gene probe.

Southern blot analysis verified that the predicted 6.0 kb fragment characteristic of correct FLP/FRT recombination at the *cbh1* locus and the predicted 4.4 kb fragment
35 characteristic of correct FLP/FRT recombination at the *cbh2* locus were both produced from the same transformant.

Example 17: Construction of plasmid pDM296

Plasmid pDM296 was constructed to insert the *hpt* (hygromycin resistance) marker between the *cbh1* flanking regions. A 0.28 kb fragment containing a portion of the *cbh1* 5' flanking region was PCR amplified from plasmid pSMai155 (U.S. Patent 7,361,495) using the primers shown below.

Forward primer

5'-AATAGAAAGAGAAGCTTAGCCAAG-3' (SEQ ID NO: 92)

Reverse primer

5'-GCCGTTTAAATGAATTCGAACCCTTAATTAAGTAGTACGCGTAGATC-3' (SEQ ID NO: 93)

The PCR was composed of 100 picomoles of each primer, 133 ng of pSMai155 DNA, 1X PHUSION™ High-Fidelity Hot Start DNA Polymerase buffer, 1 µl of a 10 mM blend of dNTPs, and 1 unit of PHUSION™ High-Fidelity Hot Start DNA Polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; 34 cycles each at 98°C for 15 seconds, 59°C for 30 seconds, and 72°C for 1 minute; and 1 cycle at 72°C for 10 minutes. The PCR product was separated by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 0.28 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

A 0.85 kb fragment containing a portion of the *hpt* marker was amplified by PCR from plasmid pSMai155 using the primers shown below.

Forward primer:

5'-GATCTACGCGTACTAGTTAATTAAGGGTTCGAATTCATTTAAACGGC-3' (SEQ ID NO: 94)

Reverse primer:

5'-GACCGATTCCTTGCGGTCCGAAT-3' (SEQ ID NO: 95)

The PCR was composed of 100 picomoles of each primer, 133 ng of pSMai155 DNA, 1X PHUSION™ High-Fidelity Hot Start DNA Polymerase buffer, 1 µl of a 10 mM blend of dNTPs, and 1 unit of PHUSION™ High-Fidelity Hot Start DNA Polymerase (New England Biolabs, Inc.) in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; 34 cycles each at 98°C for 15 seconds, 59°C for 30 seconds, and 72°C for 1 minute; and 1 cycle at 72°C for 10 minutes. The PCR product was separated by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 0.85 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

Approximately 11 µg of pJfyS139 (WO 2013/028928) DNA was digested with *Hind* III and *Rsr* II. The digested DNA was separated by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 5.8 kb fragment was excised from the gel and extracted using

a Nucleospin® Extract II Kit (Example 8).

The 0.28 and 0.85 kb PCR products were inserted into *Hind* III/*Rsr* II digested pJfyS139 using an IN-FUSION™ Advantage PCR Cloning Kit. The reaction was composed of 1X IN-FUSION™ Reaction buffer, 100 ng of the *Hind* III/*Rsr* II digested pJfyS139, 44.5 ng of the 0.28 kb PCR product, 111 ng of the 0.85 kb PCR product, and 1 µl of IN-FUSION™ enzyme in a 10 µl reaction volume. The reaction was incubated for 15 minutes at 37°C and 15 minutes at 50°C. After the incubation period the reaction was chilled on ice and then 40 µl of TE were added to the reaction. *E. coli* XL10 Gold Ultracompetent cells were transformed with the reaction. A 100 µl aliquot of cells was transferred to a tube on ice and 4 µl of beta-mercaptoethanol were added. A 2 µl aliquot of the reaction was added to the cells and the reaction was chilled on ice for 30 minutes. The reaction was heat shocked at 42°C for 30 seconds and then chilled on ice for 2 minutes. A 900 µl aliquot of NZY+ medium was added. The reaction was then incubated at 37°C with agitation at 200 rpm for 1 hour. The *E. coli* transformation reaction was transferred to a microfuge tube and centrifuged for 10 seconds at 13,000 rpm. Approximately 850 µl of supernatant were discarded and the cells were resuspended in approximately 150 µl of supernatant. The cells were spread onto a 150 mm 2XYT plus ampicillin plate. Plasmid DNA was isolated from the transformants according to Example 4. Plasmid DNA from the transformants was screened by restriction digestion with *Pac* I and *Rsr* II, which produced 0.8 and 6.1 kb fragments, respectively. DNA sequencing of one clone verified that a construct contained the correct inserts with no PCR errors. The construct was designated pDM296.

Example 18: Construction of plasmid pDM297

Plasmid pDM297 was constructed to insert the *amdS* marker between the *cbh1* flanking regions. A 2.8 kb fragment containing the *A. nidulans amdS* marker was amplified by PCR from plasmid pMJ09 (U.S. Patent 7,361,495) using the primers shown below.

Forward primer:

5'-CCGCGGACTGCGCACCATGGATGCATCTGGAAACGCAACC-3' (SEQ ID NO: 96)

Reverse primer:

5'-GGTGCGTCAGGCTTTCGCCACATTTAAATCATGCATTCTACGCCAGGACC-3' (SEQ ID NO: 97)

The PCR was composed of 100 picomoles of each primer, 100 ng of pMJ09 DNA, 1X PHUSION™ High-Fidelity Hot Start DNA Polymerase buffer, 1 µl of a 10 mM blend of dNTPs, and 1 unit of PHUSION™ High-Fidelity Hot Start DNA Polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; 34 cycles each at 98°C for 15 seconds, 59°C for 30 seconds, and 72°C for 1.5 minutes; and 1 cycle at 72°C for 10 minutes. The PCR product was separated by 0.8%

agarose gel electrophoresis in TAE buffer where an approximately 2.8 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

A 1.3 kb 3' *cbh1* flanking DNA sequence was amplified by PCR from plasmid pJfyS139 (WO 2013/028928) using the primers shown below.

5 Forward primer:

5'-TGGCGTAGAATGCATGATTAAATGTGGCGAAAGCCTGACGCACC-3' (SEQ ID NO: 98)

Reverse primer:

5'-ATGCATGCTCGAGCGGCCGCACGGCAGG-3' (SEQ ID NO: 99)

10 The PCR was composed of 100 picomoles of each primer, 100 ng of pJfyS139 DNA, 1X PHUSION™ High-Fidelity Hot Start DNA Polymerase buffer, 1 µl of a 10 mM blend of dNTPs, and 1 unit of PHUSION™ High-Fidelity Hot Start DNA Polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; 34 cycles each at 98°C for 15 seconds, 59°C for 30 seconds, and 72°C for 1.5 minutes, and 1 cycle at 72°C for 10 minutes. The PCR product was separated by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 1.3 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

15 Fifteen µg of pSMai155 (U.S. Patent 7,361,495) were digested with *Nco* I HF and *Not* I. The digested DNA was separated by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 3.7 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

25 The 2.8 kb and 1.3 kb PCR products were inserted into *Nco* I/*Not* I digested pSMai155 using an IN-FUSION™ Advantage PCR Cloning Kit. The reaction was composed of 1X IN-FUSION™ Reaction buffer, 100 ng of the *Nco* I/*Not* I digested pSMai155, 145 ng of the 2.8 kb PCR product, 67 ng of the 1.3 kb PCR product, 1 µl of IN-FUSION™ enzyme, and 2.4 µl of water in a 10 µl reaction volume. The reaction was incubated at 37°C for 15 minutes and at 50°C for 15 minutes. After the incubation period the reaction was chilled on ice and then 40 µl of TE were added to the reaction. *E. coli* XL10 Gold Ultracompetent cells were transformed with a 2 µl aliquot of the reaction according to Example 17. Plasmid DNA was isolated from the transformants according to Example 4 and screened by restriction digestion with *Nco* I HF and *Bam* HI, which produced 2.0 and 5.8 kb fragments, respectively. DNA sequencing of one clone verified that a construct contained the correct inserts with no PCR errors. The construct was designated pDM297.

35 **Example 19: Multiple site specific integrations at *Trichoderma reesei cbh1* and *cbh2* loci with pJfyS156 and pAgJg136 using FLP/FRT system**

T. reesei strain AgJg-FRT2-4B17 containing FRT sites inserted at the *cbh1* and *cbh2*

loci (Example 12) was transformed with plasmids pJfyS156 (Example 8) and pAgJg136 (Example 13) to achieve targeting of two constructs to the FRT sites at two different loci. Plasmid pJfyS156 comprises an *A. fumigatus* beta-glucosidase expression cassette and the *hpt* marker for hygromycin resistance flanked by FRT sites, and the *S. cerevisiae* flippase gene under transcriptional control of the *T. reesei cbh2* promoter. Plasmid pAgJg136 comprises an *A. fumigatus* beta-glucosidase 4M variant (WO 2013/028912) expression cassette and the *amdS* marker flanked by FRT sites, and the *S. cerevisiae* flippase gene under transcriptional control of the *T. reesei cbh2* promoter. Approximately 50 µg of plasmid pJfyS156 and approximately 80 µg of plasmid pAgJg136 were digested with *Pme* I. The digested DNA was heated for 20 minutes at 65°C to inactivate the restriction enzyme and then was ethanol precipitated. The pJfyS156 DNA and pAgJg136 DNA were resuspended in 30 µl and 50 µl of TE, respectively. Protoplasts of *T. reesei* strain AgJg-FRT2-4B17 were prepared as described in Example 1 except that 2% beta-lactose (w/v) was added to growth media and protoplast solutions. Approximately 2 µg of *Pme* I digested pJfyS156 DNA and approximately 2 µg of *Pme* I digested pAgJg136 DNA were added to 100 µl of the protoplast solution and mixed gently. PEG buffer (250 µl) supplemented with 2% beta-lactose was added, and the reaction was mixed and incubated at 34°C for 30 minutes. STC (3 ml) supplemented with 2% beta-lactose was then added and the reaction was mixed and then spread onto two COVE + 2% beta-lactose plates. Seven transformation reactions were prepared and plated. After incubation at 28°C for 16 hours, 20 ml of COVE + 2% beta-lactose overlay supplemented with 35 µg of hygromycin B per ml were added to each plate.

The transformation plates were incubated at 28°C for 9 days. Forty transformants were transferred to COVE2 plates and incubated at 30°C for 5 days. Fungal Spore PCR was utilized according to Example 9 to identify transformants with the two different plasmids integrated at the *cbh1* and *cbh2* loci. The Spore PCR was composed of 1X PHIRE® Plant Direct PCR buffer (contains dNTPs and Mg), 10 pmol each of the forward and reverse primers shown below, 0.4 µl of PHIRE® II Hot Start DNA Polymerase, and 1 µl of supernatant from the spore suspension in a final volume of 20 µl.

PCR screen of 5' end of both *cbh1* and *cbh2* loci

Forward primer (homology to 5' flanking region of *cbh1* upstream of FRT-F site):

5'-TTCCCTTCCTCTAGTGTTGAAT-3' (SEQ ID NO: 100)

Forward primer (homology to 5' flanking region of *cbh2*, upstream of FRT-F site):

5'-GTTGGTATAGAGCAGCGTTC-3' (SEQ ID NO: 101)

Reverse Primer (homology to *A. fumigatus* beta-glucosidase gene):

5'-CTATATCCGAAACAATGACG-3' (SEQ ID NO: 102)

The reactions were performed in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 15 seconds, 61°C for 15 seconds, and 72°C for 1

minute and 10 seconds; and 1 cycle at 72°C for 15 minutes.

The completed PCRs were subjected to 0.8% agarose gel electrophoresis in TBE buffer. Transformants having correct targeting of plasmid DNA to the *cbh1* locus produced a 2.0 kb fragment. Transformants having correct targeting of plasmid DNA to the *cbh2* locus produced a 1.3 kb fragment. Transformants having correct targeting to both the *cbh1* and *cbh2* loci produced both the 2.0 kb and 1.3 kb fragments.

Fungal Spore PCR was utilized as described above to identify the exact locus of integration for each of the two plasmids. Transformants identified as having integration at both the *cbh1* and *cbh2* loci by the 5' end PCR screen described above were screened with the primers shown below.

Screen for site of integration of pJfyS156

Forward primer (homology to *hpt* marker):

5'-CGTGTTCCTTCCCATTGCGATGCGACCTCGTGGTCATTGAC-3' (SEQ ID NO: 103)

Reverse primer (homology to 3' flanking region of *cbh1*, downstream of FRT-F3 site):

5'-AAAGACAGGCCAGCGACGAAG-3' (SEQ ID NO: 104)

Reverse primer (homology to 3' flanking region of *cbh2*, downstream of FRT-F3 site):

5'-GCATTGCAACCGCGGCTTTC-3' (SEQ ID NO: 105)

Screen for site of integration of pAgJg136

Forward primer (homology to the *amdS* marker):

5'-ACCTGCCGTGTCAGCCTCTACGGTTGTTA-3' (SEQ ID NO: 106)

Reverse primer (homology to 3' flanking region of *cbh1*, downstream of FRT-F3 site):

5'-AAAGACAGGCCAGCGACGAAG-3' (SEQ ID NO: 107)

Reverse primer (homology to 3' flanking region of *cbh2*, downstream of FRT-F3 site):

5'-GCATTGCAACCGCGGCTTTC-3' (SEQ ID NO: 108)

The reactions were performed in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 10 seconds, 61°C for 10 seconds, and 72°C for 30 seconds; and 1 cycle at 72°C for 1 minute.

The completed PCRs were subjected to 0.8% agarose gel electrophoresis in TBE buffer. Transformants having correct targeting of pJfyS156 DNA to the *cbh1* locus produced a 1.4 kb fragment. Transformants having correct targeting of pJfyS156 DNA to the *cbh2* locus produced a 0.75 kb fragment. Transformants having correct targeting of pAgJg136 DNA to the *cbh1* locus produced a 1.35 kb fragment. Transformants having correct targeting of pAgJg136 DNA to the *cbh2* locus produced a 0.67 kb fragment.

Transformants that produced the correct PCR fragments were chosen for spore isolation according to Example 15 except that the spores were collected from a COVE2 plate and 100 spores were spread onto COVE plates and incubated at 30°C for 4 days. Isolated colonies from each transformant were transferred with a sterile 10 µl inoculation loop to

COVE glycerol plates and incubated at 30°C for 5 days. Spore PCR using a PHIRE® Plant Direct PCR Kit and the primers and PCR conditions shown above for the PCR screen of the 5' end of both loci was used to screen the spore isolates.

The completed PCRs were subjected to 0.8% agarose gel electrophoresis in TBE buffer. Transformants having correct targeting of plasmid DNA to the *cbh1* locus produced a 2.0 kb fragment. Transformants having correct targeting of plasmid DNA to the *cbh2* locus produced a 1.3 kb fragment. Transformants having correct targeting to both the *cbh1* and *cbh2* loci produced both the 2.0 kb and 1.3 kb fragments.

Genomic DNA from the spore isolates was prepared according to Example 15. For Southern blot analysis approximately 1 µg of each genomic DNA was digested with 20 units of *Mfe* I and subjected to Southern blot analysis according to Example 15. Two blots were prepared for separate hybridizations with *hpt* and *amdS* probes.

The *hpt* gene probe was prepared according to the following protocol. Twelve µg of pDM296 (Example 17) were digested with *Nco* I and *Not* I and separated by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 1.9 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8). A 0.5 kb PCR fragment was generated from the 1.9 kb *Nco* I/*Not* I *hpt* fragment using the primers shown below.

Forward primer:

5'-ATGAAAAAGCCTGAACTCACC-3' (SEQ ID NO: 109)

Reverse primer:

5'-TCCATCACAGTTTGCCAGTGA-3' (SEQ ID NO: 110)

The PCR was composed of 50 picomoles of each of the primers, 25 ng of the 1.9 kb *Nco* I/*Not* I *hpt* fragment, 1X PHUSION™ High-Fidelity Hot Start DNA Polymerase buffer, 1 µl of a 10 mM blend of dNTPs, and 1 unit of PHUSION™ High-Fidelity Hot Start DNA Polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; 34 cycles each at 98°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and 1 cycle at 72°C for 10 minutes. The PCR product was separated by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 0.5 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

The *hpt* probe was generated using a PCR DIG Probe Synthesis Kit with the forward and reverse primers shown directly above. The PCR was composed of 1X PCR DIG Probe Synthesis mix, 50 pmol of each primer, 1X PCR buffer with MgCl₂, 45 ng purified 0.5 kb *hpt* PCR fragment (described above), and 2.6 units of EXPAND® High Fidelity DNA polymerase (Roche Applied Science Corp.) in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 45 seconds; and 1 cycle at 72°C for 7 minutes.

The *amdS* gene probe was prepared according to the following protocol. Ten µg of pDM297 (Example 18) was digested with *Nco* I and *Bam* HI. The digested DNA was separated by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 2.0 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8). A 0.59 kb PCR fragment was generated from the 2.0 kb *Nco* I/*Bam* HI *amdS* fragment using the primers shown below.

Forward primer:

5'-ACATTGCCGTCGAAGTTGTA-3' (SEQ ID NO: 111)

Reverse primer:

5'-AAGACCTCTGTCCCGCAGAC-3' (SEQ ID NO: 112)

The PCR was composed of 50 picomoles of each of the primers, 18 ng of the 2.0 kb *Nco* I/*Bam* HI *amdS* fragment, 1X PHUSION™ High-Fidelity Hot Start DNA Polymerase buffer, 1 µl of a 10 mM blend of dNTPs, and 1 unit of PHUSION™ High-Fidelity Hot Start DNA Polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; 34 cycles each at 98°C for 15 seconds, 59°C for 30 seconds, and 72°C for 1 minute; and 1 cycle at 72°C for 10 minutes. The PCR product was separated by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 0.59 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8). The *amdS* probe was generated using a PCR DIG Probe Synthesis Kit with the forward and reverse primers shown directly above.

The PCR was composed of 1X PCR DIG Probe Synthesis mix, 50 pmol of each primer, 1X PCR buffer with MgCl₂, 14 ng of the purified 0.59 kb *amdS* PCR fragment (described above), and 2.6 units of EXPAND® High Fidelity DNA polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minute; 30 cycles each at 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 45 seconds; and 1 cycle at 72°C for 7 minutes.

Each probe was boiled for 5 minutes, chilled on ice for 2 minutes, and added to 10 ml of DIG Easy Hyb to produce the hybridization solution. One blot was probed with the *hpt* probe and the other blot was probed with the *amdS* probe.

Transformants having correct integration of pJfyS156 at the *cbh1* locus produced a 2.6 kb *hpt* hybridizing fragment. Transformants having correct integration of pJfyS156 at the *cbh2* locus produced a 3.0 kb *hpt* hybridizing fragment. Transformants having correct integration of pAgJg136 at the *cbh1* locus produced a 3.4 kb *amdS* hybridizing fragment. Transformants having correct integration of pAgJg136 at the *cbh2* locus produced a 3.8 kb *amdS* hybridizing fragment. Southern blot analysis verified that transformants having one copy of pJfyS156 plus one copy of pAgJg136 were obtained and that the plasmid DNAs were correctly integrated at the *cbh1* and *cbh2* loci.

Example 20: Construction of plasmid pQM41 for targeting a non-functional *amdS* marker to *Trichoderma reesei cbh2* locus

Plasmid pQM41 was constructed by inserting an approximately 1.9 kb fragment of a non-functional *amdS* fragment 1 into an approximately 10.3 kb vector backbone from pJfyS142 (WO 2013/028912) digested with *Nco* I and *Pac* I. The non-functional *amdS* fragment 1 was amplified from pAllo1 (WO 04/111228) using the primers shown below.

Forward primer 1208222:

5'-ATCACCTCTGTGTATTGCACCAGGGCATGGGGATGACCTTG-3' (SEQ ID NO: 113)

Reverse primer 1207436:

5'-CCGGTCACGAAAGCCTTAATTAATCTACGCCAGGACCGAGCAAG-3' (SEQ ID NO: 114)

The PCR was composed of 100 ng of pAllo1, 1 µl of 10 mM dNTPs, 1 µM primers, 1X PHUSION® High-Fidelity Reaction Buffer, and 1 unit of PHUSION® Hot Start High-Fidelity DNA Polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 35 cycles each at 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 1 minute 15 seconds; and 1 cycle at 72°C for 10 minutes. The PCR product was separated by 0.7% agarose gel electrophoresis in TAE buffer where an approximately 1.9 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

The 1.9 kb PCR product was inserted into *Nco* I and *Pac* I digested pJfyS142 using an IN-FUSION™ HD Cloning Kit. The IN-FUSION™ reaction was composed of 1X IN-FUSION™ HD Enzyme Premix, 300 ng of *Nco* I and *Pac* I digested pJfyS142, and 109 ng of the 1.9 kb PCR product in a 10 µl reaction volume. The reaction was incubated at 50°C for 15 minutes. After the incubation period, a 1 µl aliquot was transformed into ONE SHOT® TOP10 Chemically Competent cells according to Example 4. Plasmid DNA was isolated from the transformants according to Example 4. The insert was confirmed by DNA sequencing. One transformant was identified as containing the insert with no PCR errors and the plasmid was designated pQM41 (Figure 3).

Example 21: Insertion of a non-functional *amdS* marker at the *cbh2* locus in *Trichoderma reesei* AgJg-FRT1-2B1A

Trichoderma reesei AgJg-FRT1-2B1A (Example 10) was transformed with 1-5 µg of *Pme* I-linearized pQM41 using hygromycin selection (Example 1) in order to insert the non-functional *amdS* fragment 1 at the *cbh2* locus. Thirty-four transformants were obtained and each one was picked and transferred to a PDA plate and incubated for 7 days at 28°C. The transformants were cultured in 2 ml of CIM at 30°C for 3 days with agitation at 250 rpm.

Supernatant from each culture was subjected to SDS-PAGE using a CRITERION® 8-16% TGX Stain-Free gel (Bio-Rad Laboratories, Inc.) and PRECISION PLUS® Protein Unstained Standards (Bio-Rad Laboratories, Inc.). Since successful targeted integration of pQM41 at the *cbh2* locus effectively disrupts the *cbh2* gene, SDS-PAGE gels were visually analyzed for loss of the CBH2 protein from the proteome. One strain designated *T. reesei* QMJi056-8, which produced no CBH2 protein, was identified and selected for genomic DNA extraction and Southern blot analysis to confirm the integration of the non-functional *amdS* fragment 1 at the *T. reesei* *cbh2* locus. Genomic DNA was isolated from *T. reesei* QMJi056-8 according to Example 2.

Plasmid pQM41 contains the *Herpes simplex* virus thymidine kinase (*tk*) gene and the *E. coli* hygromycin phosphotransferase (*hpt/hygR*) selection marker flanked by direct repeats. The direct repeats were inserted to facilitate the curing out of the *tk* and *hpt* selection markers. Spores from *T. reesei* QMJi056-8 were plated onto *Trichoderma* minimal media plates containing 1 µM 5-fluoro-2'-deoxyuridine (FdU) and incubated at 28°C for 5-7 days. Four Fdu-resistant isolates were sub-cultured onto PDA plates and incubated at 28°C for 5-7 days. Genomic DNA of those isolates were prepared as described in Example 2 and used for Southern blot analysis to confirm the absence of the *hpt* and *tk* markers.

A digoxigenin-labeled *T. reesei* *cbh2* probe hybridizing to the 3' flanking region of the *cbh2* locus was synthesized by PCR using a PCR DIG Probe Synthesis Kit and the primers shown below.

Primer 069773:

5'-CAACCAAATTTCTGTTTATAGATC-3' (SEQ ID NO: 115)

Primer 069774:

5'-GATGATATAATGGAGCAAATAAGGG-3' (SEQ ID NO: 116)

The DIG Probe Synthesis PCR was composed of approximately 10 ng of pQM22 (WO 2013/028912) as template, 1 µM primers, 5 µl of PCR DIG Synthesis Mix, 1X PCR buffer with MgCl₂, and 0.75 µl of Enzyme Mix in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 10 cycles each at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 40 seconds; 20 cycles each at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 40 seconds plus an additional 20 seconds for each successive cycle; and 1 cycle at 72°C for 7 minutes. The PCR product was separated by 1% agarose gel electrophoresis in TAE buffer where the fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

The correct integration at the *cbh2* locus was confirmed by Southern blot analysis as described in Example 3 using the *T. reesei* *cbh2* probe. Integration of the non-functional *amdS* fragment 1 with the *tk/hpt* marker at the *cbh2* locus generated a hybridization signal at approximately 7.3 kb in *Nhe* I and *Psi* I digest. Successful removal of the *tk/hpt* marker at the

cbh2 locus generated a hybridization signal at approximately 2.7 kb in the *Bam* HI digestion.

Two µg of genomic DNA of the Fdu-resistant isolates were digested with and submitted to Southern blot analysis according to Example 3 using the *T. reesei cbh2* probe. Isolates that result in a hybridized signal at recognized by the *T. reesei cbh2* probe, indicating the successful removal of the *tk/hpt* marker at the *cbh2* locus. Southern blot analysis confirmed the correct integration at the *cbh2* locus.

Example 22: Construction of plasmid pJfyS145

An empty expression vector was constructed containing the *cbh2* promoter and *amdS* marker. The *amdS* marker was PCR amplified from pMJ09 (U.S. Patent 7,361,495) using the primers shown below.

Forward primer:

5'-ATAACAACTCCGAGTGGATCCTGGAAACGCAACCCTGAAGGGATTCTTCCTT-3' (SEQ ID NO: 117)

Reverse primer:

5'-TGTTTAACTCTAGGATGCATTCTACGCCAGGACCGAGCAAGCCCA-3' (SEQ ID NO: 118)

The PCR was composed of 20 ng of pMJ09 DNA, 200 µM dNTPs, 0.4 µM primers, 1X HERCULASE® Reaction Buffer, and 2.5 units of HERCULASE® High Fidelity DNA polymerase (Stratagene Corp.) in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 3 minutes; and 1 cycle at 72°C for 7 minutes. The completed PCR was submitted to 1% agarose gel electrophoresis in TAE buffer where a 2.7 kb band was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The *amdS* PCR fragment was inserted into *Bam* HI/*Not* I digested pJfyS142-B (WO 2013/028928) using an IN-FUSION® Advantage PCR Cloning Kit. The reaction was composed of 150 ng of *Bam* HI/*Not* I digested pJfyS142-B, 100 ng of the *amdS* PCR fragment, 1X IN-FUSION® Advantage Reaction Buffer, and 1 µl of IN-FUSION® Enzyme in a 10 µl reaction volume. The reaction was incubated at 37°C for 15 minutes and then at 50°C for 15 minutes. Then 40 µl of TE were added to the reaction and 2 µl were transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Plasmid DNA was isolated from the transformants according to Example 4. The insert was confirmed by DNA sequencing. One transformant was identified as containing the insert with no PCR errors and the plasmid was designated pJfyS143.

The *A. fumigatus cbh2* coding sequence was amplified from pAILo33 (US20110111453 A1) using the primers shown below.

Forward primer:

5'-CTCTGTGTATTGCACCATGAAGCACCTTGCATCTTCCATCG-3' (SEQ ID NO: 119)

Reverse primer:

5'-CTCTGTGTATTGCACCATGAAGCACCTTGCATCTTCCATCG-3' (SEQ ID NO: 120)

The PCR was composed of 20 ng of pAILo33 (US20110111453), 200 μ M dNTPs, 0.4 μ M primers, 1X HERCULASE® Reaction Buffer, and 2.5 units of HERCULASE® High Fidelity DNA polymerase in a final volume of 50 μ l. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 2 minutes; and 1 cycle at 72°C for 7 minutes.

The completed PCR was subjected to 1% agarose gel electrophoresis in TAE buffer where a 2.7 kb band was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The *A. fumigatus cbh2* PCR fragment was inserted into *Nco* I/*Pac* I-digested pJfyS143 (described above) using an IN-FUSION® Advantage PCR Cloning Kit. The reaction was composed of 112 ng of *Nco* I/*Pac* I-digested pJfyS143, 75 ng of the *A. fumigatus cbh2* PCR, 1X IN-FUSION® Advantage Reaction Buffer, and 1 μ l of IN-FUSION® Enzyme in a 10 μ l reaction volume. The reaction was incubated at 37°C for 15 minutes and then at 50°C for 15 minutes. Then 40 μ l of TE were added to the reaction and 2 μ l were transformed into ONE SHOT® TOP10 *E. coli* chemically competent according to Example 4. Plasmid DNA was isolated from the transformants according to Example 4. The insert was confirmed by DNA sequencing. One transformant was identified as containing the insert with no PCR errors and the plasmid was designated pJfyS145 (Figure 4).

Example 23: Multiple site specific integrations at *Trichoderma reesei cbh1* and *cbh2* loci with pJfyS156 and pJfyS145 using FLP/FRT system and split-marker technology

Plasmid pJfyS145 (Example 22) was digested with *Pme* I and *Nhe* I and purified by 0.7% agarose gel electrophoresis in TAE buffer where an approximately 5.3 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8). The purified 5.3 kb fragment was used as template in PCRs with the primers shown below to obtain enough DNA for transformation.

Primer 068422:

5'-ACGAATTGTTTAAACGTCGACCCAAGTATCCAGAGGTGTATGGAAATATCAGAT-3' (SEQ ID NO: 121)

Primer 1201305:

5'-GTGCGTCAGGCTTTCGCCACGGATCCTTTCAGAGGCCGAAG-3' (SEQ ID NO: 122)

Each PCR was composed of 10 ng of pJfyS145, 1 μ l of 10 mM dNTPs, 1 μ M primers,

1X PHUSION® High-Fidelity Reaction Buffer, and 1 unit of PHUSION® Hot Start High-Fidelity DNA Polymerase in a final volume of 50 µl. Forty-eight PCRs were performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; 35 cycles each at 98°C for 15 seconds, 60°C for 30 seconds, and 72°C for 3 minutes, and 1 cycle at 72°C for 10 minutes.

5 All of the completed PCRs were combined and purified using a Nucleospin® Extract II Kit (Example 8). The resulting purified PCR fragment (approximately 5.3 kb) contains an *Aspergillus fumigatus* CBH2 expression cassette and a non-functional *amdS* fragment 2.

T. reesei strain QMJi056-8 (Example 22) was transformed with pJfyS156 (Example 8) and the 5.3 kb pJfyS145 derived fragment according to the procedure described in Example 1 except that protoplasts of *T. reesei* QMJi056-8 were re-suspended in STC solution supplemented with 2% lactose. Approximately 13 µg of pJfyS156 and approximately 4 µg of the 5.3 kb pJfyS145 derived fragment containing the *Aspergillus fumigatus* CBH2 expression cassette and the non-functional *amdS* fragment 2 were added to 100 µl of the protoplast solution and mixed gently. PEG buffer + 2% beta-lactose (250 µl) was added, and the reaction was mixed and incubated at 34°C for 30 minutes. STC + 2% beta-lactose (3 ml) was then added and the reaction was mixed and spread onto two COVE plates and incubated at 30°C for 7-14 days. Two transformation reactions were prepared and plated. Thirty transformants were obtained and each one was picked and transferred to a COVE2 plate supplemented with 10 mM uridine and incubated for 7 days at 30°C. The transformants were cultured in 25 ml of CIM and incubated at 28°C for 4 days with agitation at 200 rpm. Supernatant from each culture was subjected to SDS-PAGE using a CRITERION® 8-16% TGX Stain-Free gel and PRECISION PLUS® Protein Unstained Standards. Three transformants producing both *A. fumigatus* CBH2 and *A. fumigatus* CEL3A beta-glucosidase were selected for spore isolation according to Example 16. Eight spore isolates from each transformant were picked and transferred to COVE2 plates supplemented with 10 mM uridine and incubated for 7 days at 30°C. Spore isolates were cultured in 2 ml of CIM and incubated at 30°C for 3 days with agitation at 250 rpm. Supernatant from each culture was subjected to SDS-PAGE as described in Example 22. Genomic DNA from isolates producing both *A. fumigatus* CBH2 and *A. fumigatus* CEL3A beta-glucosidase were prepared for Southern blot analysis to evaluate the integration sites. Southern blot analysis results confirmed correct integrations at both the *cbh1* locus and the *cbh2* locus.

Example 24: Identification of *Trichoderma reesei* *adeB* gene

A BLAST search, using the *Aspergillus oryzae* phosphoribosylaminoimidazole carboxylase (AdeB) protein sequence (GenBank Accession Number AB121756) against the *Trichoderma reesei* genome database (Version 2.0) maintained by the Joint Genome Institute (<http://genome.jgi-psf.org/Trire2/Trire2.home.html>) was performed. A DNA fragment

(on scaffold 6: 149561-151463) was identified that, when translated, was found to share 74% amino acid sequence identity with *Aspergillus oryzae* AdeB.

Example 25: Construction of *adeB* deletion vector pSMai199

The 3' *adeB* flanking region was amplified from *T. reesei* RutC30 genomic DNA using the primers shown below. Underlined letters represent an added *Sbf* I site to facilitate the cloning of the amplified fragment.

Forward primer:

5'-AAAAAACCTGCAGGGGGAGTCTTGATCTGACGCTGC-3' (SEQ ID NO: 123)

Reverse primer:

5'-AAAAAACCTGCAGGCGGCCGCATATCGAACGGTAGTGAAGT-3' (SEQ ID NO: 124)

The PCR was composed of 300 ng of the *T. reesei* RutC30 genomic DNA template, 300 µM dNTPs, 50 pmol of the forward primer, 50 pmol of the reverse primer, 1X Reaction buffer (Invitrogen Corp.), 1 mM MgSO₄, and 2.5 units of PLATINUM® Pfx DNA polymerase (Invitrogen Corp.). The reaction was performed in a thermocycler programmed for 1 cycle at 94°C for 3 minutes; and 30 cycles each at 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 1 minute. After the 30 cycles, the reaction was incubated at 72°C for 15 minutes. The completed PCR was submitted to 1% agarose gel electrophoresis in TAE buffer where a 1035 bp band was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The 1035 bp PCR product was cloned into pCR2.1®TOPO® (Invitrogen Corp.). Briefly 1 µl of the gel-purified PCR was added to 1 µl of Kit-supplied salt solution and 1 µl of pCR2.1®TOPO® in a 6 µl reaction volume and incubated at room temperature for 30 seconds. Following incubation, 2 µl were transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Plasmid DNA was isolated from the transformants according to Example 4. The insert was confirmed by DNA sequencing. One transformant was identified as containing the insert with no PCR errors and the plasmid was digested with *Sbf* I to liberate the *adeB* 3' flanking region fragment. The digestion was submitted to 1% agarose gel electrophoresis where a 1.0 kb band was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4). The fragment was ligated to *Sbf* I-digested and CIP dephosphorylated pJfys1579-41-11 using a QUICK LIGATION™ Kit. The ligation reaction was composed of 1X Quick Ligation buffer, 50 ng of the 3' *adeB* fragment, and 1 µl of Quick Ligase in a 20 µl reaction volume. The reaction was incubated at room temperature for 5 minutes and 2 µl were transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Plasmid DNA was isolated from the transformants according to Example 4. The insert was confirmed by DNA sequencing. One transformant containing the insert with no PCR errors, designated

pClone1, was identified and used to insert the 5' *adeB* flanking region.

The 5' *adeB* flanking region was amplified from *T. reesei* RutC30 genomic DNA using the primers shown below. Underlined letters represent an added *Pme* I site to the sense and antisense primers to facilitate the cloning of the amplified fragment.

5 Forward primer:

5'-AAAAAAGTTTAAACGCGGCCGCGAGAATGCTTCCTAATGCTA-3' (SEQ ID NO: 125)

Reverse primer:

5'-AAAAAAGTTTAAACCTTGAACGTCGAGAGAGAGC-3' (SEQ ID NO: 126)

The PCR was composed of 300 ng of the *T. reesei* RutC30 genomic DNA, 300 µM dNTPs, 50 pmol of the forward primer, 50 pmol of the reverse primer, 1X Reaction buffer (Invitrogen Corp.), 1 mM MgSO₄, and 2.5 units of PLATINUM® *Pfx* DNA polymerase. The reaction was performed in a thermocycler programmed for 1 cycle at 94°C for 3 minutes; and 30 cycles each at 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 1 minute. After the 30 cycles, the reaction was incubated at 72°C for 15 minutes. The completed PCR was submitted to 1% agarose gel electrophoresis in TAE buffer where a 1036 bp band was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The 1036 bp PCR product was cloned into pCR2.1®TOPO® as above. Briefly 1 µl of the gel-purified PCR was added to 1 µl of Kit-supplied salt solution and 1 µl of pCR2.1®TOPO® vector in a 6 µl reaction volume and incubated at room temperature for 30 seconds. Following incubation, 2 µl were transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Plasmid DNA was isolated from the transformants according to Example 4. The insert was confirmed by DNA sequencing. One transformant was identified as containing the insert with no PCR errors and the plasmid was digested with *Pme* I to liberate the *adeB* 5' flanking fragment. The digestion was submitted to 1% agarose gel electrophoresis where a 1.0 kb band was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The 1.0 kb fragment was ligated to *Pme* I digested and CIP dephosphorylated pClone1 using a QUICK LIGATION™ Kit. The ligation reaction was composed of 1X Quick Ligation buffer, 50 ng of the *adeB* 5' flanking fragment, 100 ng of *Pme* I digested and CIP dephosphorylated pClone 1, and 1 µl of Quick Ligase in a 20 µl reaction volume. The reaction was incubated at room temperature for 5 minutes and 2 µl were transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Plasmid DNA was isolated from the transformants according to Example 4. The insert was confirmed by DNA sequencing. One transformant was identified as containing the insert with no PCR errors and the plasmid was designated pSMai199. Plasmid pSMai199 was used to delete the *T. reesei adeB* gene in *T. reesei* RutC30.

Example 26: Deletion of the *adeB* gene in *Trichoderma reesei* strain RutC30

Protoplast preparation and transformation of *Trichoderma reesei* strain RutC30 with *Not* I-linearized pSMai199 were performed as described in Example 1.

One hundred and fifty transformants were each transferred with a sterile 10 µl inoculation loop to an individual well of a 12-well plate containing PDA medium supplemented with 25 µg of hygromycin B per ml and 0.01% adenine and incubated at 28°C for 3 days. A small amount of mycelia was scraped from each well using a sterile 1 µl inoculation loop and spotted to unsupplemented TrMM medium in a 12-well plate to identify adenine auxotrophs. Two transformants displayed adenine auxotrophy.

Genomic DNA was isolated from the two transformants according to Example 2 and analyzed by Southern blot analysis as described in Example 3 except that in each case 2 µg of genomic DNA were digested with *Eco* RI and *Xho* I. The digested DNA was subjected to 0.7% agarose gel electrophoresis in TAE buffer and transferred to a NYTRAN® SuperCharge membrane according to Example 3. A probe hybridizing to the 5' flanking region of the *adeB* gene was generated using a PCR DIG Probe Synthesis Kit and the primers shown below.

Forward primer:

5'-CCACACTACAGATGCTGTCTG-3' (SEQ ID NO: 127)

Reverse primer:

5'-TCCATGACTCGCAAGATACG-3' (SEQ ID NO: 128)

The PCR was composed of 1X HERCULASE® Reaction Buffer, 400 nM each primer, 200 µM DIG-labeled dUTP-containing dNTPs, 50 ng of pSMai199 DNA, and 1.5 units of HERCULASE® DNA polymerase. The reaction was performed in a thermocycler programmed for 1 cycle at 94°C for 3 minutes; 30 cycles each at 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 30 seconds; and 1 cycle at 72°C for 5 minutes.

The digoxigenin-labeled *T. reesei adeB* DNA probe was purified by 1% agarose gel electrophoresis in TAE buffer where a 408 bp band corresponding to the probe was excised from the gel and agarose extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The Southern blot analysis indicated that one auxotroph, designated *T. reesei* SMai199-19-3, contained the deletion cassette at a single copy at the *adeB* locus.

Example 27: Construction of an expression plasmid pSMai205

Plasmid pJfyS117 (Example 31) was digested with *Spe* I and *Bam* HI and subjected to 1% agarose gel electrophoresis in TAE buffer where a 2753 bp band was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4). The 2753 bp band was then ligated into *Spe* I and *Bam* HI digested pEJG107 (WO 05/047499)

using a QUICK LIGATION™ Kit. Plasmid pEJG107 contains the *Aspergillus nidulans amdS* gene and the *Aspergillus fumigatus* beta-glucosidase gene under transcriptional control of the *Trichoderma reesei* cellobiohydrolase *cel7a* gene promoter. The ligation reaction was composed of 1X QUICK LIGATION™ buffer, 50 ng of digested pEJG107, 56 ng of the 2753 bp fragment, and 1 µl of QUICK Ligase in a 20 µl reaction volume. The reaction was incubated at room temperature for 5 minutes and 2 µl were transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Plasmid DNA was isolated from the transformants according to Example 4. The insert was confirmed by DNA sequencing. One transformant was identified as containing the insert with no PCR errors and the plasmid was designated pSMai205.

Example 28: Construction of *Trichoderma reesei adeB* based expression plasmid pSMai206 and utilization of *adeB* as a selectable marker

The full-length *Trichoderma reesei adeB* gene, including approximately 600 bp of the native promoter, was amplified by PCR from *T. reesei* RutC30 genomic DNA using a PLATINUM® Pfx DNA Polymerase Kit (Invitrogen Corp.) and the primers shown below. Underlined letters represent an added *Nsi* I site to facilitate cloning of the amplified fragment. Forward primer:

5'-CCAATGCATAGTCTGAACAGCGATAACAA-3' (SEQ ID NO: 129)

Reverse primer:

5'-CCAATGCATAGGCATTAAGTGGGTTGTAT-3' (SEQ ID NO: 130)

The PCR was composed of 270 ng of *T. reesei* RutC30 genomic DNA, 300 µM dNTPs, 50 pmol of the forward primer, 50 pmol of the reverse primer, 1X Reaction buffer (Invitrogen Corp.), 1 mM MgSO₄, and 2.5 units of PLATINUM® Pfx DNA polymerase. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 3 minutes; and 30 cycles each at 98°C for 30 seconds, 58°C for 30 seconds, and 72°C for 3 minutes. After the 30 cycles, the reaction was incubated at 72°C for 15 minutes. The completed PCR was subjected to 1% agarose gel electrophoresis in TAE buffer where a 2877 bp band was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4). The fragment was inserted into pCR2.1®TOPO® using a TOPO® TA Cloning® Kit. Briefly 1 µl of the gel-purified PCR product was added to 1 µl of Kit-supplied salt solution and 1 µl of pCR2.1®TOPO® in a 6 µl reaction volume and incubated at room temperature for 30 seconds. Following incubation 2 µl were transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Plasmid DNA was isolated from the transformants according to Example 4. The insert was confirmed by DNA sequencing. One transformant was identified as containing the insert with no PCR errors and the plasmid was digested with *Nsi* I to liberate the *adeB* fragment from the plasmid. The digestion was

submitted to 1% agarose gel electrophoresis in TAE buffer where a 2877 bp band was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

Plasmid pSMai205 (Example 27) was also digested with *Nsi* I to remove the *Aspergillus nidulans amdS* gene from the plasmid, and subsequently treated with calf intestine phosphatase (CIP) (New England Biolabs, Inc.) to dephosphorylate the ends. The digestion was submitted to 1% agarose gel electrophoresis in TAE buffer where a 7521 bp band was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The 2877 bp *Nsi* I digested *adeB* fragment was ligated to the *Nsi* I-digested and CIP-treated pSMai205 using a QUICK LIGATION™ Kit. The ligation reaction was composed of 1X Quick Ligation buffer, 50 ng of the digested pSMai205, 58 ng of the 2877 bp *adeB* fragment, and 1 µl of Quick Ligase in a 20 µl reaction volume. The reaction was incubated at room temperature for 5 minutes and 2 µl were transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Plasmid DNA was isolated from the transformants according to Example 4. The insert was confirmed by DNA sequencing. One transformant was identified as containing the insert with no PCR errors and the plasmid was designated pSMai206.

Protoplasts of *T. reesei* SMai199-19-3 were prepared as described in Example 1 and transformed with *Pme* I-linearized pSMai206. The resulting purified DNA was added to 100 µl of the protoplast solution and mixed gently followed by PEG buffer (250 µl) and mixed. The transformation reaction was incubated at 34°C for 30 minutes. STC (3 ml) was then added and mixed, and then the transformation reaction was spread onto 150 mm TrMM plates supplemented with 1 M sucrose. The transformation plates were incubated for 8 days at 28°C and 45 transformants were transferred with a sterile 10 µl inoculation loop to a 100 mm PDA plate and grown for 7 days at 28°C. Each transformant was analyzed in a small scale cultivation by transferring a small amount of spores with a sterile 10 µl inoculation loop into 25 ml of CIM in a 125 ml polycarbonate shake flask and incubating at 28°C for 5 days with agitation at 200 rpm. Supernatant from each culture was subjected to SDS-PAGE using a CRITERION® 8-16% SDS-PAGE gel and PRECISION PLUS® Protein Standards. Since the expression plasmid contained the *Aspergillus fumigatus* beta-glucosidase coding sequence as a reporter the presence of a band at approximately 150 kDa in many of the transformants indicated successful integration of the expression plasmid and utilization of *adeB* as a selectable marker.

Example 29: Construction of *hemA* deletion vector pJfyS120

The 3' flanking region of the *T. reesei* 5-aminolevulinic acid synthase (*hemA*) gene

was amplified from *T. reesei* RutC30 genomic DNA using the primers shown below.

Forward primer:

5'-TATAGCGTACCTGCAGGTGTCATGCCCCGCGGCTTTGCCTTGA-3' (SEQ ID NO: 131)

Reverse primer:

5'-ATGCTGTACCTGCAGGCGGCCGCGCTCCCGATCATCATCCCTCCGAG-3' (SEQ ID NO: 132)

The PCR was composed of 150 ng of *T. reesei* RutC30 genomic DNA, 200 μ M dNTPs, 0.4 μ M primers, 1X HERCULASE® Reaction Buffer, and 2.5 units of HERCULASE® High Fidelity DNA polymerase in a final volume of 50 μ l. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute and 45 seconds; and 1 cycle at 72°C for 7 minutes. The completed PCR was submitted to 1% agarose gel electrophoresis in TAE buffer where a 1.5 kb band was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4). The *hemA* 3' flanking region PCR fragment was inserted into pCR2.1®TOPO® using a TOPO® TA Cloning® Kit. Briefly 1 μ l of the gel-purified PCR fragment was added to 1 μ l of Kit-supplied salt solution and 1 μ l of pCR2.1®TOPO® in a 6 μ l reaction volume and incubated at room temperature for 30 seconds. Following incubation 2 μ l were transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Plasmid DNA was isolated from the transformants according to Example 4. The insert was confirmed by DNA sequencing. One transformant was identified as containing the insert with no PCR errors. The plasmid was digested with *Sbf* I to liberate the *hemA* 3' flanking region fragment. The digestion was submitted to 1% agarose gel electrophoresis in TAE buffer where a 1.5 kb band was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The fragment was ligated to *Sbf* I-digested pJfyS1579-41-11 using a QUICK LIGATION™ Kit. The ligation reaction was composed of 1X Quick Ligation buffer, 50 ng of the 3' *hemA* fragment, 50 ng of *Sbf* I-digested pJfyS1579-41-11 and 1 μ l of Quick Ligase in a 20 μ l reaction volume. The reaction was incubated at room temperature for 5 minutes and 2 μ l were transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Plasmid DNA was isolated from the transformants according to Example 4. The insert was confirmed by DNA sequencing. One transformant was identified as containing the insert with no PCR errors and the plasmid was designated pJfyS120A.

The 5' *hemA* flanking region sequence was amplified from *T. reesei* RutC30 genomic DNA using the primers shown below.

Forward primer:

5'-CATGGTTTAAACGGCGGGCGCGCCGCGGCCGCAATTCAGAGCATCACGGTTGAGGG

A-3' (SEQ ID NO: 133)

Reverse primer:

5'-CCTTGTTTTGTCGGGCGCGCCACATGGCCTTGGATTGACGCAGGAC-3' (SEQ ID NO: 134)

The PCR was composed of 150 ng of *T. reesei* RutC30 genomic DNA, 200 µM dNTPs, 0.4 µM primers, 1X HERCULASE® Reaction Buffer, and 2.5 units of HERCULASE® High Fidelity DNA polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute and 45 seconds; and 1 cycle at 72°C for 7 minutes. The completed PCR was submitted to 1% agarose gel electrophoresis in TAE buffer where a 1.0 kb band was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4). The PCR fragment was digested with Asc I after and purified by agarose gel electrophoresis as described above.

The *hemA* 5' flanking region fragment digested with Asc I was ligated to Asc I-digested pJfyS120A using a QUICK LIGATION™ Kit. The ligation reaction was composed of 50 ng of the Asc I-digested pJfyS120A, 1X Quick Ligation buffer, 50 ng of the Asc I-digested 5' *hemA* fragment, and 1 µl of Quick Ligase in a 20 µl reaction volume. The reaction was incubated at room temperature for 5 minutes and 2 µl were transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Plasmid DNA was isolated from the transformants according to Example 4. The insert was confirmed by DNA sequencing. One transformant was identified as containing the insert with no PCR errors and the plasmid was designated pJfyS120. Plasmid pJfyS120 was used to delete the *T. reesei hemA* gene in *T. reesei* RutC30.

Example 30: Deletion of the *hemA* gene in *Trichoderma reesei* strains RutC30

Protoplasts of *T. reesei* RutC30 were prepared as described in Example 1 and transformed with Not I-linearized pJfyS120 (Example 29). The resulting purified DNA was added to 100 µl of the protoplast solution and mixed gently followed by PEG buffer (250 µl) and mixed. The transformation reaction was incubated at 34°C for 30 minutes. STC (3 ml) was then added and mixed, and then the transformation reaction was spread onto PDA plates supplemented with 1 M sucrose and 5 mM 5-aminolevulinic acid (ALA) (Sigma-Aldrich Chemical Co.). After incubation at 28°C for 16 hours, 15 ml of overlay PDA medium supplemented with 100 µg of hygromycin B per ml and 5 mM 5-aminolevulinic acid were added to each plate. The plates were incubated at 28°C for 12 days. Eighty-eight transformants were each transferred with a sterile 10 µl inoculation loop to individual wells of a 12-well plate containing PDA medium supplemented with 25 µg of hygromycin B per ml and 2.5 mM ALA and incubated at 28°C for 3 days. A small amount of mycelia was scraped

from the plate using a sterile 1 µl inoculation loop and spotted onto un-supplemented TrMM in a 12-well plate to identify ALA auxotrophs. Three strains displayed ALA auxotrophy.

Genomic DNA was isolated from the three auxotrophic strains according to Example 2 and analyzed by Southern blot analysis as described in Example 3 except that in each case 2 µg of genomic DNA was digested with 33 units of *Nco* I. The digested DNA was subjected to 1% agarose gel electrophoresis in TAE buffer and transferred to a NYTRAN® SuperCharge membrane as described in Example 3. A probe hybridizing to the 3' flanking region of the *hemA* gene was generated using a PCR DIG Probe Synthesis Kit and the primers shown below.

Forward primer:

5'-GACGCATACAATACAAGCATATGCTGTTGGTGTCT-3' (SEQ ID NO: 135)

Reverse primer:

5'-AAGGCGTCTGGAAACAGAAGCTGCT-3' (SEQ ID NO: 136)

The PCR was composed of 1X HERCULASE® Reaction Buffer, 400 nM of each primer, 200 µM DIG-labeled dUTP-containing dNTPs, 150 ng of *T. reesei* RutC30 genomic DNA, and 1.5 units of HERCULASE® DNA polymerase. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 25 cycles each at 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 40 seconds; and 1 cycle at 72°C for 7 minutes.

The probe was purified by 1% agarose gel electrophoresis in TAE buffer where a band corresponding to the probe was excised from the gel and agarose extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The Southern blot analysis indicated that all three auxotrophs contained the deletion cassette as a single copy at the *hemA* locus. One strain designated *T. reesei* JfyS2010-52-65 was arbitrarily chosen for rescue of auxotrophy with a *hemA*-based expression plasmid.

Example 31: Construction of *hemA*-based expression plasmid pJfyS126 and utilization of *hemA* as a selectable marker

To facilitate selectable marker excision from expression plasmid pMJ09 (U.S. Patent 8,318,458) an additional *Nsi* I site present in the *cbh1* terminator was removed using a QUICKCHANGE® II XL Site-Directed Mutagenesis according to Example 6 and the primers shown below.

Forward primer:

5'-CGATTCCTTAGTAGCCATGCACTTTAAGATAACGGAATAGAAG-3' (SEQ ID NO: 137)

Reverse primer:

5'-CTTCTATTCCGTTATCTTAAAGTGCATGGCTACTAAGGAATCG-3' (SEQ ID NO: 138)

Plasmid DNA was isolated from the *E. coli* transformants according to Example 4. The insert was confirmed by DNA sequencing. One transformant was identified as

containing the insert with no PCR errors and the fragment was liberated from the plasmid using a *Pac* I/*Asc* I digestion. pMJ09 was also digested with *Pac* I/*Asc* I and both digestions were submitted to 1% agarose gel electrophoresis in TAE buffer where a 6.7 kb band corresponding to the pMJ09 backbone and a 0.4 kb band corresponding to the mutated region of pMJ09 were excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The two fragments were ligated using a QUICK LIGATION™ Kit. The ligation reaction was composed of 1X QUICK LIGATION™ buffer, 50 ng of the digested pMJ09, 20 ng of the mutated fragment, and 1 µl of Quick Ligase in a 20 µl reaction volume. The reaction was incubated at room temperature for 5 minutes and 2 µl were transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Plasmid DNA was isolated from the transformants according to Example 4. The insert was confirmed by DNA sequencing. One transformant was identified as containing the insert with no PCR errors and the plasmid was designated pJfyS117. Plasmid pJfyS117 was used to insert the *A. fumigatus* beta-glucosidase gene.

The *hemA* marker was then inserted into pSMai205 (Example 27). A fragment containing the *hemA* marker was PCR amplified from *T. reesei* RutC30 genomic DNA using a GC Genomic LA Polymerase Kit (Clontech Laboratories, Inc.) and the primers shown below.

Forward primer:

5'-TATCACTGTCATTCAATGCATTCTTTGTGTGTGTGTCAGCATTGTA-3' (SEQ ID NO: 139)

Reverse primer:

5'-TATCACTGTCATTCAATGCATTCTTTGTGTGTGTGTCAGCATTGTA-3' (SEQ ID NO: 140)

The PCR was composed of GC LA Reaction Buffer (Clontech Laboratories, Inc.), 1X GC Melt (Clontech Laboratories, Inc.), 400 nM of each primer, 200 µM dNTPs, 150 ng of *T. reesei* RutC30 genomic DNA, and 1.5 units of Advantage GC LA DNA polymerase (Clontech Laboratories, Inc.). The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minute; 16 cycles each at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 3 minutes; and 1 cycle at 72°C for 7 minutes. The completed PCR was subjected to 1% agarose gel electrophoresis in TAE buffer where a 3 kb band was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The 3 kb fragment was inserted into pCR2.1®TOPO® using a TOPO® TA Cloning® Kit. Briefly 1 µl of the gel-purified PCR product was added to 1 µl of Kit-supplied salt solution and 1 µl of pCR2.1®TOPO® in a 6 µl reaction volume and incubated at room temperature for 30 seconds. Following incubation 2 µl were transformed into ONE SHOT® TOP10 *E. coli*

chemically competent cells according to Example 4. Plasmid DNA was isolated from the transformants according to Example 4. The insert was confirmed by DNA sequencing. One transformant was identified as containing the insert with no PCR errors and the plasmid was digested with *Eco* RI to liberate the *hemA* fragment from the plasmid. The digestion was then treated with Klenow DNA Polymerase to fill in the overhangs generated during digestion after which the digestion was submitted to 1% agarose gel electrophoresis in TAE buffer where a 3.0 kb band was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

Plasmid pSMai205 (Example 27) was also digested with *Nsi* I to liberate the *amdS* region from the plasmid, and subsequently treated with calf intestine phosphatase (CIP) (to dephosphorylate the ends. The digestion was submitted to 1% agarose gel electrophoresis in TAE buffer where a 7.5 kb band was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4). The *Eco* RI/Klenow-treated *hemA* fragment was ligated to the *Nsi* I-digested/Klenow/ CIP-treated pSMai205 using a QUICK LIGATION™ Kit. The ligation reaction was composed of 1X Quick Ligation buffer, 50 ng of the *hemA* fragment, 50 ng of digested pSMai205, and 1 µl of Quick Ligase in a 20 µl reaction volume. The reaction was incubated at room temperature for 5 minutes and 2 µl transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Plasmid DNA was isolated from the transformants according to Example 4. The insert was confirmed by DNA sequencing. One transformant was identified as containing the insert with no PCR errors and the plasmid was designated pJfyS126.

T. reesei strain JfyS2010-52-65 protoplasts were prepared and transformed with the *Pme* I-linearized pJfyS126 as described in Example 1. The transformation reaction was spread onto 150 mm PDA plates supplemented with 1 M sucrose. The transformation plates were incubated for 7 days at 28°C and transformants were transferred with a sterile 10 µl inoculation loop to a 100 mm PDA plate and grown for 7 days at 28°C. Transformants were each analyzed in small scale cultivations by transferring a small amount of spores with a sterile 10 µl inoculation loop into 25 ml of CIM in 125 ml polycarbonate shake flasks and incubating at 28°C for 5 days with agitation at 200 rpm. Supernatant from each culture was subjected to SDS-PAGE using a CRITERION® 8-16% SDS-PAGE gel and PRECISION PLUS® Protein Standards. Since the expression plasmid contained the *Aspergillus fumigatus* beta-glucosidase gene as a reporter the presence of a band at approximately 150 kDa in many of the transformants indicated successful integration of the expression plasmid and utilization of *hemA* as a selectable marker.

Example 32: Construction of an *Aspergillus fumigatus* beta-glucosidase variant expression vector targeted to the *Trichoderma reesei cbh1* locus

Plasmid pAgJg123 was constructed to comprise an *Aspergillus fumigatus* beta-glucosidase variant under transcriptional control of the *Trichoderma reesei* cellobiohydrolase I gene promoter and terminator, with flanking sequence to target to the *T. reesei cbh1* locus. Two synthetic oligonucleotide primers shown below were designed to amplify by PCR the 3' flanking region of the *T. reesei cbh1* promoter from plasmid pJfyS139 (WO 2013/028928) and introduce flanking regions for insertion into expression vector pDFng133-3 (WO 2013/028912), which contains the *T. reesei cbh1* promoter and *A. fumigatus* beta-glucosidase variant gene. Bold letters represent sequence of the 3' *cbh1* flanking region and the remaining sequence is homologous to insertion sites of pDFng133-3.

Forward Primer (Primer 0613337):

5'-TCTGCCCCGTGTCTACGTGGCGAAAGCCTGACGCACCGGTAGATT-3' (SEQ ID NO: 141)

Reverse Primer (Primer 0613338):

5'-CACGGAGCTTACTAGACGGCACGGTTAAGCAGGGTCTTGC-3' (SEQ ID NO: 142)

The PCR was composed of 0.5 µl of plasmid pJfyS139, 10 µl of 10 mM dNTPs, 50 pmol of the forward primer, 50 pmol of the reverse primer, 1X PHUSION® GC Buffer, and 2 units of PHUSION® Hot Start DNA polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 30 seconds; 35 cycles each at 98°C for 10 seconds, 60°C for 10 seconds, and 72°C for 1 minute; and 1 cycle at 72°C for 10 minutes. The PCR products were separated by 1% agarose gel electrophoresis in TAE buffer where a 1.3 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The 1.3 kb fragment was then cloned into pDFng133-3 digested with *Spe* I using an IN-FUSION™ HD Cloning Kit. The digested vector was isolated by 1% agarose gel electrophoresis in TAE buffer where a 10.2 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4). The 1.3 kb 3' flanking region fragment and the digested vector were ligated together in a reaction resulting in the expression plasmid pAgJg123, in which the *A. fumigatus* beta-glucosidase variant could be targeted to the *T. reesei cbh1* locus, under transcriptional control of the *T. reesei cbh1* promoter and terminator. The ligation reaction was composed of 1X IN-FUSION™ HD enzyme mix, 98.9 ng of pDFng133-3 digested with *Spe* I, and 100.1 ng of the *T. reesei* 3' *cbh1* flanking PCR product in a 20 µl reaction. The reaction was incubated at 50°C for 15 minutes. Then 2.5 µl of the reaction were transformed into XL-1 Blue Subcloning cells (Agilent Technologies). Plasmid DNA was isolated using a Mini-prep Kit (QIAGEN Inc.). The insert was confirmed by DNA sequencing. The resulting plasmid was designated pAgJg123.

Example 33: Targeting the *Aspergillus fumigatus* beta-glucosidase variant gene into

the *cbh1* locus of *Trichoderma reesei* strain AgJg115-104-7B1

Protoplast preparation and transformation of *T. reesei* AgJg115-104-7B1 was performed as described in Example 1.

Plasmid pAgJg123 (240 µg; Example 32) was digested with *Pme* I and purified by 1% agarose gel electrophoresis in TAE buffer where a 8.8 kb band was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4). Approximately 3.785 µg of the resulting purified DNA fragment was added to 100 µl of the protoplast solution and spread onto COVE plates as described in Example 1. The plates were incubated at 28°C for 7-10 days. Transformants were sub-cultured onto COVE2 + 10 mM uridine plates to generate spores.

The transformants of *T. reesei* strain AgJg115-104-7B1 were screened by Fungal Spore PCR according to Example 9 for the presence of the pAgJg123 replacement vector in the *cbh1* locus, thereby deleting the coding sequence of *cbh1* and replacing it with an *A. fumigatus* beta-glucosidase variant gene. The Spore PCR was composed of 0.5 µl of spore suspension, 50 pmol of primer 069079, 50 pmol of primer 0614037, 10 µl of 2X PHIRE® Plant PCR Buffer, and 0.4 µl of PHIRE® Hot Start II DNA Polymerase in a 20 µl reaction. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 5 seconds, 68°C for 5 seconds, and 72°C for 30 seconds; 1 cycle at 72°C for 2 minutes; and a 10°C hold. Primer 069079 is located upstream of the 5' flanking region and primer 0614037 is located at the beginning of the *A. fumigatus* beta-glucosidase variant gene. If the replacement vector integrates into the *cbh1* locus, the amplified PCR fragment will be 1.4 kb in length.

Primer 069079 (forward):

5'-CAAGCAAAGCGTTCCGTCGCAGTAGCAGGC-3' (SEQ ID NO: 143)

Primer 0614037 (reverse):

5'-GCATCACAAACCTGGGCATTGGCTACAGAA-3' (SEQ ID NO: 144)

For Southern blot analysis, 2 µg of each genomic DNA were digested with 10 units of *Nco* I and 20 units of *Xba* I in a 35 µl reaction volume. The digested DNA was subjected to 0.7% agarose gel electrophoresis in TAE buffer and transferred to a NYTRAN® SuperCharge membrane according to Example 3.

The membrane was hybridized with a 500 bp digoxigenin-labeled *A. fumigatus* beta-glucosidase gene probe, which was synthesized by incorporation of digoxigenin-11-dUTP by PCR using primers 1201134 and 1201135 shown below.

Primer 1201134 (forward):

5'-ATGAGATTCGGTTGGCTCGAG-3' (SEQ ID NO: 145)

Primer 1201135 (reverse):

5'-AAAGACTCCGCGGGTATAGCTC-3' (SEQ ID NO: 146)

The PCR was composed of 5 µl of 10X *Taq* Buffer, 2.5 µl of PCR DIG Labeling Mix, 20 ng of pAgJg123, 50 pmol of primer 1201134, 50 pmol of primer 1201135, 2.5 µl of 10 mM dNTPs, and 5 units of *Taq* DNA polymerase in a 50 µl reaction. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 40 seconds; 1 cycle at 72°C for 15 minutes; and a 10°C hold. The probe was purified by 1% agarose gel electrophoresis in TAE buffer, excised from the gel, and extracted using a QIAQUICK® Gel Extraction Kit.

Southern blot analysis identified several transformants having the *cbh1* coding sequence replaced with a single copy of the coding sequence of the *A. fumigatus* beta-glucosidase variant. One such transformant was designated *T. reesei* AgJg123.

Example 34: Construction of an *Aspergillus fumigatus* beta-glucosidase variant expression vector targeted to a *Trichoderma reesei* protease locus

Plasmid pAgJg124 was constructed to comprise an *Aspergillus fumigatus* beta-glucosidase variant under transcriptional control of the *Trichoderma reesei* cellobiohydrolase I gene promoter and terminator, with flanking sequence to target a locus encoding a *T. reesei* protease (JGI Protein ID: 70962), a locus that receives a moderate amount of translation signal as found via mRNA analysis. Two synthetic oligonucleotide primers shown below were designed to amplify by PCR the 5' flanking region of the *T. reesei* protease from genomic DNA and introduce flanking regions for insertion into expression vector pMJ05 (U.S. Patent 8,497,115). Bold letters represent sequence of the 5' protease flanking region and the remaining sequence is homologous to insertion sites of pMJ05.

Forward Primer (Primer 0613544):

5'-CGAATTGTTTAAACGGGCTCCTATGTTCAATCATGGCACA-3' (SEQ ID NO: 147)

Reverse Primer (Primer 0613545):

5'-**CCTACATT**CGGTCGACTTGACGATTCAGTGTCTTCTCTTG-3' (SEQ ID NO: 148)

The PCR was composed of 118 ng of *T. reesei* genomic DNA, 10 µl of 10 mM dNTPs, 50 pmol of primer 0613544, 50 pmol of primer 0613545, 1X PHUSION® GC Buffer, and 2 units of PHUSION® Hot Start DNA polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 30 seconds; 30 cycles each at 98°C for 10 seconds, 55°C for 30 seconds, and 72°C for 1 minute; and 1 cycle at 72°C for 10 minutes. The PCR products were separated by 1% agarose gel electrophoresis in TAE buffer where a 1.5 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The 1.5 kb fragment was then cloned into pMJ05 using an IN-FUSION™ HD Cloning Kit. Plasmid pMJ05 was digested with *Sa*/I and isolated by 1% agarose gel electrophoresis in TAE buffer where a 7.9 kb fragment was excised from the gel and agarose was extracted

using a MINELUTE® Gel Extraction Kit (Example 4). The 1.5 kb 5' flanking region fragment and the digested vector were ligated together in a reaction resulting in expression plasmid pAgJg124A, in which the 5' flanking region of the *T. reesei* protease gene was cloned upstream of the *T. reesei cbh1* promoter in pMJ05. The ligation reaction was composed of 1X IN-FUSION™ HD enzyme mix, 100 ng of pMJ05 digested with *Sa*I, and 150 ng of the *T. reesei* 5' protease flanking region fragment in a 10 µl reaction. The reaction was incubated at 50°C for 15 minutes. Then 2.5 µl of the reaction were transformed into SoloPack® Gold Supercompetent cells (Stratagene). One aliquot of SoloPack® cells were thawed on ice. Then 2.5 µl of the reaction were added to the competent cells in a tube and incubated on ice for an additional 30 minutes. The tube was incubated at 42°C for 30 seconds and immediately placed on ice for 2 minutes after which 250 µl of SOC medium were added. The tube was then incubated at 37°C with agitation at 200 rpm for 1 hour and transferred to 150 mm 2XYT plus ampicillin plates. The plates were incubated at 37°C overnight. Plasmid DNA was isolated using a Mini-prep Kit (QIAGEN Inc.). The insert was confirmed by DNA sequencing where the resulting plasmid was designated pAgJg124A.

To amplify the 3' flanking region of the *T. reesei* protease, two synthetic oligonucleotide primers shown below were designed for insertion into expression vector pAgJg124A. Bold letters represent sequence of the 3' protease flanking region and the remaining sequence is homologous to insertion sites of pAgJg124A.

Forward Primer (Primer 0613546):

5'-AAACGATTCCTTAATA**AAAACAATGCTTGGACTCCGAAAGT**-3' (SEQ ID NO: 149)

Reverse Primer (Primer 0613547):

5'-CAGTCACCTCTAGTTAT**GCAATTAAACAAATGCCCCACCTAG**-3' (SEQ ID NO: 150)

The PCR was composed of 118 ng of *T. reesei* genomic DNA, 10 µl of 10 mM dNTPs, 50 pmol of primer 0613546, 50 pmol of primer 0613547, 1X PHUSION® GC Buffer, and 2 units of PHUSION® Hot Start DNA polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 30 seconds; 30 cycles each at 98°C for 10 seconds, 55°C for 30 seconds, and 72°C for 1 minute; and 1 cycle at 72°C for 10 minutes. The PCR products were separated by 1% agarose gel electrophoresis in TAE buffer where a 1.5 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The 1.5 kb fragment was then cloned into pAgJg124A using an IN-FUSION™ HD Cloning Kit. The vector was digested with *Pac*I in a reaction consisting of 40 units of *Pac*I and isolated by 1% agarose gel electrophoresis in TAE buffer where a 9.4 kb fragment was excised from the gel and agarose was extracted using a QIAQUICK® Gel Extraction Kit (Example 9). The 1.5 kb 3' flanking region fragment and the digested vector were ligated together in a reaction resulting in the expression plasmid pAgJg124B, in which the 3'

flanking region of the *T. reesei* protease was cloned downstream of the *T. reesei cbh1* terminator in pAgJg124A. The ligation reaction was composed of 1X IN-FUSION™ HD enzyme mix, 100 ng of pAgJg124A digested with *Pac* I, and 150 ng of the *T. reesei* 3' protease flanking region fragment in a 20 µl reaction. The reaction was incubated at 50°C for 15 minutes. Then 2.5 µl of the reaction were transformed into SoloPack® Gold Supercompetent cells as described above. Plasmid DNA was isolated using a Mini-prep Kit (QIAGEN Inc.). The insert was confirmed by DNA sequencing. The resulting plasmid was designated pAgJg124B.

Finally, two synthetic oligonucleotide primers shown below were designed to amplify a portion of the *T. reesei cbh1* promoter and the entire coding sequence of the *A. fumigatus* beta-glucosidase variant for insertion into expression vector pAgJg124B. Bold letters represent sequence of the *T. reesei cbh1* promoter and *A. fumigatus* beta-glucosidase fragment, while the remaining sequence is homologous to insertion sites of pAgJg124B.

Forward Primer (Primer 0613727):

5'-CTAAGATCTCGGGCCCTCGGGCCTTTCGGCCTTTGGGTGTACATGT-3' (SEQ ID NO: 151)

Reverse Primer (Primer 0613728):

5'-GCTACTAGTACGCGTCTAGTAGACACGGGGCAGAGGCGCT-3' (SEQ ID NO: 152)

The PCR was composed of 200 ng of pDFng133-3 DNA, 10 µl of 10 mM dNTPs, 50 pmol of primer 0613727, 50 pmol of primer 0613728, 1X PHUSION® GC Buffer, and 2 units of PHUSION® Hot Start DNA polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 30 seconds; 30 cycles each at 98°C for 10 seconds, 60°C for 30 seconds, and 72°C for 2 minutes; and 1 cycle at 72°C for 7 minutes. The PCR products were separated by 1% agarose gel electrophoresis in TAE buffer where a 3.5 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The 3.5 kb fragment was then cloned into pAgJg124B using an IN-FUSION™ HD Cloning Kit. The vector was digested with *Apa* I and *Mlu* I and isolated by 1% agarose gel electrophoresis in TAE buffer where a 10.9 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4). The 3.5 kb *T. reesei cbh1* promoter and *A. fumigatus* beta-glucosidase variant fragment and the digested vector were ligated together in a reaction resulting in the expression plasmid pAgJg124, in which the *A. fumigatus* beta-glucosidase variant, under transcriptional control by the *T. reesei cbh1* promoter and terminator, has 5' and 3' flanking regions for targeting to the *T. reesei* protease locus. The ligation reaction was composed of 1X IN-FUSION™ HD enzyme mix, 100 ng of pAgJg124B digested with *Apa* I and *Mlu* I, and 150 ng of the *T. reesei cbh1* promoter and *A. fumigatus* beta-glucosidase variant fragment in a 20 µl reaction. The

reaction was incubated at 50°C for 15 minutes. Then 2.5 µl of the reaction were transformed into SoloPack® Gold Supercompetent cells as described above. Plasmid DNA was isolated using a Mini-prep Kit (QIAGEN Inc.). The insert was confirmed by DNA sequencing. The resulting plasmid was designated pAgJg124.

5

Example 35: Targeting the *Aspergillus fumigatus* beta-glucosidase variant gene into a protease locus of *Trichoderma reesei* strain AgJg115-104-7B1

Protoplast preparation and transformation of *Trichoderma reesei* AgJg115-104-7B1 were performed as described in Example 1.

10 Plasmid pAgJg124 (66 µg; Example 34) was digested with *Pme* I and purified by 1% agarose gel electrophoresis in TAE buffer where a 10.3 kb band was excised from the gel, and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4). Approximately 2.5 µg of the resulting purified DNA fragment were added to 100 µl of the protoplast solution as described in Example 1. The transformation reaction was spread onto
15 COVE plates. The plates were incubated at 28°C for 7-10 days. The transformants were sub-cultured onto COVE2 + 10 mM uridine plates to generate spores.

The transformants of *T. reesei* strain AgJg115-104-7B1 were screened by Fungal Spore PCR according to Example 9 for the presence of the pAgJg124 replacement vector at the *T. reesei* protease locus, thereby deleting the coding sequence of the protease and
20 replacing it with the *A. fumigatus* beta-glucosidase variant gene. The Spore PCRs were composed of 0.5 µl of each spore suspension, 50 pmol of primer 0614119, 50 pmol of primer 0614037, 10 µl of 2X PHIRE® Plant PCR Buffer, and 0.4 µl of PHIRE® Hot Start II DNA Polymerase in a 20 µl reaction. The reactions were performed in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 5 seconds, 65°C
25 for 5 seconds, and 72°C for 50 seconds; 1 cycle at 72°C for 1 minute; and a 10°C hold. Primer 0614119 is located upstream of the 5' flanking region and primer 0614037 is located at the beginning of the *A. fumigatus* beta-glucosidase variant gene. If the replacement vector integrates into the protease locus, the amplified PCR fragment will be 2.7 kb in length.

Primer 0614119 (forward):

30 5'-CTGAGGAAAGGCAGTCTTCACATTC-3' (SEQ ID NO: 153)

Primer 0614037 (reverse):

5'-GCATCACAAACCTGGGCATTGGCTACAGAA-3' (SEQ ID NO: 154)

For Southern blot analysis, 2 µg of each genomic DNA were digested with 10 units of *Nru* I and 20 units of *Bam* HI in a 35 µl reaction volume. The digested DNA was subjected to
35 0.7% agarose gel electrophoresis in TAE buffer and transferred to a NYTRAN® SuperCharge membrane according to Example 3. The membrane was hybridized with a 500 bp digoxigenin-labeled *A. fumigatus* beta-glucosidase probe (Example 33).

The Southern blot analysis identified several transformants containing a single copy of the *A. fumigatus* beta-glucosidase variant coding sequence at the *T. reesei* protease locus. One such transformant was designated *T. reesei* AgJg124.

5 Example 36: Construction of an *Aspergillus fumigatus* beta-glucosidase variant expression vector targeted to a *Trichoderma reesei* homolog of dihydroflavonol-4-reductase locus

Plasmid pAgJg125 was constructed to comprise an *Aspergillus fumigatus* beta-glucosidase variant coding sequence under transcriptional control of the *Trichoderma reesei* cellobiohydrolase I gene promoter and terminator, with flanking sequence to target to a locus encoding a *T. reesei* homolog of dihydroflavonol-4-reductase (DHR) (JGI Protein ID: 111716), a locus that receives no translation signal as found via mRNA analysis. Two synthetic oligonucleotide primers shown below were designed to amplify by PCR the 5' flanking region of the *T. reesei* DHR gene from genomic DNA and introduce flanking regions for insertion into expression vector pMJ05 (U.S. Patent 8,497,115). Bold letters represent sequence of the 5' DHR flanking region and the remaining sequence is homologous to insertion sites of pMJ05.

Forward Primer (Primer 0613552):

5'-CGAATTGTTTAAACGAAGTCTGCTTCCTTGCAATTATGCA-3' (SEQ ID NO: 155)

Reverse Primer (Primer 0613553):

5'-CCTACATTCGGTCGAAACGAGAAGTTCTCAACTAAGGGCT-3' (SEQ ID NO: 156)

The PCR was composed of 118 ng of *T. reesei* genomic DNA, 10 µl of 10 mM dNTPs, 50 pmol of primer 0613552, 50 pmol of primer 0613553, 1X PHUSION® GC Buffer, and 2 units of PHUSION® Hot Start DNA polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 30 seconds; 30 cycles each at 98°C for 10 seconds, 55°C for 30 seconds, and 72°C for 1 minute; and 1 cycle at 72°C for 10 minutes. The PCR products were separated by 1% agarose gel electrophoresis in TAE buffer where a 1.5 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The 1.5 kb fragment was then cloned into pMJ05 using an IN-FUSION™ HD Cloning Kit. The vector was digested with *Sa*I and isolated by 1% agarose gel electrophoresis in TAE buffer where a 7.9 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4). The 1.5 kb 5' flanking region fragment and the digested vector were ligated together in a reaction resulting in expression plasmid pAgJg125A, in which the 5' flanking region of the *T. reesei* DHR gene was cloned upstream of the *T. reesei cbh1* promoter in pMJ05. The ligation reaction was composed of 1X IN-FUSION™ HD enzyme mix, 100 ng of pMJ05 digested with *Sa*I, and 150 ng of the *T. reesei*

5' DHR flanking region fragment in a 10 µl reaction. The reaction was incubated at 50°C for 15 minutes. Then 2.5 µl of the reaction were transformed into SoloPack® Gold Supercompetent cells as described in Example 34. Plasmid DNA was isolated using a Mini-prep Kit (QIAGEN Inc.). The insert was confirmed by DNA sequencing. The resulting plasmid was designated pAgJg125A.

To amplify the 3' flanking region of the *T. reesei* DHR gene, two synthetic oligonucleotide primers shown below were designed for insertion into expression vector pAgJg125A. Bold letters represent sequence of the 3' DHR flanking region and the remaining sequence is homologous to insertion sites of pAgJg125A.

Forward Primer (Primer 0613554):

5'-AAACGATTCCTTAAT**GTTATCTGTACGCTCTGATGAGAAG**-3' (SEQ ID NO: 157)

Reverse Primer (Primer 0613555):

5'-CAGTCACCTCTAGTTAG**GACAGCTGGCCTAGAGCGCTCAGCAGACTCCTTT**-3' (SEQ ID NO: 158)

The PCR was composed of 118 ng of *T. reesei* genomic DNA, 10 µl of 10 mM dNTPs, 50 pmol of primer 0613554, 50 pmol of primer 0613555, 1X PHUSION® GC Buffer, and 2 units of PHUSION® Hot Start DNA polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 30 seconds; 30 cycles each at 98°C for 10 seconds, 55°C for 30 seconds, and 72°C for 1 minute; and 1 cycle at 72°C for 10 minutes. The PCR products were separated by 1% agarose gel electrophoresis in TAE buffer where a 1.5 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The 1.5 kb fragment was then cloned into pAgJg125A using an IN-FUSION™ HD Cloning Kit. The vector was digested with *Pac* I and isolated by 1% agarose gel electrophoresis in TAE buffer where a 9.4 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4). The 1.5 kb 3' flanking region fragment and the digested vector were ligated together in a reaction resulting in expression plasmid pAgJg125B, in which the 3' flanking region of the *T. reesei* DHR gene was cloned downstream of the *T. reesei cbh1* terminator in pAgJg125A. The ligation reaction was composed of 1X IN-FUSION™ HD enzyme mix, 100 ng of pAgJg125A digested with *Pac* I, and 150 ng of the *T. reesei* 3' DHR flanking region fragment in a 20 µl reaction. The reaction was incubated at 50°C for 15 minutes. A 2.5 µl volume of the cloning reaction was transformed into SoloPack® Gold Supercompetent cells as described in Example 34. Plasmid DNA was isolated using a Mini-prep Kit (QIAGEN Inc.). The insert was confirmed by DNA sequencing. The resulting plasmid was designated pAgJg125B.

Finally, two synthetic oligonucleotide primers shown below were designed to amplify a portion of the *T. reesei cbh1* promoter and the entire coding sequence of the *A. fumigatus*

beta-glucosidase variant for insertion into expression vector pAgJg125B. Bold letters represent sequence of the *T. reesei cbh1* promoter and *A. fumigatus* beta-glucosidase variant fragment, while the remaining sequence is homologous to insertion sites of pAgJg125B.

5 Forward Primer (Primer 0613727):

5'-CTAAGATCTCGGGCCCTCGGGCCTTCGGCCTTTGGGTGTACATGT-3' (SEQ ID NO: 159)

Reverse Primer (Primer 0613728):

5'-GCTACTAGTACGCGTCTAGTAGACACGGGGCAGAGGCGCT-3' (SEQ ID NO: 160)

10 The PCR was composed of 200 ng of pDFng133-3 DNA, 10 µl of 10 mM dNTPs, 50 pmol of primer 0613727, 50 pmol of primer 0613728, 1X PHUSION® GC Buffer, and 2 units of PHUSION® Hot Start DNA polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 30 seconds; 30 cycles each at 98°C for 10 seconds, 60°C for 30 seconds, and 72°C for 2 minutes; and 1 cycle at 72°C
15 for 7 minutes. The PCR products were separated by 1% agarose gel electrophoresis in TAE buffer where a 3.5 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 9).

The 3.5 kb fragment was then cloned into pAgJg125B using an IN-FUSION™ HD Cloning Kit. The vector was digested with *Apa* I and *Mlu* I and isolated by 1% agarose gel
20 electrophoresis in TAE buffer where a 10.9 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4). The 3.5 kb *T. reesei cbh1* promoter and *A. fumigatus* beta-glucosidase variant fragment and the digested vector were ligated together in a reaction resulting in the expression plasmid pAgJg125, in which the *A. fumigatus* beta-glucosidase variant coding sequence, under transcriptional
25 control of the *T. reesei cbh1* promoter and terminator, has 5' and 3' flanking regions for targeting to the *T. reesei* DHR gene locus. The ligation reaction was composed of 1X IN-FUSION™ HD enzyme mix, 100 ng of pAgJg125B digested with *Apa* I and *Mlu* I, and 150 ng of the *T. reesei cbh1* promoter and *A. fumigatus* beta-glucosidase variant fragment in a 20 µl reaction. The reaction was incubated at 50°C for 15 minutes. Then 2.5 µl of the reaction
30 were transformed into SoloPack® Gold Supercompetent cells as described in Example 34. Plasmid DNA was isolated using a Mini-prep Kit (QIAGEN Inc.). The insert was confirmed by DNA sequencing. The resulting plasmid was designated pAgJg125.

**Example 37: Targeting the *Aspergillus fumigatus* beta-glucosidase variant gene into a
35 *Trichoderma reesei* homolog of dihydroflavonal-4-reductase locus of *Trichoderma reesei* strain AgJg115-104-7B1**

Protoplast preparation and transformation of *Trichoderma reesei* AgJg115-104-7B1

were performed as described in Example 1.

A total of 112 µg of the transforming plasmid pAgJg125 (Example 36) was digested with *Pme* I and purified by 1% agarose gel electrophoresis in TAE buffer where a 10.3 kb band was excised from the gel, and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4). Approximately 3.377 µg of the resulting purified DNA fragment were added to 100 µl of the protoplast solution according to Example 1. The transformation reaction was spread onto COVE plates. The plates were incubated at 28°C for 7-10 days. Transformants were sub-cultured onto COVE2 + 10 mM uridine plates to generate spores.

The transformants of *T. reesei* strain AgJg115-104-7B1 were screened by Fungal Spore PCR according to Example 9 for the presence of the pAgJg125 replacement vector at the *T. reesei* DHR gene locus, thereby deleting the coding sequence of the DHR gene and replacing it with the *A. fumigatus* beta-glucosidase variant coding sequence. The Spore PCRs were composed of 0.5 µl of spore suspension, 50 pmol of primer 0614118, 50 pmol of primer 0614037, 10 µl of 2X PHIRE® Plant PCR Buffer, and 0.4 µl of PHIRE® Hot Start II DNA Polymerase in a 20 µl reaction. The reactions were performed in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 5 seconds, 68°C for 5 seconds, and 72°C for 50 seconds; 1 cycle at 72°C for 1 minute; and a 10°C hold. Primer 0614118 is located upstream of the 5' flanking region and primer 0614037 is located at the beginning of the *A. fumigatus* beta-glucosidase variant gene. If the replacement vector integrates into the DHR gene locus, the amplified PCR fragment will 2.7 kb in length.

Primer 0614118 (forward):

5'-ATCTCATCCCACGAGAAGGTTATGC-3' (SEQ ID NO: 161)

Primer 0614037 (reverse):

5'-GCATCACAAACCTGGGCATTGGCTACAGAA-3' (SEQ ID NO: 162)

For Southern blot analysis, 2 µg of each genomic DNA was digested with 10 units of *Nco* I and 10 units of *Sca* I. The digested DNA was subjected to 0.7% agarose gel electrophoresis in TAE buffer and transferred to a NYTRAN® SuperCharge membrane according to Example 3. The membrane was hybridized with a 500 bp digoxigenin-labeled *A. fumigatus* beta-glucosidase probe (Example 32).

The Southern blot analysis identified one transformant designated *T. reesei* AgJg125 as containing a single copy of the *A. fumigatus* beta-glucosidase variant coding sequence replacing the *T. reesei* DHR coding sequence.

Example 38: Construction of plasmid pAgJg133

Plasmid pAgJg133 was constructed to comprise the *Trichoderma reesei* endoglucanase I (EG1) gene promoter, the *T. reesei* cellobiohydrolase I gene (*cbh1*) terminator, and restriction sites to clone in a gene of interest. Two synthetic oligonucleotide

primers shown below were designed to amplify by PCR the *T. reesei* EG1 promoter region (1 kb sequence upstream of the EG1 coding sequence) from genomic DNA and introduce flanking regions for insertion into expression vector pSMai226 (WO 2012/083081). Bold letters represent coding sequence and the remaining sequence is homologous to insertion sites of pSMai226. The reverse primer also includes a *Spe* I site and *Pac* I site for cloning in a gene of interest.

Forward Primer:

5'-GTTTAAACGTCGACGCATGGAAGTGGTATGTACCATCGTGCTCTGT-3' (SEQ ID NO: 163)

Reverse Primer:

5'-CTTTCGCCACGGAGCTTAATTAATAGTACGCGTAGATCTGCGGACTAGTTTGGGAC AACAAGAAGGAC-3' (SEQ ID NO: 164)

The PCR was composed of 90 ng of *T. reesei* genomic DNA, 1 µl of 10 mM dNTPs, 50 pmol of the forward primer, 50 pmol of the reverse primer, 1X PHUSION® HF buffer, and 2 units of PHUSION® Hot Start DNA polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 30 seconds; 30 cycles each at 98°C for 10 seconds, 59°C for 10 seconds, and 72°C for 30 seconds; and 1 cycle at 72°C for 10 minutes. The PCR products were separated by 1% agarose gel electrophoresis in TAE buffer where a 1 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4).

The 1 kb fragment was then cloned into pSMai226 using an IN-FUSION™ HD Cloning Kit. The vector was digested with *Sph* I and *Spe* I and isolated by 1% agarose gel electrophoresis in TAE buffer where a 6.2 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit (Example 4). The 1 kb fragment and the digested vector were ligated together in a reaction resulting in expression plasmid pAgJg133. The ligation reaction (20 µl) was composed of 1X IN-FUSION™ HD enzyme mix, 186 ng of pSMai226 digested with *Spe* I and *Sph* I, and 190 ng of the *T. reesei* EG1 promoter purified PCR product. The reaction was incubated at 50°C for 15 minutes. Four µl of the reaction were transformed into ONE SHOT® TOP10 competent cells according to Example 4. Plasmid DNA was isolated using a Mini-prep Kit (QIAGEN Inc.). The insert was confirmed by DNA sequencing. The resulting plasmid was designated pAgJg133.

Example 39: Construction of plasmid pGMEr189

Plasmid pGMEr189 is the expression plasmid for the *T. reesei* codon optimized cDNA for the *Talaromyces leycettanus cbh2* gene. Such cDNA for the *T. leycettanus cbh2* gene was obtained by designing three 500 bp DNA fragments (G-blocks) with 5' and 3'

homology with one another to facilitate the correct assembly of the gene cDNA. The sequence of the three G-blocks used are listed below and the regions highlighted in *italics* and in underlined characters show the regions of homology between the DNA blocks:

G-block 1:

5 **TGTAAGATCACCTCTGTGTATTGCACCAT**GC GGTCTCTTCTCGCCCTTGACCTACTC
TACTCGCGCCCGTTGTGCAGGCCAGCAGACCATGTGGGGCCAATGTGGCGGCCAAG
GCTGGACCGGCCGACGATCTGTGTTGCCGGCGCAACATGTAGCACACAGAATCCCT
GGTACGCTCAGTGTACCCCGGCACCTACCGCGCCGACGACTTTGCAAACGACGACGA
CGACGAGCTCGAAATCGTCCACGACCACCAGCTCGAAGTCGTCCACTACCACAGGTGG
10 AAGTGGCGGTGGTACTACTACATCCACGTCAGCCACCATCACCGCGGCACCATCCGGT
AACCTTACAGCGGCTACCAGCTGTATGTGAACCAGGAATACTCCTCCGAGGTCTACG
CGTCTGCCATTCTTCTCTGACCGGCACTCTGGTCGCGAAGGCTAGTGCTGCGGCTGA
AGTGCCTCATTCTGTGGCTGGACACTGCCTCCAAGG (SEQ ID NO: 165)

G-block 2:

15 GAAGTGCCCTCATTCTGTGGCTGGACACTGCCTCCAAGGTGCCCTGATGGGAACCT
ACCTGCAGGACATCCAGGCCAAGAACGCTGCCGGCGCCAACCCTCCTTACGCTGGTC
AATTCGTGGTCTACGACTTGCCGGACCGTGACTGCGCCGCTCTGGCCAGTAATGGCGA
GTACTCAATTGCCAACACGGTGTGGCCAACTACAAGGCGTACATTGACTCCATCCGTG
CTCTTCTGGTGCAATACTCTAACGTTACGTCATCCTCGTCATCGAACCCGACAGCTTG
20 GCCAACCTGGTGACCAACCTCAACGTCCAGAAATGCGCCAACGCCAGAGCGCCTACC
TGGAGTGTATCAACTATGCTCTGACTCAGCTCAACCTGAAGAACGTCGCCATGTACATC
GACGCAGGCCATGCGGGCTGGCTCGGATGGCCCGCCAACCTTGAGCCCCGCCGCACAA
CTCTTCGCCTCCGTCTACCAGAATGCCAGTTCCCC (SEQ ID NO: 166)

G-block 3:

25 CGCCTCCGTCTACCAGAATGCCAGTTCCCCCGCGGCTGTTCTGTGGCCTGGCCACCAAC
GTCGCCAACTACAACGCCTGGTTCGATCGCCACCTGCCCCCTCCTACACCCAGGGAGACC
CCAACTGCGACGAGCAGAAGTACATCAACGCCCTGGCCCCCTTCTCCAGCAACAGGG
CTGGTCATCAGTTCACTTCATCACCGATAACGGCCGGAATGGCGTCCAGCCCACGAAG
CAAAACGCCTGGGGTGAAGTGGTGAACGTCATCGGCACCGGCTTCGGTGTTGCCCC
30 ACCACGAACACCGGCGATCCGCTCGAGGATGCCTTTGTGTGGGTCAAGCCCGGTGGA
GAGAGTGATGGCACGTCCAACCTCGACTTCCCCTCGGTATGACGCCCACTGCGGATATA
GTGATGCTCTGCAGCCTGCTCCTGAGGCAGGTACTTGGTTCGAGGCCTACTTTGAGCA
GCTTCTGACCAACGCTAACCCGTCCTTTTAATAGTTAA (SEQ ID NO: 167)

The sequence highlighted in bold at the beginning of G-block 1 and at the end of G-block 2 indicates the homology with the sense PCR primer 1203972 and the antisense PCR primer 1203973 used in the G-blocks assembly reaction:

Primer 1203972 (sense):

5'-GCAGCTCACCTGAAGAGGCTTGTAAGATCACCTCTGTGTATTGCACCAT-3' (SEQ ID NO: 168)

Primer 1203973 (antisense):

5'-CCGGTCACGAAAGCCTTAATTAATACTATTAAGGACGGGTTAGCGTTGG-3' (SEQ ID NO: 169)

The three G-blocks were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa) and received as dry DNA fragments. The 500 bp G-block 1, the 500 bp G-block 2, and the 500 bp G-block 3 were assembled together by PCR using primer 1203972 (sense) and primer 1203973 (antisense), resulting in a 1469 bp fragment comprising the entire cDNA for the *T. leycettanus cbh2* gene. The PCR (50 µl) was composed of an equimolecular ratio of all the three G-block fragments for a total amount of DNA of approximately 120 ng, 1X PHUSION® HF buffer, 50 pmol of primer 1203972, 50 pmol of primer 1203973, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µl of 100% DMSO, and 1 unit of PHUSION® High Fidelity DNA polymerase. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 35 cycles each at 98°C for 30 seconds, 60°C for 30 seconds, and 72°C for 2 minutes and 30 seconds; and a final extension cycle at 72°C for 7 minutes. The completed PCR was submitted to 0.8% agarose gel electrophoresis in TBE buffer where an approximately 1469 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit.

The resulting 1469 bp fragment, comprising the complete *T. leycettanus cbh2* gene cDNA, was cloned after PCR and gel purification into plasmid pCR®4-blunt TOPO® (Life Technologies Corp.) and transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Transformants were spread onto 2XYT plus ampicillin plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired fragment by sequencing analysis. One transformant containing the correct plasmid was identified and the plasmid was designated pGMEr187.

The *T. leycettanus cbh2* cDNA was PCR cloned out of plasmid pGMEr187 using primer 1203972 (sense) and primer 1203973 (antisense). The PCR (50 µl) was composed of an equimolecular ratio of about 100 ng of plasmid pGMEr187, 1X PHUSION® HF buffer, 50 pmol of primer 1203972, 50 pmol of primer 1203973, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µl of 100% DMSO, and 1 unit of PHUSION® High Fidelity DNA polymerase. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 35 cycles each at 98°C for 30 seconds, 60°C for 30 seconds, and 72°C for 50 seconds; and a final extension cycle at 72°C for 7 minutes. The completed PCR was submitted to 0.8% agarose gel electrophoresis in TBE buffer where an approximately 1469 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit.

The resulting 1469 bp PCR fragment (*T. leycettanus cbh2* cDNA) was inserted into *Nco* I/*Pac* I-linearized plasmid pJfyS142 (PCT/US2012/052143, WO2013028912 A2) using an IN-FUSION® Advantage PCR Cloning Kit. The reaction was composed of 1X IN-FUSION® Reaction Buffer, 50 ng of *Nco* I/*Pac* I-linearized pJfyS142, 100 ng of the *T. leycettanus cbh2* cDNA fragment (1469 bp), and 1 µl of IN-FUSION® Enzyme in a 10 µl reaction volume. The reaction was incubated for 15 minutes at 50°C. Then 40 µl of TE were added to the reaction and a 2 µl aliquot of the reaction was transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Transformants were spread onto 2XYT plus ampicillin plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired insert by *Sac* I restriction digestion. Plasmid DNA was extracted and purified using a QIAGEN® Plasmid Mini Kit. A transformant containing a plasmid yielding the desired band sizes of 4223 bp, 3912 bp, 2741 bp and 874 bp was isolated and the plasmid was designated pGMEr189.

Example 40: Construction of plasmid pECW1

Plasmid pECW1 was constructed to comprise the 5' and the 3' flanking regions of the *T. reesei* strain AgJg115-104-7B1 (WO 2011/075677) *cbh1* locus to facilitate homologous recombination at the *cbh1* locus.

The *cbh1* locus 5' flanking region was PCR amplified from *T. reesei* strain AgJg115-104-7B1 genomic DNA using the primers shown below. Genomic DNA was prepared according to Example 2.

Primer 1205799 (sense):

Pme I

5'-CGGTTTAAACGGGAGAGCAACAACAATCATTCTGCTGTCG-3' (SEQ ID NO: 170)

Primer 1205800 (anti-sense):

Pac I

Nco I

5'-TTATTAATTAAGTAGTACGCGTAGATCTGCGGCCATGGGCTTCGAACAGCCCCAGTCGGTCAAGCAGGCA-3' (SEQ ID NO: 171)

The sense primer added a *Pme* I restriction site at the 5' end of the fragment while the anti-sense primer added *Pac* I and *Nco* I restriction sites at its 3' end.

The PCR (50 µl) was composed of 100 ng of *T. reesei* strain AgJg115-104-7B1 genomic DNA, 1X PHUSION® HF buffer, 50 pmol of primer 1205799, 50 pmol of primer 1205800, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µl of 100% DMSO, and 1 unit of PHUSION® High Fidelity DNA polymerase. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 30 seconds, 58°C for 30 seconds, and 72°C for 3 minutes; and a final extension cycle at 72°C for 7 minutes. The completed PCR was submitted to 0.8% agarose gel electrophoresis in TBE buffer where

an approximately 1550 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit (Macherey-Nagel Inc.).

A second PCR fragment containing 5' homology to the PCR product above, including *Pac* I and the *Nco* I sites, was generated to amplify the *cbh1* locus 3' flanking region from *T. reesei* AgJg115-104-7B1 genomic DNA using the following primers:

Primer 1205801 (sense):

Nco I

Pac I

5'-GCCCATGGCCGCAGATCTACGCGTACTAGTTAATTAATAAGCTCCGTGGCGAAAGC
CTGACGCACCGGT-3' (SEQ ID NO: 172)

Primer 1205802 (anti-sense):

Pme I

5'-CGGTTTAAACGGACTTCGGTGGAGGTGTCGAGTACGAGT-3' (SEQ ID NO: 173)

The sense primers added *Nco* I and a *Pac* I restriction sites at the 5' end of the fragment while the anti-sense primer added a *Pme* I restriction site at its 3' end.

The PCR (50 µl) was composed of about 100 ng of *T. reesei* strain AgJg115-104-7B1 genomic DNA, 1X PHUSION® HF buffer, 50 pmol of primer 1205801, 50 pmol of primer 1205802, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µl of 100% DMSO, and 1 unit of PHUSION® High Fidelity DNA polymerase. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 30 seconds, 58°C for 30 seconds, and 72°C for 3 minutes; and a final extension cycle at 72°C for 7 minutes. The completed PCR was submitted to 0.8% agarose gel electrophoresis in TBE buffer where an approximately 1549 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit.

The 1550 bp and 1549 bp fragments were fused together by PCR using primer 1205799 (sense) and primer 1205802 (anti-sense), resulting in a 3049 bp fragment in which the *cbh1* locus 5' and 3' flanking regions were joined together. The PCR (50 µl) was composed of about 80 ng of each of the fragments, 1X PHUSION® HF buffer, 50 pmol of primer 1205799, 50 pmol of primer 1205802, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µl of 100% DMSO, and 1 unit of PHUSION® High Fidelity DNA polymerase. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 3 minutes; 40 cycles each at 98°C for 30 seconds, 60°C for 30 seconds, and 72°C for 3 minutes; and a final extension cycle at 72°C for 7 minutes. The completed PCR was submitted to 0.8% agarose gel electrophoresis in TBE buffer where an approximately 3049 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit.

The resulting 3049 bp fragment was digested with *Pme* I and ligated to the 2647 bp *Pme* I fragment from plasmid pGMEr189 (Example 39) bearing the ampicillin resistance gene and the *E. coli* origin of replication. About 20 µg of plasmid pGMEr189 were digested

with *Pme* I at 37°C for 3 hours. One hour before the end of the digestion, 1 µl of calf intestinal alkaline phosphatase was added to the pGMEr189-*Pme* I digestion in order to dephosphorylate the ends and prevent self-ligation. The resulting digestion was submitted to 0.8% agarose gel electrophoresis in TBE buffer where a 2647 bp vector fragment, containing the ampicillin resistance gene and the *E. coli* origin of replication, was excised from the gel and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit.

The ligation reaction was performed using a QUICK LIGATION™ Kit. The ligation reaction was composed of 2 µl of pGMEr189 vector fragment, 3 µl of the *cbh1* locus 5' flanking region/3' flanking region insert fragment, 5 µl of sterile deionized water, 10 µl of 2X Quick Ligation Buffer, and 1 µl of Quick T4 Ligase. The ligation reaction was incubated for 1 hour at room temperature. A 5 µl aliquot of the ligation reaction was transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Transformants were spread onto 2XYT plus ampicillin plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired insert by *Pme* I restriction digestion. Plasmid DNA was extracted and purified using a Plasmid Mini Kit (QIAGEN Inc.). A transformant containing a plasmid yielding the desired band sizes of 2647 bp and of 3047 bp was isolated and the plasmid was designated pECW1.

Plasmid pECW1 comprises the *cbh1* locus 5' flanking region/3' flanking region, and restriction sites for *Nco* I and *Pac* I in the sequence spacer between the two flanking regions.

Example 41: Construction of plasmid pSaMe-TsGH10

Plasmid pSaMe-TsGH10 was constructed to comprise the *Trichoderma reesei* cellobiohydrolase I gene promoter and terminator and the *Trichophaea saccata* GH10 xylanase coding sequence. Two synthetic oligonucleotide primers shown below were designed to PCR amplify the *Trichophaea saccata* GH10 xylanase gene from plasmid pDAu81#5 (WO 2011/057083) and introduce flanking regions for insertion into expression vector pMJ09 (WO 2005/056772). Bold letters represent coding sequence and the remaining sequence is homologous to the insertion sites of pMJ09.

Forward Primer:

5'-cggactg~~cg~~caccat**gcgtac**cttctcgtctctt-3' (SEQ ID NO: 174)

Reverse Primer:

5'-tcgccacggagcttat**caagccgcaagagcagacg**-3' (SEQ ID NO: 175)

Cloning of the *Trichophaea saccata* xylanase followed the overall expression cloning protocol described below.

Fifty picomoles of each of the primers above were used in a PCR composed of 50 ng of plasmid pDAu81#5 DNA, 1 µl of 10 mM blend of dATP, dTTP, dGTP, and dCTP, 5 µl of 10X PLATINUM® *Pfx* DNA Polymerase Buffer, and 1 unit of PLATINUM® *Pfx* DNA

Polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 94°C for 2 minutes; and 30 cycles each at 94°C for 15 seconds, 55°C for 30 seconds, and 68°C for 1 minute. After the 30 cycles, the reaction was incubated at 72°C for 10 minutes and then cooled to 4°C until further processing.

5 The reaction products were isolated by 1.0% agarose gel electrophoresis in TAE buffer where a 1.2 kb product band was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Example 9).

The 1.2 kb fragment was then cloned into pMJ09 using an IN-FUSION® Advantage PCR Cloning Kit. The vector was digested with *Nco* I and *Pac* I and purified by agarose gel electrophoresis as described above. The gene fragment and the digested vector were ligated together in a reaction resulting in the expression plasmid pSaMe-TsGH10 in which transcription of the xylanase gene was under the control of the *T. reesei cbh1* gene promoter. The ligation reaction (50 µl) was composed of 1X IN-FUSION® Reaction Buffer, 1X BSA, 1 µl of IN-FUSION® Enzyme (diluted 1:10), 100 ng of pMJ09 digested with *Nco* I and *Pac* I, and 100 ng of the *Trichophaea saccata* xylanase purified PCR product. The reaction was incubated at room temperature for 30 minutes. One µl of the reaction was used to transform *E. coli* XL10 SOLOPACK® Gold cells. An *E. coli* transformant containing pSaMe-AfGH10 was detected by restriction enzyme digestion and plasmid DNA was prepared using a BIOROBOT® 9600 (QIAGEN Inc.). DNA sequencing of the *Trichophaea saccata* xylanase gene from pSaMe-TsGH10 confirmed the correct sequence of plasmid pSaMe-TsGH10.

Example 42: Construction of plasmid pECW2

Plasmid pECW2 was constructed from plasmid pECW1 to comprise the *cbh1* locus 5' and 3' flanking regions upstream and downstream, respectively, of the *A. nidulans amdS* gene expression cassette with TP901-1 *attP* sites added at its 5' and 3' ends.

The TP901-1 *attP* site is 56 bp long and its sequence is shown below. The portions of the *attP* sequence in *italics* and underlined highlight the fragments included in the PCR primers used to add the TP901-1 *attP* site at each end of the *amdS* gene expression cassette.

attP site (56 bp):

TCCAACTCGCTTAATTGCGAGTTTTTATTTCGTTTATTTCAATTAAGGTAACTAAA (SEQ ID NO: 176)

The *amdS* expression cassette was PCR amplified from plasmid pSaMe-TsGH10 using the primers shown below.

Primer 1205803 (sense):

5'-CGTTTATTTCAATTAAGGTAACTAAATTCTACGCCAGGACCGAGCAAGCCCAGATGAG

AA-3' (SEQ ID NO: 177)

Primer 1205804 (anti-sense):

5'-ATAAAACTCGCAATTAAGCGAGTTGGAATGCATCTGGAAACGCAACCCTGAAGGGA
TTC-3' (SEQ ID NO: 178)

5 The sense primer added the second half of the TP901-1 *attP* site at the 5' end of the *amdS* expression cassette (underlined sequence), while the anti-sense primer added the first half of the *attP* site at its 3' end (sequence in italics).

The PCR (50 µl) was composed of 25 ng of pSaMe-TsGH10 DNA, 1X PHUSION® HF buffer, 50 pmol of primer 1205803, 50 pmol of primer 1205804, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µl of 100% DMSO, and 1 unit of PHUSION® High Fidelity DNA polymerase. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 30 seconds, 58°C for 30 seconds, and 72°C for 3 minutes; and a final extension cycle at 72°C for 7 minutes. The completed PCR was submitted to 0.8% agarose gel electrophoresis in TBE buffer where an approximately 2780 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit.

To complete the TP901-1 *attP* sites at the 5' and 3' ends of the *amdS* expression construct a second PCR was performed using the PCR product described above as template DNA and the primers shown below.

20 Primer 1205805 (sense):

Nco I

5'-GCCCATGGTCCAACCTCGCTTAATTGCGAGTTTTTATTTCTTTATTTCAATTAAGGT
AACTAAATTCTACGCCAG-3' (SEQ ID NO: 179)

Primer 1205806 (anti-sense):

25 *Pac* I

5'-CGTTAATTAA**TTTAGTTACCTTAATTGAAATAAACGAAATAAA**ACTCGCAATTAAG
CGAGTTGGAATGCATCTG-3' (SEQ ID NO: 180)

30 The sense and antisense primers completed the TP901-1 *attP* sites flanking the *amdS* expression construct and added an *Nco* I restriction site at the 5' end of the construct and a *Pac* I site at its 3' end. The bold portion of the sequence in both primers highlights the complete TP901-1 *attP* site.

35 The PCR (50 µl) was composed of 25 ng of the 2780 bp PCR product, 1X PHUSION® HF buffer, 50 pmol of primer 1205805, 50 pmol of primer 1205806, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µl of 100% DMSO, and 1 unit of PHUSION® High Fidelity DNA polymerase. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 30 seconds, 58°C for 30 seconds, and 72°C for 3 minutes; and a final extension cycle at 72°C for 7 minutes. The completed

PCR was submitted to 0.8% agarose gel electrophoresis in TBE buffer where an approximately 2856 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit.

The resulting 2856 bp fragment was digested with *Pac* I and *Nco* I and ligated to *Pac* I/*Nco* I digested plasmid pECW1. About 20 µg of plasmid pECW1 was double digested with *Pac* I and *Nco* I at 37°C for 4 hours. One hour before the end of the digestion reaction, 1 µl of calf intestinal alkaline phosphatase was added to the pECW1-*Pac* I/*Nco* I digestion in order to de-phosphorylate the ends to prevent self-ligation. The resulting digestion was submitted to 0.8% agarose gel electrophoresis in TBE buffer where a 5663 bp vector fragment was excised from the gel and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit.

The ligation reaction was performed using a QUICK LIGATION™ Kit. The ligation reaction was composed of 2 µl of vector fragment, 3 µl of the insert fragment, 5 µl of sterile deionized water, 10 µl of 2X Quick Ligase Buffer, and 1 µl of Quick T4 Ligase. The ligation reaction was incubated for 1 hour at room temperature. A 5 µl aliquot of the ligation reaction was transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Transformants were spread onto 2XYT plus ampicillin plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired insert by *Nco* I and *Sca* I double digestion. Plasmid DNA was extracted and purified using a QIAGEN® Plasmid Mini Kit. One transformant containing a plasmid yielding restriction fragments of 2425 bp and 6086 bp was identified and the plasmid was designated pECW2 (Figure 5).

Plasmid pECW2 comprises the *amdS* expression cassette flanked by the TP901-1 *attP* sites, the *cbh1* locus 5' flanking region upstream, and the *cbh1* locus 3' flanking region downstream.

Example 43: Construction of *Trichoderma reesei* strain ECW1

When inserting the TP901-1 *attP* sites at the *cbh1* locus in a *Trichoderma reesei* host strain, the *amdS* marker gene was chosen as a reporter gene to track the integration event. In plasmid pECW2, the *amdS* expression cassette, flanked by the *attP* sites, was inserted between the *cbh1* locus 5' and 3' flanking regions, allowing homologous recombination at the target locus with a gene replacement.

Protoplast preparation and transformation of *T. reesei* AgJg115-104-7B1 were performed according to the protocol described in Example 1.

Approximately 100 µg of plasmid pECW2 were digested with *Pme* I, which excised a 5864 bp fragment comprising the *cbh1* flanking regions, and the *amdS* selection marker flanked by the *attP* sites. The 5864 bp fragment was separated by 0.8% agarose gel

electrophoresis in TBE buffer, excised from the gel, and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit.

Approximately 800 ng of the 5864 bp *Pme* I digested pECW2 fragment were added to 100 µl of the protoplast solution and mixed gently. PEG buffer (250 µl) was added, and the reaction was mixed and incubated at 34°C for 30 minutes. STC (3 ml) was then added and mixed and then the reaction was spread onto COVE plates for *amdS* selection. The plates were incubated at 28°C for 6-11 days.

Thirty primary transformants were identified and insertion of the *attP-amdS-attP* construct at the *cbh1* locus of *T. reesei* AgJg115-104-7B1 was verified by PCR analysis of the upstream and downstream integration sites. Each putative primary transformant was patched onto COVE2 plates supplemented with 10 mM uridine and incubated at 30°C for 2-3 days. Transformants were screened using a PHIRE® Plant Direct PCR Kit. Briefly, a small amount of spores from each transformant was resuspended in 30 µl of Dilution Buffer (provided with the Kit) and incubated at room temperature for 10 minutes. The samples were then centrifuged using a table top microcentrifuge and 1 µl of each supernatant was used in the PCRs as template DNA.

The 5' integration site was confirmed using the primers shown below.

Primer 1206299 (sense):

5'-ACAGCACTCTCTCGCCCAATGATG-3' (SEQ ID NO: 181)

Primer 1206000 (anti-sense):

5'-GGGCGAACTTGACTGTCTGTC-3' (SEQ ID NO: 182)

The 3' integration site was confirmed using the primers shown below.

Primer 1205994 (sense):

5'-TTCCATCTCTCAAAGGAAGA-3' (SEQ ID NO: 183)

Primer 1206301 (anti-sense):

5'-CAGTTTCAGCCCTAGAAGCGCC-3' (SEQ ID NO: 184)

The total lengths of the resulting PCR fragments were approximately 1906 bp for the 5' integration site and 1672 bp for the 3' integration site.

The PCR (20 µl) was composed of 1 µl of genomic DNA, 1X PHIRE® Plant PCR Buffer, 0.5 µM of primer 1206299 or 1205994, 0.5 µM of primer 1206000 or 1206301, and 0.4 µl of PHIRE® Hot Start II DNA Polymerase. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 3 minutes; 40 cycles each at 98°C for 30 seconds, 57°C for 20 seconds, and 72°C for 1 minute and 15 seconds; and a final extension cycle at 72°C for 7 minutes. Following thermocycling, the PCR products were visualized by 0.8% agarose gel electrophoresis in TBE buffer.

Transformants showing the correct PCR products by both PCRs were chosen and grown in shake flasks by inoculating 25 ml of CIM in a 125 ml polycarbonate non-baffled shake flask with spores collected using a 10 µl inoculation loop. The flasks were incubated at 28°C for 5 days with agitation at 200 rpm. The shake flasks were harvested by pouring samples of the cultures into 1.5 ml microcentrifuge tubes and centrifuging the samples for 10 minutes at 13,000 rpm in a SORVALL® Biofuge Pico (Kendro Laboratory Products/Thermo Scientific). Samples were analyzed by SDS-PAGE using a CRITERION® 8-16% Tris-HCl Gel and 10 µl of PRECISION PLUS™ All Blue Protein Standards (Bio-Rad Laboratories, Inc.). Gels were stained with BIO-SAFE® Coomassie Stain (Bio-Rad Laboratories, Inc.). The SDS-PAGE analysis revealed that the positively identified primary transformants had lost the CBH I band, as a consequence of replacement of the *cbh1* gene with the *amdS* gene.

Sixteen positive transformants were spore purified by adding spores collected on a 10 µl inoculation loop to 1.5 ml of 0.01% TWEEN® 20. Spore dilutions of 1:1500 and 1:150 were spread onto 150 mm PDA plates and incubated at 28°C for 3 days. Two spore isolates per transformant were obtained and transferred to COVE2 plates supplemented with 10 mM uridine and incubated at 30°C for 2-3 days. Each spore isolate was analyzed again by PCR as described above to confirm the upstream and downstream sites of integration. Of the thirty-two spore isolates, seven were analyzed by Southern blotting to confirm correct integration of the *attP-amdS-attP* construct at the *cbh1* locus with consequent replacement of the *cbh1* gene. For Southern analysis genomic DNA was extracted according to Example 2.

Two µg of genomic DNA from each transformant were digested with *Nhe* I. The digestions were submitted to 0.7-0.8% agarose gel electrophoresis in TAE buffer and blotted onto a HYBOND® N+ blotting membrane using a TURBOBLOTTER® for approximately 1-2 hours. The membrane was hybridized with a digoxigenin-labeled probe specific for the 3' flanking region of the *cbh1* locus, which was synthesized using a PCR DIG Probe Synthesis Kit.

A probe hybridizing to the 3' flanking region of the *cbh1* gene was generated using a PCR DIG Probe Synthesis Kit with the forward and reverse primers shown below. The PCR (50 µl) was composed of 1X *Taq* DNA Polymerase Buffer, 50 pmol of each primer, 0.5X PCR DIG Probe Synthesis mix (Kit), 0.5X dNTP stock solution (Kit), 100 ng *T. reesei* RutC30 genomic DNA, and 2.5 units of *Taq* DNA polymerase. The PCR was incubated in a thermocycler programmed for 1 cycle at 95°C for 2 minute; 30 cycles each at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 40 seconds; and 1 cycle at 72°C for 15 minutes.

Forward primer:

5'-AATGACCCATAGGGAGACAAACAGCATAAT-3' (SEQ ID NO: 185)

Reverse primer:

5'-TGTTGGACGCAGGATTTTGA-3' (SEQ ID NO: 186)

The 0.56 kb probe was purified by 1% agarose gel electrophoresis in TAE buffer where a band corresponding to the probe was excised from the gel and extracted using a QIAQUICK® Gel Extraction Kit (Example 9).

Hybridization was performed as described in Example 3.

Transformants with the correct integration of pECW2 DNA produced a 3.5 kb hybridizing fragment. The untransformed strain produced a 6.0 kb hybridizing fragment. One transformant with correct integration of pECW2 was chosen and designated *T. reesei* ECW1.

Example 44: Construction of plasmid pECW4

Plasmid pECW4 was constructed to comprise the temperate *Lactococcus lactis* bacteriophage TP901-1 integrase expression cassette, where the integrase gene was under transcriptional control of a *T. reesei* glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) promoter and terminator and the SV40 virus nuclear localization signal (NLS) (SEQ ID NO: 187) added at the 5' end of the gene just after the ATG codon. To ensure maximum expression and activity in *T. reesei* the TP901-1 integrase coding sequence was codon optimized (SEQ ID NO: 188 [native DNA sequence], SEQ ID NO: 189 [codon-optimized DNA sequence], and SEQ ID NO: 190 [amino acid sequence]) and synthesized by GENEART® Gene Synthesis (Life Technologies).

The *Trichoderma reesei gpd* promoter fragment was PCR amplified from *Trichoderma reesei* strain AgJg115-104-7B1 genomic DNA using the primers shown below.

Primer 1205807 (sense):

5'-GGTTAATTAAGTACGTCAATGTAACGTCAAAGCCGCCCTC-3' (SEQ ID NO: 191)

Pac I

Primer 1205808 (anti-sense):

5'-CATTTTGTATCTGCGAATTGAGCTTGCGTGAGTCG-3' (SEQ ID NO: 192)

The sense primer added a *Pac I* restriction site at the 5' end of the fragment.

The PCR (50 µl) was composed of 100 ng of *T. reesei* AgJg115-104-7B1 genomic DNA, 1X PHUSION® HF buffer, 50 pmol of primer 1205807, 50 pmol of primer 1205808, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µl of 100% DMSO, and 1 unit of PHUSION® High Fidelity DNA polymerase. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 3 minutes; 40 cycles each at 98°C for 30 seconds, 57°C for 30 seconds, and 72°C for 1 minute and 30 seconds; and a final extension cycle at 72°C for 7 minutes. The completed PCR was submitted to 0.8% agarose gel electrophoresis in

TBE buffer where an approximately 995 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit.

The *Trichoderma reesei* *gpd* terminator fragment was PCR amplified from *Trichoderma reesei* strain AgJg115-104-7B1 genomic DNA using the primers shown below.

5 Primer 1205809 (sense):

5'-TAATAAGTGCTGTGTTCTCAGCAATGGGCCCCAGAAGGG-3' (SEQ ID NO: 193)

Primer 1205810 (anti-sense):

5'-CGCTTAATTAACGGCCTCTTGAGATCATTCTTCTTGCTCCTTTTC-3' (SEQ ID NO: 194)

10 *Pac* I

The anti-sense primer added a *Pac* I restriction site at the 3' end of the fragment.

The PCR (50 µl) was composed of 100 ng of *T. reesei* AgJg115-104-7B1 genomic DNA, 1X PHUSION® HF buffer, 50 pmol of primer 1205809, 50 pmol of primer 1205810, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µl of 100% DMSO, and 1 unit of PHUSION® High Fidelity DNA polymerase. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 3 minutes; 40 cycles each at 98°C for 30 seconds, 57°C for 30 seconds, and 72°C for 1 minute and 30 seconds; and a final extension cycle at 72°C for 7 minutes. The completed PCR was submitted to 0.8% agarose gel electrophoresis in TBE buffer where an approximately 614 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit.

The TP901-1 integrase codon optimized CDS plus the SV40 virus NLS was amplified by PCR from the GENEART® vector pMA and the primers shown below.

Primer 1205812 (sense):

5'-GCTCAGCACTCACGCAAGCTCAATTCGCAGATACAAAATGTCGGGTCTCCGTTCTCG
25 TCGCGACCCCAAGAAGAAGCGCAAGGTC-3' (SEQ ID NO: 195)

Primer 1205813 (anti-sense):

5'-CCCTTCTGGGGCCATTCTGAGGAACACAGCACTTATTAAGCGAGCTGAACTTGAA
GATAATGTCGACGTTGTCGGCAGTCACG-3' (SEQ ID NO: 196)

The sense primer added 37 bp of homologous sequence to the 3' end of the *gpd* promoter fragment upstream of the ATG codon of the codon optimized integrase coding sequence, and the anti-sense primer added 36 bp of homologous sequence to the 5' end of the *gpd* terminator fragment downstream of the stop codon of the integrase coding sequence.

The PCR (50 µl) was composed of 10 ng of the GENEART® vector pMA containing the codon optimized integrase coding sequence, 1X PHUSION® HF buffer, 50 pmol of primer 1205812, 50 pmol of primer 1205813, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µl of 100% DMSO, and 1 unit of PHUSION® High Fidelity DNA polymerase. The reaction

was performed in a thermocycler programmed for 1 cycle at 98°C for 3 minutes; 40 cycles each at 98°C for 30 seconds, 57°C for 30 seconds, and 72°C for 1 minute and 30 seconds; and a final extension cycle at 72°C for 7 minutes. The completed PCR was submitted to 0.8% agarose gel electrophoresis in TBE buffer where an approximately 1582 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit.

The 995 bp *gpd* promoter fragment and the 1585 bp TP901-1 NLS-integrase fragment were fused together by PCR using primer 1205807 (sense) and primer 1205813 (anti-sense), resulting in a 2540 bp fragment in which the *T. reesei gpd* promoter was placed upstream of the codon optimized integrase. The PCR (50 µl) was composed of an equimolecular ratio of both fragments for a total amount of DNA of approximately 125 ng, 1X PHUSION® HF buffer, 50 pmol of primer 1205807, 50 pmol of primer 1205813, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µl of 100% DMSO, and 1 unit of PHUSION® High Fidelity DNA polymerase. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 3 minutes; 40 cycles each at 98°C for 30 seconds, 60°C for 30 seconds, and 72°C for 2 minutes and 30 seconds; and a final extension cycle at 72°C for 7 minutes. The completed PCR was submitted to 0.8% agarose gel electrophoresis in TBE buffer where an approximately 2540 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit.

The 2540 bp PCR fragment comprising the *gpd* promoter fragment and TP901-1 NLS-integrase fragment were fused together by PCR to the 614 bp *gpd* terminator fragment using primer 1205807 (sense) and primer 1205810 (anti-sense), resulting in a 3115 bp fragment in which the *T. reesei gpd* terminator was placed downstream of the codon optimized integrase. The PCR (50 µl) was composed of an equimolecular ratio of both fragments for a total amount of DNA of approximately 200 ng, 1X PHUSION® HF buffer, 50 pmol of primer 1205807, 50 pmol of primer 1205810, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µl of 100% DMSO, and 1 unit of PHUSION® High Fidelity DNA polymerase. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 3 minutes; 40 cycles each at 98°C for 30 seconds, 60°C for 30 seconds, and 72°C for 3 minutes; and a final extension cycle at 72°C for 7 minutes. The completed PCR was submitted to 0.8% agarose gel electrophoresis in TBE buffer where an approximately 3115 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit.

The resulting 3115 bp fragment, comprising the complete TP901-1 integrase expression cassette, was cloned after PCR and gel purification into plasmid pCR®2.1-TOPO® (Life Technologies Corp.) and transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Transformants were spread onto 2XYT

plus ampicillin plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired fragment by *Eco* RI digestion and sequencing analysis. Plasmid DNA was extracted and purified using a QIAGEN® Plasmid Mini Kit. One transformant containing a plasmid yielding *Eco* RI restriction fragments of 3939 bp and 3133 bp was identified and the plasmid was designated pECW4.

Plasmid pECW4 comprises the TP901-1 integrase expression cassette, in which gene transcription is regulated by the *T. reesei gpd* promoter and terminator, and the SV40 virus NLS added after the ATG of the codon optimized integrase CDS.

10 Example 45: Construction of plasmid pECW5

Plasmid pECW5 was constructed to comprise the *hpt-tk* dual selectable marker system (WO 2010/039889) flanked by *attB* sites shown below.

CTGATAATTGCCAACACAATTAACATCTCAATCAAGGTAAATGCTTT (SEQ ID NO: 197)

The *tk* fragment was PCR amplified from plasmid pGMEr189 (Example 39) using the primers shown below. The underlined sequences in the primers highlight the portion of the *attB* site the primer will add to the 5' end of the *tk* fragment.

Primer 1206177 (sense):

5'-TAACATCTCAATCAAGGTAAATGCTTTGAGCTGAACCCGTACTACTACCAAGTGTGTTGT
GATTACATTAAGC-3' (SEQ ID NO: 198)

20 Primer 1206331 (anti-sense):

5'-GGCCATGGTGCCCGTGAAGCCGTTTAAATGAATTCGAACCC-3' (SEQ ID NO: 199)

Nco I

The sense primer added the 3' half of the *attB* site at the 5' end of the fragment; while the anti-sense primer added an *Nco* I restriction site at its 3' end.

25 The PCR (50 µl) was composed of 25 ng of pGMEr189 DNA, 1X PHUSION® HF buffer, 50 pmol of primer 1206177, 50 pmol of primer 1206331, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µl of 100% DMSO, and 1 unit of PHUSION® High Fidelity DNA polymerase. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 3 minutes; 40 cycles each at 98°C for 30 seconds, 57°C for 30 seconds, and 72°C for 2 minutes; and a final extension cycle at 72°C for 7 minutes. The completed PCR was submitted to 0.8% agarose gel electrophoresis in TBE buffer where an approximately 2906 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit.

35 The resulting gel purified PCR product was used as template DNA in a second PCR to complete the *attB* site (underlined sequence in primer 1206178) upstream of the *tk* construct, using the primers shown below.

Primer 1206178 (sense):

Pme I*attB*

5'-CGGTTTAAACCTGATAATTGCCAACACAATTAACATCTCAATCAAGGTAAATGCTTTG
AGCTGAAC-3' (SEQ ID NO: 200)

Primer 1206331 (anti-sense):

5

Nco I

5'-GGCCATGGTGCCCGTGAAGCCGTTTAAATGAATTCGAACCC-3' (SEQ ID NO: 201)

The sense primer completed the *attB* site upstream of the *tk* construct and added a *Pme* I restriction site at the 5' end of the fragment, while the anti-sense primer conserved an *Nco* I restriction site at its 3' end.

10

The PCR (50 µl) was composed of 25 ng of the 2906 kb fragment, 1X PHUSION® HF buffer, 50 pmol of primer 1206178, 50 pmol of primer 1206331, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µl of 100% DMSO, and 1 unit of PHUSION® High Fidelity DNA polymerase. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 3 minutes; 40 cycles each at 98°C for 30 seconds, 57°C for 30 seconds, and 72°C for 2 minutes; and a final extension cycle at 72°C for 7 minutes. The completed PCR was submitted to 0.8% agarose gel electrophoresis in TBE buffer where an approximately 2936 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit.

15

The *hpt* gene construct was PCR amplified from plasmid pGMEr189 (Example 39) using the following primers:

20

Primer 1206330 (sense):

5'-GCCCATGGGTTCGAATTCATTTAAACGGCTTCACGGGCA-3' (SEQ ID NO: 202)

Primer 1206180 (anti-sense):

Pme I*Pac* I*attB*

25

5'-CGGTTTAAACTTAATTAAAGCATTACCTTGATTGAGATGTTAATTGTGTTGGCAATTA
TCAGGCCGTGGTACTGGG-3' (SEQ ID NO: 203)

The anti-sense primer added the *attB* site downstream of the *hpt* gene (underlined sequence) and *Pac* I and *Pme* I restriction sites at the 3' end of the fragment.

30

The PCR (50 µl) was composed of 25 ng of pGMEr189 DNA, 1X PHUSION® HF buffer, 50 pmol of primer 1206330, 50 pmol of primer 1206180, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µl of 100% DMSO, and 1 unit of PHUSION® High Fidelity DNA polymerase. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 3 minutes; 40 cycles each at 98°C for 30 seconds, 57°C for 30 seconds, and 72°C for 2 minutes; and a final extension cycle at 72°C for 7 minutes. The completed PCR was submitted to 0.8% agarose gel electrophoresis in TBE buffer where an approximately 2349 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit.

35

The resulting 2936 bp fragment (comprising the *attB-tk* gene construct) and the 2349 bp fragment (comprising the *hpt* gene-*attB* construct) were double digested with *Pme* I and *Nco* I and ligated to the 2647 bp *Pme* I fragment from plasmid pGMEr189 bearing the ampicillin resistance gene and the *E. coli* origin of replication. About 20 µg of plasmid pGMEr189 were digested with *Pme* I at 37°C for 3 hours. One hour before the end of the digestion, 1 µl of calf intestinal alkaline phosphatase was added to the pGMEr189-*Pme* I digestion to de-phosphorylate the ends to prevent self-ligation. The resulting digestion was submitted to 0.8% agarose gel electrophoresis in TBE buffer where a 2647 bp vector fragment, containing the ampicillin resistance gene and the *E. coli* origin of replication, was excised from the gel and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit.

The ligation reaction was performed using a QUICK LIGATION™ Kit. The ligation reaction was composed of 1.5 µl of the pGMEr189 vector fragment, 2 µl of the *attB-tk* gene fragment, 2 µl of the *hpt* gene-*attB* fragment, 4 µl of sterile deionized water, 10 µl of 2X Quick Ligation Buffer, and 1 µl of Quick T4 Ligase. The ligation reaction was incubated for 1 hour at room temperature. A 5 µl aliquot of the ligation reaction was transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Transformants were spread onto 2XYT plus ampicillin plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired inserts by *Pme* I restriction digestion and sequencing analysis. Plasmid DNA was extracted and purified using a QIAGEN® Plasmid Mini Kit. One transformant containing a plasmid yielding *Pme* I restriction fragments of approximately 2.6 kb and of 5.2 kb was identified and the plasmid was designated pECW5.

Plasmid pECW5 comprises the *hpt-tk* dual selectable marker system flanked by the TP901-1 *attB* sites.

Example 46: Construction of plasmid pECW7

Plasmid pECW5 and plasmid pECW4 were digested with *Pme* I. The restriction reaction products were submitted to 0.8% agarose gel electrophoresis in TBE buffer where the 5226 bp pECW5 fragment and the 7072 bp pECW4 fragment were excised from the gels and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit. The 5226 bp *Pme* I fragment from plasmid pECW5, comprising the *hpt-tk* dual selectable marker system flanked by the TP901-1 *attB* sites, was ligated to the 7072 bp *Pme* I linearized pECW4 fragment. The ligation reaction was performed using a 1:3 vector:insert ratio. The reaction was composed of 2 µl of the 7072 bp linearized vector, 5 µl of the 5226 bp insert fragment, 3 µl of sterile deionized water, 9 µl of 2X Reaction Buffer, and 1 µl of Quick T4 DNA ligase. The ligation reaction was incubated for 1 hour at room temperature.

Five µl of the ligation reaction were transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Transformants were spread onto 2XYT plus ampicillin plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired insert by *Nco* I and *Pac* I restriction digestions. Plasmid DNA was extracted and purified using a QIAGEN® Plasmid Mini Kit. One transformant containing a plasmid yielding *Nco* I restriction fragments of approximately 5.4 kb and 6.9 kb and *Pac* I restriction fragments of approximately 9.2 kb, 3.1 kb, and 0.03 kb was identified and the plasmid was designated pECW7 (Figure 6).

Plasmid pECW7 harbors the *hpt-tk* dual selectable marker system flanked by the TP901-1 *attB* sites upstream of the TP901-1 integrase expression cassette.

Example 47: Construction of *Trichoderma reesei* strain TrGMEr60

Plasmid pECW7 was transformed into *Trichoderma reesei* strain ECW1 (Example 43) to show that the TP901-1 integrase can be expressed, is functional in filamentous fungal host cells, and able to promote a site-specific recombination event between the *attP* sites present in the recipient host strain and the *attB* sites in the transforming construct.

Plasmid pECW7 was linearized with *Sbf* I and transformed into *T. reesei* ECW1. *T. reesei* ECW1 harbors TP901-1 and two *attP* sites at the *cbh1* locus, one upstream and the other one downstream of the *amdS* marker gene. *T. reesei* ECW1 was able to grow on COVE2 plates supplemented with 10 mM uridine. To track the *attP/attB* recombination, mediated by the TP901-1 integrase, the transforming construct comprised two *attB* sites at each ends of the *hpt-tk* dual selectable marker system fragment.

Protoplast preparation and transformation of *Trichoderma reesei* ECW1 were performed according to the protocol described in Example 1.

Approximately 100 µg of plasmid pECW7 were digested with *Sbf* I. The linearized pECW7 plasmid was submitted to 0.8% agarose gel electrophoresis in TBE buffer, excised from the gel, and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit.

Approximately 2 µg of the *Sbf* I digested pECW7 were added to 100 µl of the protoplast solution and mixed gently. PEG buffer (250 µl) was added, and the reaction was mixed and incubated at 34°C for 30 minutes. STC (3 ml) was then added and mixed and then the mixture was spread onto PDA plates for protoplast regeneration selection. The plates were incubated at 28°C overnight. After overnight incubation the transformation plates were overlaid with PDA medium supplemented with 35 µg of hygromycin B per ml for transformant selection. The plates were incubated at 28°C for 6-9 days.

Thirty-eight primary transformants were identified and insertion of the *amdS* gene with the *hpt-tk* construct at the *cbh1* locus was verified by PCR analysis of the upstream and downstream integration sites as described below. In particular each putative primary

transformant was patched onto COVE2 plates supplemented with 10 mM uridine selection plates and incubated at 30°C for 2-3 days. Transformants were screened using a PHIRE® Plant Direct PCR Kit.

The 5' integration site was confirmed using the primers shown below.

5 Primer 1205989 (sense):

5'-CACCTCTTCTCAACCTTTGG-3' (SEQ ID NO: 204)

Primer 1206548 (anti-sense):

5'-ACGGGGCAAAGCTGCCTACC-3' (SEQ ID NO: 205)

The 3' integration site was confirmed using the primers shown below.

10 Primer 1206537 (sense):

5'-GCAGGGTCGATGCGACGCAA-3' (SEQ ID NO: 206)

Primer 1202659 (anti-sense):

5'-TACCATGACTGTCACGATAG-3' (SEQ ID NO: 207)

The total length of the resulting PCR fragments were approximately 1174 bp and
15 2422 bp for the 5' integration site and the 3' integration site, respectively.

The PCRs (20 µl) were composed of 1 µl of spore purified genomic DNA, 1X PHIRE® Plant PCR Buffer, 0.5 µM of primer 1205989 or 1206537, 0.5 µM of primer 1206548 or 1202659, and 0.4 µl of PHIRE® Hot Start II DNA Polymerase. The reaction was
20 performed in a thermocycler programmed for 1 cycle at 98°C for 3 minutes; 40 cycles each at 98°C for 30 seconds, 57°C for 20 seconds, and 72°C for 1 minute and 15 seconds; and a final extension cycle at 72°C for 7 minutes. Following thermocycling, the PCR products were visualized by 0.8% agarose gel electrophoresis in TBE buffer.

The thirty-eight primary transformants were patched onto COVE2 plates supplemented with 10 mM uridine to test for the *amdS* minus phenotype. The plates were
25 incubated at 28°C for 6-9 days.

Five transformants showing the correct PCR products by both PCRs and a negative *amdS* phenotype were spore purified by adding spores collected on a 10 µl inoculation loop to 1.5 ml of 0.01% TWEEN® 20. Spore dilutions of 1:1500 and 1:150 were spread onto 150 mm PDA plates and cultured for 3 days at 28°C. Four spore isolates per strain were
30 obtained and transferred to COVE2 plates supplemented with 10 mM uridine and cultivated at 30°C for 2-3 days. Each spore isolate was tested again by PCR to check the upstream and downstream sites of integration as described above. Of the twenty spore isolated transformants, eight were analyzed by Southern blotting according to Example 3 to confirm the correct recombination event between the *attP* and the *attB* sites mediated by the TP901-
35 1 integrase. A correct recombination event led to the replacement of the *amdS* marker with the *hpt-tk* expression cassettes flanked by the *attR* (upstream) and the *attL* (downstream) sites.

Two µg of genomic DNA from each transformant were digested with *Nhe* I. The digestions were submitted to 0.7-0.8% agarose gel electrophoresis in TAE buffer and blotted onto a HYBOND® N+ blotting membrane using a TURBOBLOTTER® according to Example 3. The membrane was hybridized with a digoxigenin-labeled probe specific for the *hpt* marker gene, which was synthesized using a PCR DIG Probe Synthesis Kit. Plasmid pJfyS1579-41-11 (WO 2011/075677) was digested with *Bsp* HI and purified by 0.8% agarose gel electrophoresis in TAE buffer. A 2.7 kb *Bsp* HI fragment containing the *hpt* gene was excised and the DNA was recovered using a Nucleospin® Extract II Kit (Example 8). This 2.7 kb fragment was used as the *hpt* probe template.

A probe hybridizing to the *hpt* gene was generated using a PCR DIG Probe Synthesis Kit with the forward and reverse primers indicated below. The 50 µl PCR contained 1X PCR DIG Probe Synthesis mix, 50 pmol each primer, 1X PCR buffer with MgCl₂, approximately 100 ng of purified 2.7 kb probe template (described above), and 2.6 units of EXPAND® High Fidelity DNA polymerase. The PCR was incubated in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 45 seconds; and 1 cycle at 72°C for 7 minutes.

Primers for 0.5 kb *hpt* probe:

Primer 0614123 (sense):

5'-ATGAAAAAGCCTGAACTCACC-3' (SEQ ID NO: 208)

Primer 0614124 (anti-sense):

5'-TCCATCACAGTTTGCCAGTGA-3' (SEQ ID NO: 209)

Hybridization was performed as described in Example 3.

One transformant with the correct upstream and downstream sites of integration was chosen and designated *T. reesei* TrGMEr60. Furthermore the presence of a correct *attR* site (SEQ ID NO: 210 below) and *attL* site (SEQ ID NO: 211 below), respectively, upstream and downstream of the *hpt-tk* marker genes were confirmed by sequencing analysis.

attR: TCCAACCTCGCTTAATTGCGAGTTTTTATTTCTTTATTTCAATCAAGGTAAATG (SEQ ID NO: 210)

attL: CTGATAATTGCCAACACAATTAACATCTCAATTAAGGTAAGTAACTAAA (SEQ ID NO: 211)

The results demonstrated that a site specific recombination event between the *attP* sites in the recipient strain ECW1 and the *attB* sites in the transforming construct, mediated by the TP901-1 integrase, led to the replacement of the *amdS* expression cassette with the *hpt-tk* dual selectable marker system, resulting in *T. reesei* strain TrGMEr60, which is able to grow on medium supplemented with hygromycin B and unable to grow on COVE2 plates supplemented with 10 mM uridine.

Example 48: Construction of plasmid pGMEr193

Plasmid pGMEr193 was constructed to comprise the *hpt-tk* dual selectable marker system flanked by the TP901-1 *attB* sites upstream of the TP901-1 integrase expression cassette. The only difference between plasmid pGMEr193 and pECW7 is that the TP901-1 integrase gene doesn't include the SV40 virus nuclear localization signal (NLS).

The *T. reesei gpd* promoter fragment was PCR amplified from plasmid pECW7 using the primers shown below:

Primer 1209171 (sense):

A/e I

5'-AGCATGAATGTCGCTCATCCGATGCCGCATCACCGTTGTGTCAG-3' (SEQ ID NO: 212)

Primer 1208898 (anti-sense):

5'-TAGTATAGATGGCGACCTTCTTGGTCATTTTGTATCTGCGAATTGAGCTTGCGTGAGT CGTGAGCTTCC-3' (SEQ ID NO: 213)

The sense primer added an *A/e* I restriction site at the 5' end of the fragment while the anti-sense primer added the ATG start codon and the first nine amino acids of the TP901-1 CDS without the NLS at the 3' end of the fragment.

The PCR (50 µl) was composed of 50 ng of plasmid pECW7 DNA, 1X PHUSION® HF buffer, 50 pmol of primer 1209171, 50 pmol of primer 1208898, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µl of 100% DMSO, and 1 unit of PHUSION® High Fidelity DNA polymerase. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 3 minutes; 40 cycles each at 98°C for 30 seconds, 57°C for 30 seconds, and 72°C for 1 minutes; and a final extension cycle at 72°C for 7 minutes. The completed PCR was submitted to 0.8% agarose gel electrophoresis in TBE buffer where an approximately 551 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit.

A second PCR fragment, containing 5' homology to the PCR product above, comprising the first 266 amino acids of the TP901-integrase without the SV40 virus NLS sequence was generated using the following primers:

Primer 1208899 (sense):

5'-TTCGCAGATACAAAATGACCAAGAAGGTCGCCATCTATACTAGAGTCTCGACGACCA ACCAG-3' (SEQ ID NO: 214)

Primer 1209170 (anti-sense):

A/f II

5'-CTGCTGCTGGCGCTCTTCCAATTCCTTTTGGACCTTAAGATAC-3' (SEQ ID NO: 215)

The sense primer is reverse and complementary to the antisense primer 1208898 used in the PCR described above to create the *gpd* promoter fragment. The anti-sense primer added an *A/f* II restriction site at the fragment 3' end.

The PCR (50 µl) was composed of 50 ng of plasmid pECW7 DNA, 1X PHUSION® HF buffer, 50 pmol of primer 1209171, 50 pmol of primer 1208898, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µl of 100% DMSO, and 1 unit of PHUSION® High Fidelity DNA polymerase. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 3 minutes; 40 cycles each at 98°C for 30 seconds, 57°C for 30 seconds, and 72°C for 1 minutes; and a final extension cycle at 72°C for 7 minutes. The completed PCR was submitted to 0.8% agarose gel electrophoresis in TBE buffer where an approximately 812 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit.

The resulting 551 bp *gpd* promoter fragment was digested with *A*/e I and the 812 bp TP901-1 integrase fragment was digested with *A*/f II for two hours at 37°C. Both digested fragments were then inserted into *A*/e I/*A*/f II-linearized pECW7 using an IN-FUSION® Advantage PCR Cloning Kit. The reaction was composed of 1X IN-FUSION® Reaction Buffer, 50 ng of *A*/e I/*A*/f II-linearized pECW7, 100 ng of the *A*/e I-*cbh1* promoter fragment (~551 bp), and 100 ng of the TP901-1 integrase *A*/f II fragment (~812 bp), and 1 µl of IN-FUSION® Enzyme in a 10 µl reaction volume. The reaction was incubated for 15 minutes at 50°C. Then 40 µl of TE were added to the reaction and a 2 µl aliquot of the reaction was transformed into ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Transformants were spread onto 2XYT plus ampicillin plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired inserts by *A*/e I and *A*/f II double restriction digestion. Plasmid DNA was extracted and purified using a QIAGEN® Plasmid Mini Kit. A transformant containing a plasmid yielding the desired band sizes of 10999 bp and 1248 bp was isolated and the plasmid was designated pGMEr193 (Figure 7).

Plasmid pGMEr193 comprises the *hpt-tk* dual selectable marker system flanked by the TP901-1 *attB* sites upstream of the TP901-1 integrase expression cassette without the SV40 virus NLS.

Example 49: Testing the integration efficiency of the TP901-1 integrase with and without the SV40 virus NLS Comparison Construction of *Trichoderma reesei* strain TrGMEr60

Plasmid pECW7 and pGMEr193 were transformed into *Trichoderma reesei* strain ECW1 (Example 43) to test whether the TP901-1 integrase needs a nuclear localization signal (NLS) sequence to successfully promote a site-specific recombination event between the *attP* sites present in the recipient host strain and the *attB* sites in the transforming construct.

Plasmid pECW7 and pGMEr193 were linearized with *Sbf* I and transformed into *T. reesei* ECW1. *T. reesei* ECW1 harbors TP901-1 and two *attP* sites at the *cbh1* locus, one upstream and the other one downstream of the *amdS* marker gene. *T. reesei* ECW1 was able to grow on COVE2 plates supplemented with 10 mM uridine. To track the *attP/attB* recombination, mediated by the TP901-1 integrase, the transforming construct comprised two *attB* sites at each end of the *hpt-tk* dual selectable marker fragment.

Protoplast preparation and transformation of *Trichoderma reesei* ECW1 were performed according to the protocol described in Example 1.

Approximately 100 µg of plasmids pECW7 and pGMEr193 were digested and linearized with *Sbf* I. The linearized pECW7 and pGMEr193 plasmids were submitted to 0.8% agarose gel electrophoresis in TBE buffer, excised from the gel, and purified using a NUCLEOSPIN® Extract II Gel and PCR Clean-up Kit.

Approximately 2 µg of the *Sbf* I digested pECW7 and pGMEr193 were added to 100 µl of the protoplast solution, respectively, and mixed gently. PEG buffer (250 µl) were added, and the reaction was mixed and incubated at 34°C for 30 minutes. STC (3 ml) was then added and mixed and then the mixture was spread onto PDA plates for protoplast regeneration selection. The plates were incubated at 28°C overnight. After overnight at 28°C the transformation plates were overlaid with PDA medium supplemented with 35 µg of hygromycin B per ml for transformant selection. The plates were incubated 28°C for 6-9 days.

Totally 10-11 primary transformants/µg of plasmid DNA were obtained with both linearized plasmid pECW7 and pGMEr193 confirming that both plasmids had retained the same transformation efficiency. After PCR checking of all the primary transformants it was shown that 46% of the pECW7 transformants integrated at the expected *cbh1* locus while only 13% for plasmid pGMEr193.

At the same time, approximately 4 µg of circular pECW7 and pGMEr193 were added to 100 µl of the protoplast solution, respectively, and mixed gently. PEG buffer (250 µl) were added, and the reaction was mixed and incubated at 34°C for 30 minutes. STC (3 ml) was then added and mixed and the mixture was spread onto PDA plates for protoplast regeneration selection. The plates were incubated at 28°C overnight. After overnight at 28°C the transformation plates were overlaid with PDA medium supplemented with 35 µg of hygromycin B per ml for transformant selection. The plates were incubated 28°C for 6-9 days.

About 11 primary transformants/µg of plasmid DNA were obtained with circular plasmid pGMEr193, and about 4 primary transformants/µg of plasmid DNA were obtained with circular plasmid pECW7, showing that uncut plasmid pGMEr193 has a higher

transformation efficiency than uncut plasmid pECW7. Although, PCR checking of all the primary transformants showed that pECW7 retains the best targeting efficiency of the construct at the desired locus, in particular about 60% of the pECW7 (circular) transformants integrated at the expected *cbh1* locus while only 40% for plasmid pGMEr193 (circular).

In all the primary transformants the insertion of the *hpt-tk* construct at the *cbh1* locus was verified by PCR analysis of the upstream and downstream integration sites using a PHIRE® Plant Direct PCR Kit as described below.

The 5' integration site was confirmed using the primers shown below.

Primer 1203893 (sense):

5'-GTAATTTGCCTGCTTGACCG-3' (SEQ ID NO: 216)

Primer 1208272 (anti-sense):

5'-CACCAGCCTTTCCACTTCGG-3' (SEQ ID NO: 217)

The 3' integration site was confirmed using the primers shown below.

Primer 1208273 (sense):

5'-CCTTCTGGCATGACCTTTTG-3' (SEQ ID NO: 218)

Primer 1202659 (anti-sense):

5'-TACCATGACTGTCACGATAG-3' (SEQ ID NO: 219)

The total lengths of the resulting PCR fragments were approximately 620 bp and 1959 bp for the 5' integration site and the 3' integration site, respectively.

The PCRs (20 µl) were composed of 1 µl of spore purified genomic DNA, 1X PHIRE® Plant PCR Buffer, 0.5 µM of primer 1203893 or 1208273, 0.5 µM of primer 1208272 or 1202659, and 0.4 µl of PHIRE® Hot Start II DNA Polymerase. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 3 minutes; 40 cycles each at 98°C for 30 seconds, 57°C for 20 seconds, and 72°C for 1 minute and 15 seconds; and a final extension cycle at 72°C for 7 minutes. Following thermocycling, the PCR products were visualized by 0.8% agarose gel electrophoresis in TBE buffer.

This experiment showed that adding the SV40 virus NLS is not essential for a proper site directed integration mediated by the TP901-1 integrase, but the NLS increases the success rate of the integration event.

Example 50: Generation of *Trichoderma reesei* strain attP-FRT

This Example describes the introduction of FRT sites at the *cbh2* locus of *T. reesei* strain ECW1 (Example 43) to create a strain with TP901-1 attP recognition sites at the *cbh1* locus and FRT sites at the *cbh2* locus.

Seventy µg of pAgJg137 (Example 11) were digested with *Pme* I. The digested DNA was ethanol precipitated and the DNA was resuspended in TE buffer. *T. reesei* strain ECW1 protoplasts were prepared as described in Example 1. The protoplasts were transformed

with *Pme* I digested pAgJg137 DNA selecting for the hygromycin resistance marker (*hpt*) as described in Example 1. Transformation plates were incubated 5 days at 28°C. Transformants were transferred to PDA plates and incubated for 3-5 days at 30°C. A spore PCR using a PHIRE® Plant Direct PCR Kit was utilized to identify transformants which had the correct integration of the pAgJg137 DNA at the *cbh2* locus. Briefly, spores from the transformants were collected with a sterile 1 µl inoculation loop and transferred to 15 µl of Kit-supplied dilution buffer in microfuge tubes and incubated for 5 minutes at room temperature. Each spore suspension was centrifuged at 13,000 rpm for 10 seconds and 1 µl of supernatant was used in the spore PCR. The 20 µl spore PCR contained 1X PHIRE® Plant Direct PCR buffer (contains dNTPs and Mg), 10 pmol of the forward primer and 10 pmol of the reverse primer listed below, 0.4 µl of PHIRE® II Hot Start DNA Polymerase and 1 µl of each supernatant from the spore suspensions.

Forward primer (homology to 5' *cbh2* flank):

5'-CTGATCGAGAAGATTAGCATG-3' (SEQ ID NO: 220)

Reverse primer (homology to *hpt* marker):

5'-GTTTCAGGCAGGTCTTGCAACG-3' (SEQ ID NO: 221)

Reverse primer (homology to *cbh2* gene):

5'-GATCAGTGATGAAGAAGGCG-3' (SEQ ID NO: 222)

The PCRs were incubated in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 5 seconds, 60°C for 5 seconds, and 72°C for 1 minute and 15 seconds; and 1 cycle at 72°C for 1 minute and 15 seconds.

The completed PCRs were subjected to 0.8% agarose gel electrophoresis in TAE buffer. Transformants with correct targeting of plasmid DNA to the *cbh2* locus produced a 2.6 kb fragment. Transformants that did not have correct targeting of plasmid DNA to the *cbh2* locus produced a 3.5 kb fragment.

Transformants with correct targeting of plasmid DNA to the *cbh2* locus were chosen for spore isolation. Spores from a PDA plate culture were collected using a 1 µl inoculation loop and added to 2 ml of 0.01% TWEEN® 20. One µl of each spore suspension and 100 µl 0.01% TWEEN® 20 were spread onto 150 mm PDA plates. The plates were incubated for 2 days at 30°C. Isolated colonies were picked with a sterile 10 µl inoculation loop and transferred to PDA plates and incubated 3-5 days at 30°C. A spore PCR using a PHIRE® Plant Direct PCR Kit and the primers and PCR conditions shown above was performed to identify spore isolates that had the correct integration at the *cbh2* locus.

Correct spore isolates were chosen and the *hpt/tk* markers were looped out using 5-fluorodeoxyuridine (FdU) counter-selection as described below. Spores from 6 day old PDA plates were collected in 4 ml of 0.01% TWEEN® 20 and the spore concentration was determined using a hemocytometer. Approximately 10⁶ and 10⁵ spores from each isolate

were plated to 150 mm FdU plates. The FdU plates were incubated at 30°C for 7 days. Eight to nine FdU colonies were selected for each strain and transferred to PDA plates. The plates were incubated at 30°C for 3 days. Spore PCRs using the PHIRE® Plant Direct PCR Kit were performed as described above using the primers shown below.

5 Forward primer 1 (homology to 5' flanking region *cbh2* locus):

5'-CTCAGGCCATCGTAGGAAAT-3' (SEQ ID NO: 223)

Reverse primer 1 (homology to 3' flanking region *cbh2* locus):

5'-CTAGGTAGGTAGGTAGTATA-3' (SEQ ID NO: 224)

Forward primer 2 (homology to *hpt* marker):

10 5'-CGTGTTCCTTCCCATTGCGATGCGACCTCGTGGTCATTGAC-3' (SEQ ID NO: 225)

Reverse primer 2 (homology to 3' flanking region *cbh2* locus):

5'-CTCCTGTCACGACGTGCTTTT-3' (SEQ ID NO: 226)

The PCRs were performed in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 5 seconds, 55°C for 5 seconds, and 72°C for 1 minute
15 45 seconds; and 1 cycle at 72°C for 1 minute 15 seconds.

The completed PCRs were subjected to 0.8% agarose gel electrophoresis in TBE buffer. Isolates with correct loopout of the *hpt/tk* markers produced a 2 kb fragment using the first set of primers and did not produce a 3.2 kb fragment with the second set of primers. Isolates retaining the *hpt/tk* markers produced a 6.6 kb fragment using the first set of primers
20 and a 3.2 kb fragment using the second set of primers.

An additional round of spore isolation was performed with isolates containing the correct *hpt/tk* marker loopout. Spores from a 6 day old PDA plate were collected in 4 ml of 0.01% TWEEN® 20 and the spore concentration was determined using a hemocytometer. Spores were diluted appropriately to a concentration of 10³ spores per ml using sterile water
25 and approximately 50 spores were spread onto PDA plates. The plates were incubated for 2 days at 30°C. Isolated colonies were picked with a sterile 10 µl inoculation loop, transferred to PDA plates, and incubated at 30°C. A spore PCR using a PHIRE® Plant Direct PCR Kit and forward primer 1 and reverse primer 1 described above was utilized to identify final spore isolates with the correct *hpt/tk* marker loopout.

30 Genomic DNA from the final spore isolates was prepared and subjected to Southern blot analysis as described in Example 15.

For Southern blot analysis approximately 1 µg of genomic DNA was digested with 20 units of *Eco* RI and subjected to 0.8% agarose electrophoresis in TAE buffer. The DNA in the gel was depurinated, denatured, neutralized, and transferred to a NYTRAN®
35 Supercharge membrane according to Example 3. The DNA was UV cross-linked to the membrane using a UV STRATALINKER™ (Stratagene) and prehybridized for 1 hour at 42°C in 20 ml of DIG Easy Hyb.

A template for a probe hybridizing to the 3' flanking region of the *cbh2* gene was generated using PHUSION™ High Fidelity Hot Start DNA Polymerase (New England Biolabs, Inc.) and gene-specific forward and reverse primers shown below.

Fifty picomoles of each of the primers were used in a PCR containing 100 ng of *T. reesei* RutC30 genomic DNA, 1X PHUSION™ High Fidelity Hot Start DNA Polymerase buffer, 1 µl of a 10 mM blend of dATP, dTTP, dGTP, and dCTP, and 1 unit of PHUSION™ High Fidelity Hot Start DNA Polymerase in a final volume of 50 µl. The PCR was performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; 34 cycles each at 98°C for 15 seconds, 60°C for 30 seconds, and 72°C for 1 minute and 30 seconds; and 1 cycle at 72°C for 10 minutes. The completed PCR was purified by 0.8% agarose gel electrophoresis in TAE buffer where a 0.46 kb DNA band was excised from the gel and the DNA was extracted using a Nucleospin® Extract II Kit (Example 8).

A probe hybridizing to the 3' flanking region of the *cbh2* gene was generated using a PCR DIG Probe Synthesis Kit with the forward and reverse primers shown below. The 50 µl PCR contained 1X PCR DIG Probe Synthesis mix, 50 pmol of each primer, 1X PCR buffer with MgCl₂, 24 ng of purified probe template (described above), and 2.6 units of EXPAND® High Fidelity DNA polymerase. The PCR was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minute; 30 cycles each at 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 45 seconds; and 1 cycle at 72°C for 7 minutes.

Primers for *cbh2* 3' flanking region probe

Forward primer:

5'-TCTTGAGCCGCATCGCATAGA-3' (SEQ ID NO: 227)

Reverse primer:

5'-TACGGTCAGCGCTCATGCGAA-3' (SEQ ID NO: 228)

The probe was boiled for 5 minutes, chilled on ice for 2 minutes, and added to 10 ml of DIG Easy Hyb to produce the hybridization solution. Hybridization was performed as described in Example 3.

Southern blot analysis identified several final spore isolates that produced a 2.1 kb hybridizing fragment, verifying the correct integration of FRT sites at the *cbh2* locus with loopout of the *hpt/tk* markers. The native *T. reesei cbh2* locus produced a 4.1 kb hybridizing fragment. One spore isolate was chosen as the final strain and designated *T. reesei attP-frt*.

Example 51: Construction of plasmid pCKle137 for removal of the *amdS* marker from the *cbh1* locus of *Trichoderma reesei* strain *attP-FRT*

Plasmid pCKle137 was constructed so that the *amdS* marker could be removed from the *cbh1* locus in *T. reesei attP-FRT* via homologous recombination. Plasmid pCKle137 contains *cbh1* flanking regions, *attP* sites, and the *hpt/tk* selectable markers, which can be

looped out to generate a marker-free strain. The TP901-1 *attP* site was inserted downstream of the 5' *cbh1* flanking region using PCR. The 5' *cbh1* flanking region and *attP* site were amplified together from pJfyS147 (Example 4) plasmid DNA using PHUSION™ Hot Start II High Fidelity DNA Polymerase (Thermo Scientific).

5 Forward primer:

5'-CATGGTTTAAACGGCGCGCCGGTGAAACACCGCCCCCTTC-3' (SEQ ID NO: 229)

Reverse primer (*attP* site is underlined):

5'-CCTTGTTTTGTCGTTTAGTTACCTTAATTGAAATAAACGAAATAAAACTCGCAATT
AAGCGAGTTGGATTCGAACAGCCCC-3' (SEQ ID NO: 230)

10 Ten picomoles of each of the primers were used in a PCR composed of 7.4 ng of pJfyS147 DNA, 10 µl of 5X PHUSION™ HF buffer, 1 µl of a 10 mM blend of dATP, dTTP, dGTP, and dCTP, and 1.5 units of PHUSION™ Hot Start II DNA Polymerase in a final volume of 50 µl. The PCR was performed in a thermocycler programmed for 1 cycle at 98°C for 30 seconds; 30 cycles each at 98°C for 7 seconds, 55°C for 15 seconds, and 72°C for 25
15 seconds; and 1 cycle at 72°C for 5 minutes.

The completed PCR was submitted to 0.65% agarose gel electrophoresis in TBE buffer where a 1.58 kb band was excised from the gel and agarose was extracted using a Nucleospin® PCR Cleanup and Gel Extraction Kit (Macherey-Nagel Inc.).

The 1.58 kb purified fragment was inserted into *Asc* I-digested pJfyS1579-41-11 (WO
20 2011/075677) using an IN-FUSION® HD PCR Cloning Kit. The reaction was composed of 2 µl of 5X IN-FUSION® HD Enzyme Premix, 100 ng of *Asc* I-digested pJfyS1579-41-11, and 40 ng of the purified PCR product in a 10 µl reaction volume. The reaction was incubated for 15 minutes at 50°C. Then 1 µl was used to transform 50 µl of Stellar™ *E. coli* chemically competent cells (Clontech Laboratories, Inc.) by addition to a tube containing the competent
25 cells and incubating the cells on ice for 30 minutes. The tube was incubated at 42°C for 45 seconds after which 450 µl of SOC medium were added. The tube was then incubated at 37°C with agitation at 250 rpm for 1 hour. Volumes of 50 µl and 150 µl were transferred to two 150 mm 2XYT plus ampicillin plates. The plates were incubated overnight at 37°C. *E. coli* transformants were used to inoculate 3 ml of LB plus ampicillin medium in a 14 ml
30 Falcon round-bottom polypropylene tube and incubated at 37°C overnight with agitation at 200 rpm. Plasmid DNA was isolated using a QIAprep® Spin Miniprep Kit (QIAGEN Inc.). Plasmid DNA from the transformants was screened by restriction digestion with *Stu* I FD for production of 1.4 kb, 3.5 kb, and 4.6 kb fragments. Sequencing of one of the plasmids verified that the plasmid contained the correct inserts with no PCR errors. The plasmid was
35 designated pCKle136.

The 3' *cbh1* flanking region and *attP* site were amplified together from pJfyS147 plasmid DNA using PHUSION™ Hot Start II High Fidelity DNA Polymerase (Thermo Scientific, Pittsburgh, PA, USA).

Forward primer (*attP* site is underlined):

5 5'-GTATTCCTGCAGGTCCAACTCGCTTAATTGCGAGTTTTTATTTGTTTATTTCCAATTAA
GGTAACTAAAGATAACGGAATAG-3' (SEQ ID NO: 231)

Reverse primer:

5'-TGGCCATATTTAAATAGTCAACACGTCTCCTATGTCT-3' (SEQ ID NO: 232)

Twenty-five picomoles of each of the primers were used in a PCR composed of 7.4
10 ng of pJfyS147 DNA, 10 µl of 5X PHUSION™ HF buffer, 1 µl of a 10 mM blend of dATP, dTTP, dGTP, and dCTP, and 1 unit of PHUSION™ Hot Start II DNA Polymerase in a final volume of 50 µl. The PCR was performed in a thermocycler programmed for 1 cycle at 98°C for 30 seconds; 30 cycles each at 98°C for 7 seconds, 55°C for 15 seconds, and 72°C for 25 seconds, and 1 cycle at 72°C for 5 minutes.

15 The completed PCR was submitted to 0.65% agarose gel electrophoresis in TBE buffer where a 1.6 kb band was excised from the gel and agarose was extracted using a Nucleospin® PCR Cleanup and Gel Extraction Kit.

The purified fragment was inserted into *Sbf* I-digested pCKle136 using an IN-FUSION® HD PCR Cloning Kit. The reaction was composed of 2 µl of 5X IN-FUSION® HD
20 Enzyme Premix, 100 ng of *Sbf* I-digested pCKle136, and 33.6 ng of the 1.6 kb purified PCR product in a 10 µl reaction volume. The reaction was incubated for 15 minutes at 50°C. Then 1 µl was used to transform 50 µl of Stellar™ *E. coli* chemically competent cells as described above. Plasmid DNA was isolated using a QIAprep® Spin Miniprep Kit. Plasmid DNA from the transformants was screened by restriction digestion with *Stu* I FD for production of 1.4
25 kb, 4.6 kb, and 5.1 kb fragments. Sequencing of one of the plasmids verified that the plasmid contained the correct inserts with no PCR errors. The plasmid was designated pCKle137 (Figure 8).

Example 52: Removal of the *amdS* marker from the *cbh1* locus of *Trichoderma reesei* strain *attP*-FRT to generate strain CKle215.
30

Trichoderma reesei strain *attP*-FRT (Example 50) was transformed with *Pme* I-linearized pCKle137 selecting for hygromycin resistance as described in Example 1. Transformation plates were incubated at 28°C for 5 days. Fifty transformants were obtained and each one was picked and transferred to a PDA plate and incubated for 6 days at 30°C.
35 Spore PCR using a PHIRE® Plant Direct PCR Kit was utilized to identify transformants which had the correct integration of the pCKle137 DNA at the 3' end of the *cbh1* locus. Briefly, spores from each of the transformants were collected with a sterile 1 µl inoculation

loop and transferred to 15 µl of Kit-supplied dilution buffer in microfuge tubes and incubated for 5 minutes at room temperature. The 20 µl spore PCR contained 1X PHIRE® Plant Direct PCR buffer (contains dNTPs and Mg), 10 pmol of forward and reverse primers listed below, 0.4 µl of PHIRE® II Hot Start DNA Polymerase, and 1 µl of supernatant from each spore suspension.

Forward primer (homology to *tk* marker):

5'-TACCTCCGGGATGGTCCAGA-3' (SEQ ID NO: 233)

Forward primer (homology to *amdS* marker):

5'-TTCCATCTCTCAAAGGAAGA-3' (SEQ ID NO: 234)

Reverse primer (homology to outside 3' *cbh1* flank):

5'-TTGCTCTTATTGAGACCATGCG-3' (SEQ ID NO: 235)

The PCRs were incubated in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 5 seconds, 60°C for 5 seconds, and 72°C for 38 seconds; and 1 cycle at 72°C for 1 minute.

The completed PCRs were subjected to 0.8% agarose gel electrophoresis in TBE buffer. Transformants with correct targeting of plasmid DNA to the *cbh1* locus produced a 2.5 kb fragment. Transformants that did not have correct targeting of plasmid DNA to the *cbh1* locus produced a 1.9 kb fragment.

Spore PCR was also performed as described above for the 5' end of the *cbh1* locus.

Forward primer (homology to outside 5' *cbh1* flank):

5'-GTACAAACAACCTACCTGGTG-3' (SEQ ID NO: 236)

Reverse primer (homology to *hpt* marker):

5'-GTCAGCTTCATTTTCCGTGT-3' (SEQ ID NO: 237)

Reverse primer (homology to *amdS* marker):

5'-CTCGAATCGAGCCACCGATA-3' (SEQ ID NO: 238)

The PCRs were incubated in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 5 seconds, 55°C for 5 seconds, and 72°C for 1 minute 5 seconds; and 1 cycle at 72°C for 1 minute.

The completed PCRs were subjected to 0.8% agarose gel electrophoresis in TBE buffer. Transformants with correct targeting of plasmid DNA to the *cbh1* locus produced a 2 kb fragment. Transformants that did not have the correct targeting of plasmid DNA to the *cbh1* locus produced a 3.2 kb fragment.

Transformants with correct targeting of plasmid DNA to the *cbh1* locus were chosen for spore isolation. Spores from PDA plates were collected using a 1 µl inoculation loop and added to 2 ml of 0.01% TWEEN® 20. One µl of each spore suspension and 200 µl of 0.01% TWEEN® 20 were spread onto 150 mm PDA + 1M sucrose plates. The plates were incubated for 2 days at 30°C. Isolated colonies were picked with a sterile metal spear and

transferred to PDA plates and incubated at 30°C for 5 days. Spore PCR using a PHIRE® Plant Direct PCR Kit and the primers and PCR conditions shown above was performed to identify spore isolates with correct integration at the *cbh1* locus.

Correct spore isolates were chosen and the *hpt/tk* markers were looped out using 5-fluorodeoxyuridine (FdU) counter-selection as described below. Spores from a 6 day old PDA plate were collected in 3 ml of 0.01% TWEEN® 20 and the spore concentration was determined using a hemocytometer. Approximately 10⁵ spores from each isolate were spread onto 150 mm FdU plates. The FdU plates were incubated at 30°C for 6-7 days. Two to eight FdU colonies were selected for each strain and transferred to PDA plates. The plates were incubated at 30°C for 5-6 days. Spore PCRs using a PHIRE® Plant Direct PCR Kit were performed as described above using the primers shown below.

Forward primer (homology to *tk* marker):

5'-TACCTCCGGGATGGTCCAGA-3' (SEQ ID NO: 239)

Reverse primer (homology to 3' *cbh1* flank):

5'-ATGGGTCATTACCAATTGGC-3' (SEQ ID NO: 240)

Forward primer (homology to 5' *cbh1* flank):

5'-GTAATTTGCCTGCTTGACCG-3' (SEQ ID NO: 241)

The PCRs were incubated in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 5 seconds, 63°C for 5 seconds, and 72°C for 25 seconds; and 1 cycle at 72°C for 1 minute.

The completed PCRs were subjected to 0.8% agarose gel electrophoresis in TBE buffer.

Isolates with loopout of the *hpt/tk* markers produced a 0.6 kb fragment and isolates that retained the *hpt/tk* markers produced a 1.2 kb fragment.

An additional round of spore isolation was performed with isolates with the *hpt/tk* marker looped-out as described above. Plates were incubated for 2 days at 30°C. Isolated colonies were picked with a sterile metal spear, transferred to PDA plates, and incubated at 30°C. Spore PCR using the PHIRE® Plant Direct PCR Kit and forward primers and reverse primers was utilized to identify final spore isolates with the correct *hpt/tk* marker loopout as described above.

PCRs using the primers and PCR conditions shown below were used to generate a PCR fragment from nine spore isolates for sequencing.

Forward primer (homology to 5' *cbh1* flank, upstream of *attP* site):

5'-TTTGAGCTACAAGAACCTGTGGGG-3' (SEQ ID NO: 242)

Reverse primer (homology to 3' *cbh1* flank, downstream of *attP* site):

5'-CGAAAAGGCCACCTGTTGAGAGGCT-3' (SEQ ID NO: 243)

Spore PCR was performed using a PHIRE® Plant Direct PCR Kit. The PCRs (20 µl) were composed of 1X PHIRE® Plant Direct PCR buffer (contains dNTPs and Mg), 10 pmol of forward and reverse primers listed above, 0.4 µl of PHIRE® II Hot Start DNA Polymerase, and 1 µl of supernatant from a spore suspension. The PCRs were performed in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 5 seconds, 72°C for 28 seconds, and 1 cycle at 72°C for 1 minute. The 1.37 kb PCR fragments were purified using a USB® ExoSAP-IT® PCR Product Cleanup Kit (Affymetrix). Sequencing of the PCR fragments verified that integration of pCKle137 at the *cbh1* locus had occurred by TP901-1 recombinase-mediated recombination between the host *attP* sites and plasmid *attB* sites.

Genomic DNA from the final spore isolates was prepared and subjected to Southern blot analysis. *Trichoderma reesei* strains were grown in 25 ml of YP medium supplemented with 2% glucose (w/v) in a 125 ml baffled shake flask at 28°C for 2 days with agitation at 200 rpm. The mycelia were harvested by filtration using a MIRACLOTH® lined funnel and frozen in liquid nitrogen. The frozen mycelia were disrupted by quickly smashing with a hammer while wrapped inside the MIRACLOTH®. Approximately 2 g of the disrupted mycelia were then transferred to a 50 ml polypropylene conical centrifuge tube containing 10 ml of 1X lysis buffer (100 mM EDTA, 10 mM Tris pH 8.0, 1% TRITON® X-100, 0.5 M Guanidine-HCl, 200 mM NaCl) and 3 µl of a 100 mg RNase A (QIAGEN Inc.) per ml stock solution. The tube was mixed by gentle vortexing, and then incubated at room temperature for 5 minutes after which was added 150 µl of a Proteinase K (QIAGEN Inc.) stock solution containing 20 mg/ml. The tube was mixed by inversion and incubated at 50°C for 1 hour. The tube was then centrifuged at 7240 x g for 20 minutes. The supernatant was then added to a pre-equilibrated QIAGEN-tip 100 (QIAGEN Inc.) and the remaining DNA extraction steps were performed according to the manufacturer's instructions for a Plasmid Midi Kit. The DNA was re-suspended in 200 µl of TE buffer.

For Southern blot analysis approximately 2 µg of genomic DNA was digested with 1 µl of *Nco* I and subjected to 0.8% agarose electrophoresis in TBE buffer. The DNA in the gel was depurinated, denatured, neutralized, and transferred to a NYTRAN® Supercharge membrane as described in Example 3. The DNA was UV cross-linked to the membrane using a CL-1000 UV Crosslinker (UVP, LLC) and prehybridized for 1 hour at 42°C in 20 ml of DIG Easy Hyb.

Templates for probes hybridizing to the 5' and 3' flanks of the *cbh1* gene were generated using PHUSION™ Hot Start II High Fidelity DNA Polymerase and gene-specific forward and reverse primers shown below.

Twenty-five picomoles of each of the primers were used in PCRs composed of 100 ng of *T. reesei* 981-0-8 genomic DNA, 10 µl of 5X PHUSION™ HF buffer, 1 µl of a 10 mM

blend of dATP, dTTP, dGTP, and dCTP, and 1 unit of PHUSION™ Hot Start II DNA Polymerase in a final volume of 50 µl. The PCRs were performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; 35 cycles each at 98°C for 10 seconds, 72°C for 30 seconds, and 1 cycle at 72°C for 5 minutes. The completed PCRs were purified by 0.65% agarose gel electrophoresis in TBE buffer where a 0.55 kb DNA band (3' flank) and a 0.35 kb band (5' flank) were excised from the gels and the DNA was extracted using a Nucleospin® PCR Cleanup and Gel Extraction Kit.

Probes hybridizing to the 5' and 3' flanks of the *cbh1* gene were generated using a PCR DIG Probe Synthesis Kit with the forward and reverse primers indicated below. The 50 µl PCRs were composed of 1X PCR DIG Probe Synthesis mix, 25 pmol of each primer, 1X PCR buffer with MgCl₂, 80 ng of purified probe template (described above), and 2.6 units of EXPAND® High Fidelity DNA polymerase. The PCRs were performed in a thermocycler programmed for 1 cycle at 95°C for 2 minute; 30 cycles each at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 40 seconds; and 1 cycle at 72°C for 7 minutes.

Primers for *cbh1* 5' flanking region probe

Forward primer:

5'-TAGGGTCGGCAACGGCAAAA-3' (SEQ ID NO: 244)

Reverse primer:

5'-CCCACATTGCTCCAAACCCC-3' (SEQ ID NO: 245)

Primers for *cbh1* 3' flanking region probe

Forward primer:

5'-AATGACCCATAGGGAGACAAACAGCATAAT-3' (SEQ ID NO: 246)

Reverse primer:

5'-TGTTGGACGCAGGATTTTGGA-3' (SEQ ID NO: 247)

Hybridization was performed as described in Example 3.

Southern blot analysis identified several final spore isolates that produced a 5.4 kb hybridizing fragment, verifying the correct removal of the *amdS* marker at the *cbh1* locus and loopout of the *hpt/tk* markers. The parent *T. reesei* (*attP*-FRT) *cbh1* locus produced 1.6 kb and 6.4 kb hybridizing fragments while strains containing the *hpt/tk* marker produced 2.7 kb and 7.3 kb hybridizing fragments. One spore isolate was chosen as the final strain and designated *T. reesei* CKle215.

Example 53: Construction of TP901-1 integration plasmid pDM315

Expression plasmid pDM315 was constructed to target a *Humicola insolens* endoglucanase V (EGV) (U.S. Patent 8,735,549) expression cassette to TP901-1 recombinase recognition sites at the *cbh1* locus in *T. reesei* CKle215. Plasmid pDM315 has *attB* sites which can recombine with the *attP* sites at the *cbh1* locus.

A 0.25 kb PCR fragment was generated from pECW7 (Example 46) using PHUSION™ Hot Start II DNA Polymerase and the forward and reverse primers shown below.

Forward primer:

5 5'-GCAATTAATGTGAGTTAGCT-3' (SEQ ID NO: 248)

Reverse primer:

5'-TAACAATCCTACATTCGGTCGAAAAGCATTTACCTTGATTGAGAT-3' (SEQ ID NO: 249)

Fifty picomoles of each of the primers were used in a PCR composed of 700 ng of pECW7 plasmid DNA, 1X PHUSION™ HF Buffer, 1 µl of a 10 mM blend of dATP, dTTP, dGTP, and dCTP, and 1 unit of PHUSION™ Hot Start II DNA Polymerase in a final volume of 50 µl. The PCR was performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; 34 cycles each at 98°C for 15 seconds, 59°C for 30 seconds, and 72°C for 3 minutes, and 1 cycle at 72°C for 10 minutes. The completed PCR was purified by 0.8% agarose gel electrophoresis in TAE buffer where a 0.25 kb DNA band was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

A 5.3 kb PCR fragment containing the *Humicola insolens* endoglucanase V expression cassette and *amdS* marker was generated from plasmid pMJ05 (U.S. Patent 8,735,549) using the forward and reverse primers shown below.

20 Forward primer:

5'-ATCTCAATCAAGGTAAATGCTTTTCGACCGAATGTAGGATTGTTA-3' (SEQ ID NO: 250)

Reverse primer:

25 5'-GTTAATTGTGTTGGCAATTATCAGTCTAGGATGCATTCTACGCCAG-3' (SEQ ID NO: 251)

Fifty picomoles of each of the primers were used in a PCR composed of 500 ng of pMJ05 plasmid DNA, 1X PHUSION™ HF Buffer, 1 µl of a 10 mM blend of dATP, dTTP, dGTP, and dCTP, and 1 unit of PHUSION™ Hot Start II DNA Polymerase in a final volume of 50 µl. The PCR was performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; 34 cycles each at 98°C for 15 seconds, 59°C for 30 seconds, and 72°C for 3 minutes, and 1 cycle at 72°C for 10 minutes. The completed PCR was purified by 0.8% agarose gel electrophoresis in TAE buffer where a 5.3 kb DNA band was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

35 A 0.2 kb PCR fragment was generated from pECW7 using PHUSION™ Hot Start II DNA Polymerase and the forward and reverse primers shown below.

Forward primer:

5'-CTGGCGTAGAATGCATCCTAGACTGATAATTGCCAACACAATTAAC-3' (SEQ ID NO: 252)

Reverse primer:

5'-GCCATAATGCATAGGTAGGT-3' (SEQ ID NO: 253)

5 Fifty picomoles of each of the primers were used in a PCR composed of 700 ng of pECW7 plasmid DNA, 1X PHUSION™ HF buffer, 1 µl of a 10 mM blend of dATP, dTTP, dGTP, and dCTP, and 1 unit of PHUSION™ Hot Start II DNA Polymerase in a final volume of 50 µl. The PCR was performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; 34 cycles each at 98°C for 15 seconds, 59°C for 30 seconds, and 72°C for 3 minutes, and 1 cycle at 72°C for 10 minutes. The completed PCR was purified by 0.8% agarose gel electrophoresis in TAE buffer. A 0.2 kb DNA band was excised from the gel, and extracted using a Nucleospin® Extract II Kit (Example 8).

The three purified PCR fragments described above were joined together using SOE PCR and the forward and reverse primers shown below.

15 Forward primer:

5'-CTCAGAATTAACCCTCACTAA-3' (SEQ ID NO: 254)

Reverse primer:

5'-GTACTIONAATTAACCAAGGGCG-3' (SEQ ID NO: 255)

20 Fifty picomoles of each of the primers were used in a PCR composed of 24 ng of the 0.25 kb PCR fragment, 30 ng of the 5.3 kb PCR fragment, 26 ng of the 0.2 kb PCR fragment, 1X PHUSION™ HF buffer, 1 µl of a 10 mM blend of dATP, dTTP, dGTP, and dCTP, and 1 unit of PHUSION™ Hot Start II DNA Polymerase in a final volume of 50 µl. The PCRs were performed in triplicate. The primers were omitted until after cycle 6 as indicated below. The thermocycler was programmed for 1 cycle at 95°C for 2 minutes; and 5 cycles each at: 95°C for 30 seconds, 1 minute ramping down from 95°C and decreasing by 1°C/second down to 35°C, and 72°C for 4 minutes. The PCR samples were then cooled to 4°C and held at 4°C until the primers were added. After primer addition the program continued for 10 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 4 minutes. Then the program proceeded for 20 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C starting at 4 minutes with the addition of 20 seconds for each successive cycle. The three identical 50 µl reactions were combined and purified by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 5.4 kb DNA band was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

35 Approximately 25 µg of pECW7 DNA was digested with *Sbf* I and *Mss* I (also called *Pme* I). The digested DNA was separated by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 7.1 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

The 5.4 kb SOE PCR fragment was inserted into *Sbf* I and *Mss* I digested pECW7 using an IN-FUSION® HD Cloning Kit. The reaction was composed of 50 ng of *Sbf* I and *Mss* I digested pECW7, 111 ng of the 5.4 kb SOE PCR product, and 4 µl of IN-FUSION® buffer plus Enzyme in a 20 µl reaction volume. The reaction was incubated for 15 minutes at 50°C and then held on ice. A 2 µl aliquot was used to transform 50 µl of Stellar™ *E. coli* chemically competent cells by addition to a tube containing the competent cells and incubating the cells on ice for 30 minutes. The tube was incubated at 42°C for 45 seconds after which 450 µl of SOC medium were added. The tube was then incubated at 37°C with agitation at 200 rpm for 1 hour. The reaction was transferred to a microfuge tube and was centrifuged at 13,000 rpm for 10 seconds. Three hundred µl supernatant was discarded, the cells were resuspended in 200 µl supernatant and were transferred to two 150 mm 2XYT plus ampicillin plates. The plates were incubated overnight at 37°C. *E. coli* colonies were used to inoculate 3 ml of LB + Amp in a 14 ml Falcon round-bottom polypropylene tube and incubated 37°C overnight with agitation at 200 rpm. Plasmid DNA was isolated using a QIAprep® Spin Miniprep Kit. The plasmid DNA from the transformants was screened by restriction digestion with *Nco* I HF for production 3.8 and 8.6 kb fragments. Sequencing of one of the plasmids verified that the construct contained the correct inserts with no PCR errors. The plasmid was designated pDM315 (Figure 9).

Example 54: Multiple site specific integrations at *Trichoderma reesei cbh1* and *cbh2* loci using TP901-1 integrase and FLP/FRT simultaneously

In this Example *T. reesei* strain CKle215 (Example 52) which has *attP* sites inserted at the *cbh1* locus and FRT sites inserted at the *cbh2* locus was transformed with plasmids pDM313 (Example 14) and pDM315 (Example 53) to achieve targeting of constructs to the *attP* sites and FRT sites at two different loci. Plasmid pDM313 has an *A. fumigatus* beta-glucosidase expression cassette and the *hpt* marker for hygromycin resistance flanked by FRT sites, and the *S. cerevisiae* flippase gene under the control of the *T. reesei gpdA* promoter. Plasmid pDM315 has a *Humicola insolens* endoglucanase V expression cassette and the *amdS* marker flanked by *attB* sites, and the TP901-1 recombinase gene under the control of the *T. reesei gpdA* promoter.

Protoplasts of CKle215 were prepared as described in Example 1. Approximately 1.5 µg of pDM313 circular DNA and approximately 3.2 µg of pDM315 circular DNA were added to 100 µl of the protoplast solution and mixed gently. PEG buffer (250 µl) was added, and the reaction was mixed and incubated at 34°C for 30 minutes. STC (3 ml) was then added and the reaction was mixed and then spread onto two COVE + 2% glucose transformation plates. Ten transformation reactions were prepared and plated. After incubation at 28°C for

16 hours, 20 ml of a COVE + 2% glucose overlay supplemented with 35 µg of hygromycin B per ml were added to each plate.

The transformation plates were incubated at 28°C for 9 days and at room temperature for 5 days. Twenty-eight transformants were transferred to COVE + 2% glucose plates and incubated at 30°C for 3-5 days. Spore PCR using a PHIRE® Plant Direct PCR Kit was utilized to identify transformants that had integrated the two different plasmids at the *cbh1* and *cbh2* loci. Briefly, spores from each transformant were collected with a sterile 1 µl inoculation loop and transferred to 15 µl of Kit-supplied dilution buffer in a 96-well plate and incubated for 5 minutes at room temperature. The spore suspension was centrifuged briefly in a plate spinner and 1 µl of supernatant was used in the spore PCR. The 20 µl spore PCR was composed of 1X PHIRE® Plant Direct PCR buffer (contains dNTPs and Mg), 10 pmol of the forward primers shown below, 10 pmol of the reverse primers shown below, 0.4 µl of PHIRE® II Hot Start DNA Polymerase, and 1 µl of supernatant from the spore suspension.

PCR screen of 5' end of *cbh1* locus (primer set 1)

Forward primer (homology to 5' flanking region of *cbh1* upstream of *attP* site):

5'-GAGCTTGGACATAACTGTTCC-3' (SEQ ID NO: 256)

Reverse primer (homology to *H. insolens* EGV gene):

5'-AAAAGACAGGCTGGTTCACG-3' (SEQ ID NO: 257)

PCR screen of 5' end of *cbh2* locus (primer set 2)

Forward primer (homology to 5' flanking region of *cbh2* upstream of FRT-F site):

5'-GTTGGTATAGAGCAGCGTTC-3' (SEQ ID NO: 258)

Reverse primer (homology to 3' flanking region of *cbh2*):

5'-CTAGGTAGGTAGGTAGTATA-3' (SEQ ID NO: 259)

Reverse primer (homology to *A. fumigatus* beta-glucosidase):

5'-TGGTGGAGAGAAAGCCAATT-3' (SEQ ID NO: 260)

The PCRs were incubated in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 10 seconds, 60°C for 10 seconds, and 72°C for 1 minute and 10 seconds; and 1 cycle at 72°C for 1 minute and 10 seconds.

The completed PCRs were subjected to 0.8% agarose gel electrophoresis in TAE buffer. Transformants having the correct targeting of plasmid DNA to the *cbh1* locus produced a 1.4 kb fragment with primer set 1. Transformants having the correct targeting of plasmid DNA to the *cbh2* locus produced a 1.35 kb fragment with primer set 2. Transformants having the correct targeting to both the *cbh1* and *cbh2* loci produced the 1.4 kb fragment with primer set 1 and the 1.35 kb fragment with primer set 2.

Spore PCR using a PHIRE® Plant Direct PCR Kit, as described above, was utilized to verify correct integration at the 3' end of both the *cbh1* and *cbh2* loci. Transformants

identified as having integration at both the *cbh1* and *cbh2* loci by the 5' end PCR screen described above were screened with the primers listed below.

PCR screen of 3' end of *cbh1* locus

Forward primer (homology to *amdS* marker):

5 5' AATAACGCTGTCTTCCGCAG-3' (SEQ ID NO: 261)

Reverse primer (homology to 3' flanking region of *cbh1*, downstream of FRT-F3 site):

5' ATGGGTCATTACCAATTGGC-3' (SEQ ID NO: 262)

PCR screen of 3' end of *cbh2* locus

Forward primer (homology to *hpt* marker):

10 5' CGTGTTCCTTCCCATTGCGATGCGACCTCGTGGTCATTGAC-3' (SEQ ID NO: 263)

Reverse primer (homology to 3' flanking region of *cbh2*, past FRT-F3 site):

5' GCATTGCAACCGCGGCTTTC-3' (SEQ ID NO: 264)

The PCRs were incubated in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 10 seconds, 60°C for 10 seconds, and 72°C for 1 minute and 10 seconds; and 1 cycle at 72°C for 1 minute and 10 seconds.

The completed PCRs were subjected to 0.8% agarose gel electrophoresis in TAE buffer. Transformants having the correct targeting of plasmid DNA to the *cbh1* locus produced a 1.0 kb fragment. Transformants having the correct targeting of plasmid DNA to the *cbh2* locus produced a 0.75 kb fragment. Transformants having the correct targeting to both the *cbh1* and *cbh2* loci produced the 1.0 kb fragment and the 0.75 kb fragment.

A transformant that produced the correct PCR fragments was chosen for spore isolation. Spores from a COVE + 2% glucose plate culture were collected using a 1 µl inoculation loop and added to 2 ml of 0.01% TWEEN® 20. One µl of the spore suspension and 100 µl of 0.01% TWEEN® 20 were spread onto a 150 mm PDA plate. The plate was incubated at 30°C for 2 days. Isolated colonies were picked with a sterile 10 µl inoculation loop and transferred to COVE2 plates and incubated at 30°C for 5 days. Spore PCR using a PHIRE® Plant Direct PCR Kit and the primers and PCR conditions shown above for the PCR screen of the 5' end of both loci and the 3' end of the *cbh1* locus was used to screen the spore isolates.

The completed PCRs were subjected to 0.8% agarose gel electrophoresis in TBE buffer. Spore isolates having the correct targeting of plasmid pDM315 to the *cbh1* locus produced a 1.4 kb fragment from the 5' end of the locus and a 1.0 kb fragment from the 3' end of the locus. Transformants having the correct targeting of plasmid pDM313 to the *cbh2* locus produced a 1.35 kb fragment from the 5' end of the locus. Two spore isolates having the correct targeting to both the *cbh1* and *cbh2* loci were chosen for further analysis. Genomic DNA from these two spore isolates was prepared as described in Example 15.

PCRs using the primers and PCR conditions shown below were used to generate PCR fragments from genomic DNA of the two spore isolates.

PCR fragment 1 (2.0 kb):

Forward primer (homology to 5' *cbh1* flank, upstream of *attP* site):

5 5'-TTTGAGCTACAAGAACCTGTGGGG-3' (SEQ ID NO: 265)

Reverse primer (homology to *H. insolens* EGV gene):

5'-ATGTTGAGATCGAAGTGGTT-3' (SEQ ID NO: 266)

PCR fragment 2 (1.3 kb):

Forward primer (homology to *amdS* marker):

10 5'-AATAACGCTGTCTTCCGCAG-3' (SEQ ID NO: 267)

Reverse primer (homology to 3' *cbh1* flank, downstream of *attP* site):

5'-CGAAAAGGCCACCTGTTGAGAGGCT-3' (SEQ ID NO: 268)

To amplify PCR fragments 1 and 2, twenty-five picomoles of each of the primers were used in a PCR composed of 100 ng of genomic DNA, 1X PHUSION™ HF buffer, 1 µl of a 10 mM blend of dATP, dTTP, dGTP, and dCTP, and 2 units of PHUSION™ Hot Start II DNA Polymerase in a final volume of 50 µl. The PCRs were performed in a thermocycler programmed for 1 cycle at 98°C for 30 seconds; 30 cycles each at 98°C for 7 seconds, 60°C for 20 seconds, and 72°C for 1 minute, and 1 cycle at 72°C for 5 minutes. The PCR fragments were purified using a Nucleospin® Extract II Kit (Example 8).

20 Sequencing of PCR fragments 1 and 2 verified that the integration of pDM315 at the *cbh1* locus had occurred by TP901-1 recombinase mediated recombination between the host *attP* sites and plasmid *attB* sites. The recombined *attR* site was present just upstream of the EGV expression cassette and the recombined *attL* site was present just downstream of the *amdS* marker.

25 PCR fragment 3 (1.35 kb):

Forward primer (homology to 5' *cbh2* flank, upstream of FRT-F site):

5'-GTTGGTATAGAGCAGCGTTC-3' (SEQ ID NO: 269)

Reverse primer (homology to *A. fumigatus* beta-glucosidase gene):

5'-TGGTGGAGAGAAAGCCAATT-3' (SEQ ID NO: 270)

30 PCR fragment 4 (0.75 kb):

Forward primer (homology to *hpt* marker):

5'-CGTGTTCCTTCCCATTGCGATGCGACCTCGTGGTCATTGAC-3' (SEQ ID NO: 271)

Reverse primer (homology to 3' flanking region of *cbh2*, downstream of FRT-F3 site):

5'-GCATTGCAACCGCGGCTTTC-3' (SEQ ID NO: 272)

35 To amplify PCR fragments 3 and 4, the 40 µl PCR contained 1X PHIRE® Plant Direct PCR buffer (contains dNTPs and Mg), 20 pmol each of the forward and reverse

primers listed above, 0.8 µl of PHIRE® II Hot Start DNA Polymerase, and 100 ng of genomic DNA.

The PCRs were performed in a thermocycler programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 10 seconds, 60°C for 10 seconds, and 72°C for 1 minute 10 seconds, and 1 cycle at 72°C for 1 minute 10 seconds. The PCR fragments were purified using a Nucleospin® Extract II Kit (Example 8).

Sequencing of PCR fragments 3 and 4 verified that integration of pDM313 at the *cbh2* locus had occurred by flippase mediated recombination between the host FRT sites and plasmid FRT sites.

Genomic DNA from the final spore isolates was prepared and subjected to Southern blot analysis. *Trichoderma reesei* strains were grown in 25 ml of YP medium supplemented with 2% glucose (w/v) in a 125 ml baffled shake flask at 28°C for 3 days with agitation at 200 rpm. Mycelia samples were harvested by filtering over Whatman 55 mm filter paper, rinsed with deionized water, and transferred to a pre-chilled mortar and pestle. Each mycelia preparation was ground into a fine powder and kept frozen with liquid nitrogen. A total of 0.2-0.3 g of powder was transferred to a 50 ml tube and genomic DNA was extracted using a DNEASY® Plant Maxi Kit.

For Southern blot analysis approximately 1 µg of genomic DNA was digested with of *Sca* I + *Cla* I + *Hpa* I (for *cbh1* locus) and *Nru* I + *Mlu* I (for *cbh2* locus) and subjected to 0.8% agarose electrophoresis in TBE buffer. The DNA in the gels was depurinated, denatured, neutralized, and transferred to NYTRAN® Supercharge membranes as described in Example 3. The DNA was UV cross-linked to the membranes using a CL-1000 UV Crosslinker and prehybridized for 1 hour at 42°C in 20 ml of DIG Easy Hyb.

The probe for the 3' flank of *cbh1* was produced as described in Example 10. Templates for probes hybridizing to the 3' flanks the *cbh2* gene, as well as the *H. insolens* endoglucanase V (EGV) and *A. fumigatus* beta-glucosidase (BG) genes, were generated using PHUSION™ Hot Start II High Fidelity DNA Polymerase or PHUSION™ High-Fidelity Hot Start DNA Polymerase and gene-specific forward and reverse primers shown below.

3' *cbh2* flank: Fifty picomoles of each of the primers were used in a PCR composed of 100 ng of *T. reesei* RutC30 genomic DNA, 1X PHUSION™ High-Fidelity Hot Start DNA Polymerase buffer, 1 µl of a 10 mM blend of dATP, dTTP, dGTP, and dCTP, and 1 unit of PHUSION™ High-Fidelity Hot Start DNA Polymerase in a final volume of 50 µl. The PCR was performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; 34 cycles each at 98°C for 15 seconds, 60°C for 30 seconds, and 72°C for 1 minute 30 seconds, and 1 cycle at 72°C for 10 minutes. The completed PCR was purified by 0.8% agarose gel electrophoresis in TAE buffer. A 0.46 kb DNA band was excised from the gel and the DNA was extracted using a Nucleospin® PCR Cleanup and Gel Extraction Kit.

A probe hybridizing to the 3' flanking region of the *cbh2* gene was generated using a PCR DIG Probe Synthesis Kit with the forward and reverse primers indicated below. The 50 µl PCR was composed of 1X PCR DIG Probe Synthesis mix, 50 pmol of each primer, 1X PCR buffer with MgCl₂, 24 ng of purified probe template (described above), and 2.6 units of EXPAND® High Fidelity DNA polymerase. The PCR was incubated in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 45 seconds; and 1 cycle at 72°C for 7 minutes.

Forward primer:

5'-TCTTGAGCCGCATCGCATAGA-3' (SEQ ID NO: 273)

Reverse primer:

5'-TACGGTCAGCGCTCATGCGAA-3' (SEQ ID NO: 274)

EGV and BG probes: To make probe templates, 25 picomoles of each of the primers listed below were used in a PCR composed of 5 ng of either pDM315 (EGV) or pDM313 (BG) DNA, 10 µl of 5X PHUSION™ HF buffer, 1 µl of a 10 mM blend of dATP, dTTP, dGTP, and dCTP, and 1 unit of PHUSION™ Hot Start II DNA Polymerase in a final volume of 50 µl. For EGV the PCR was performed in a thermocycler programmed for 1 cycle at 98°C for 30 seconds; 35 cycles each at 98°C for 7 seconds, 72°C for 15 seconds; 1 cycle at 72°C for 5 minutes and 1 cycle at 98°C for 30 seconds. For BG the thermocycler was programmed for 1 cycle at 98°C for 30 seconds; 35 cycles each at 98°C for 7 seconds, 66°C for 15 seconds, 72°C for 15 seconds; and 1 cycle at 72°C for 5 minutes. The completed PCRs were purified by 0.65% agarose gel electrophoresis in TBE buffer. A 0.54 kb DNA band (EGV) and 0.5 kb band (BG) were excised from the gels and the DNA was extracted using a Nucleospin® PCR Cleanup and Gel Extraction Kit.

Probes hybridizing to the EGV and BG genes were generated using a PCR DIG Probe Synthesis Kit with the forward and reverse primers indicated below. The 50 µl PCR contained 1X PCR DIG Probe Synthesis mix, 50 pmol of each primer, 1X PCR buffer with MgCl₂, 80-90 pg of purified probe template (described above), and 2.6 units of EXPAND® High Fidelity DNA polymerase. The PCRs were performed in a thermocycler programmed for 1 cycle at 95°C for 2 minute; 30 cycles each at 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 40 seconds; and 1 cycle at 72°C for 7 minutes.

Primers for EGV probe

Forward primer:

5'-TCCGGTCCTGTTGCTGGCAA-3' (SEQ ID NO: 275)

Reverse primer:

5'-TTCGTGCAAGTGCTGCCAGC-3' (SEQ ID NO: 276)

Primers for BG probe

Forward primer:

5'-ATGAGATTCGGTTGGCTCGAG-3' (SEQ ID NO: 277)

Reverse primer:

5'-AAAGACTCCGCGGGTATAGCTC-3' (SEQ ID NO: 278)

Southern hybridization was performed as described in Example 3.

5 The Southern blot containing the *Sca* I/*Cla* I/*Hpa* I digested DNA was probed with both the 3' *cbh1* flanking region and EGV probes. Southern blot analysis identified final spore isolates that produced 6 kb and 4.3 kb hybridizing fragments, verifying the correct integration of pDM315 DNA at the *attP* sites at the *cbh1* locus. The parent *T. reesei* (CKle215) *cbh1* locus produced a 5.5 kb hybridizing fragment with these probes. The blot
10 containing the *Nru* I/*Mlu* I digested DNA was probed with both the 3' *cbh2* flanking region and BG probes. Southern blot analysis identified final spore isolates that produced 6.9 kb and 5.4 kb hybridizing fragments, verifying the correct integration of pDM313 DNA at the FRT sites of the *cbh2* locus. The parent *T. reesei* (CKle215) *cbh2* locus produced a 6.4 kb hybridizing fragment with these probes. Combining the results of the two Southern blots
15 indicates the final spore isolates had the correct simultaneous integration of pDM315 DNA at the *attP* sites of the *cbh1* locus and pDM313 DNA at the FRT sites of the *cbh2* locus.

Example 55: Construction of plasmid pQM43 for targeting a FRT-F site containing non-functional *amdS* marker to *Trichoderma reesei cbh1* locus

20 Plasmid pQM43 was constructed for targeting a non-functional *amdS* fragment (designated "non-functional *amdS* fragment 3") flanked at its 5' by a FRT-F site to the *T. reesei cbh1* gene locus. In the construct, the FRT-F site (49 bp) was added within the first intron of an approximately 1 kb non-functional *amdS* fragment as described below. An approximately 400 bp fragment containing the *T. reesei cbh1* 5' flanking region and FRT-F
25 fragment was amplified from *T. reesei* RutC30 genomic DNA using primers 1208187 and 1208194 shown below. The non-functional *amdS* fragment 3 flanked by a FRT-F site was amplified from pAllo1 (WO 04/111228) using primers 1208195 and 1208196 shown below.

Primer 1208187:

5'-GATTGAGTTGAACTGCCTAAGATCTCG-3' (SEQ ID NO: 279)

30 Primer 1208194:

5'-CTATACTTTCTAGAGAATAGGAACTCGGAATAGGAACTTCAAGGTGCGCAGTC
CGCGGTTGAC-3' (SEQ ID NO: 280)

Primer 1208195:

5'-CTATTCCGAGTTCCTATTCTCTAGAAAGTATAGGAACTTCGGCGTTGTTACATC
35 TCCCTGAG-3' (SEQ ID NO: 281)

Primer 1208196:

5'-GCGTCAGGCTTTCGCCACGTCTACGCCAGGACCGAGCAAG-3' (SEQ ID NO: 282)

The first PCR was composed of 100 ng of *T. reesei* RutC30 genomic DNA, 1 µl of 10 mM dNTPs, 1 µM primers, 1X PHUSION® High-Fidelity Reaction Buffer, and 1 unit of PHUSION® Hot Start High-Fidelity DNA Polymerase in a final volume of 50 µl. The second PCR was composed of 100 ng of pAllo1, 1 µl of 10 mM dNTPs, 1 µM primers, 1X PHUSION® High-Fidelity Reaction Buffer, and 1 unit of PHUSION® Hot Start High-Fidelity DNA Polymerase in a final volume of 50 µl. Both reactions were performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 35 cycles each at 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 1 minute and 15 seconds; and 1 cycle at 72°C for 10 minutes. The PCR products were separated by 0.7% agarose gel electrophoresis in TAE buffer where fragments of approximately 400 bp and 1 kb were excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

The third PCR was composed of 100 ng of each of the 400 bp and 1 kb purified PCR products, 1 µl of 10 mM dNTPs, 1X PHUSION® High-Fidelity Reaction Buffer, and 1 unit of PHUSION® Hot Start High-Fidelity DNA Polymerase in a final volume of 48 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; and 5 cycles each at 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 2 minutes. Primers 1208187 and 1208196 were then added at final concentrations of 1 µM and continued for 30 cycles each at 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 2 minutes, and 1 cycle at 72°C for 10 minutes. The PCR product was separated by 0.7% agarose gel electrophoresis in TAE buffer where a fragment of approximately 1.4 kb were excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

The 1.4 kb PCR product was inserted into an approximately 9.3 kb *Bgl* II/*Pac* I digested pJfyS139 using an IN-FUSION™ HD Cloning Kit. The reaction was composed of 1X IN-FUSION™ HD Enzyme Premix, 200 ng of *Bgl* II/*Pac* I digested pJfyS139, and 61 ng of the 1.4 kb PCR product in a 10 µl reaction volume. The reaction was incubated at 50°C for 15 minutes. After the incubation period, a 1 µl aliquot was transformed into ONE SHOT® TOP10 Chemically Competent cells according to Example 4. Plasmid DNA was isolated from the transformants according to Example 4. The insert was confirmed by DNA sequencing. One transformant was identified as containing the insert with no PCR errors and the plasmid was designated pQM43 (Figure 10).

Example 56: Construction of *Trichoderma reesei* strain QMJi057 for targeting a non-functional *amdS* fragment 3 flanked by a FRT-F site to the *Trichoderma reesei cbh1* locus

Trichoderma reesei AgJg115-118-1H (Example 9) was transformed with 1-5 µg of *Pme* I digested pQM43 to insert the non-functional *amdS* fragment 3 flanked at its 5' by a FRT-F site at the *cbh1* gene locus. Twenty-six transformants were obtained and each one

was picked and transferred to a PDA plate and incubated for 7 days at 30°C. The transformants were cultured in 2 ml of CIM and incubated at 30°C for 3 days with agitation at 250 rpm. Supernatant from each culture was subjected to SDS-PAGE using a CRITERION® 8-16% TGX Stain-Free gel and PRECISION PLUS® Protein Unstained Standards. Since successful targeted integration of pQM43 at the *cbh1* locus effectively disrupts the *cbh1* gene, SDS-PAGE gels were visually analyzed for loss of the CBH1 protein from the proteome. *T. reesei* QMJi057-5 was identified as producing no CBH1 protein and was selected for genomic DNA extraction and Southern blot analysis to confirm the integration of the FRT-F site containing the non-functional *amdS* fragment 3 at the *T. reesei cbh1* gene locus. Genomic DNA was isolated from the transformants according to the procedure described in Example 2. Genomic DNA was digested with *Nhe* I for Southern blot analysis with a digoxigenin-labeled *T. reesei cbh1* 3' probe synthesized by PCR using a PCR DIG Probe Synthesis Kit and the primers shown below.

Primer 0610249:

5'-GAGAACACAGTGAGACCATAGC-3' (SEQ ID NO: 283)

Primer 0610250:

5'-TCTCAACCCAATCAGCAACATG-3' (SEQ ID NO: 284)

The DIG Probe Synthesis PCR was composed of approximately 100 pg of a PCR fragment containing the *T. reesei cbh1* 3' flanking region used to make pQM21 (WO 2013/028912) as template, 1 µM primers, 5 µl of PCR DIG Synthesis Mix, 1X PCR buffer with MgCl₂, and 0.75 µl of Enzyme Mix in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 95°C for 2 minutes; 10 cycles each at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 40 seconds; 20 cycles each at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 40 seconds plus an additional 20 seconds for each successive cycle; and 1 cycle at 72°C for 7 minutes. The PCR product was separated by 1% agarose gel electrophoresis in TAE buffer where a 720 bp band was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

Transformant QMJi057-5 was confirmed by Southern blot analysis to contain the non-functional *amdS* fragment 3 flanked at its 5' by a FRT-F site at the *cbh1* locus, which resulted in a hybridized signal at approximately 7.1 kb recognized by the *T. reesei cbh1* 3' probe.

Example 57: Site specific integrations at *Trichoderma reesei cbh1* locus in strain QMJi057-5

An approximately 3 kb DNA fragment containing the coding sequence of an *A. niger* mannosidase (SEQ ID NO: 285 for the DNA sequence and SEQ ID NO: 286 for the amino acid sequence) was amplified from *A. niger* Bo-1 derivative strain CKle47 with primer

0614762 and 0614763 and cloned into *Nco* I and *Pac* I digested pMJ09 (US8318458 B2; approximately 7.2 kb), resulting in plasmid pQM27 (SEQ ID NO: 287). The *A. niger* mannosidase expression cassette in pQM27 is followed by a functional *amdS* marker.

An approximately 6.7 kb DNA fragment containing an *A. niger* mannosidase expression cassette, a non-functional *amdS* fragment 4, and the FRT-F site was amplified from pQM27 with primer 1201956 and 1201606. The PCR was composed of 100 ng of pQM27, 200 µM dNTPs, 0.4 µM primers, 1X PHUSION® Reaction Buffer (Thermo Fisher Scientific, Inc.), and 1 units of PHUSION® High Fidelity DNA polymerase (Thermo Fisher Scientific, Inc.) in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; 35 cycles each at 98°C for 15 seconds, 60°C for 30 seconds, and 72°C for 3.5 minutes; and 1 cycle at 72°C for 10 minutes. The 6.7 kb PCR product was separated by 0.7% agarose gel electrophoresis in TAE, excised from the gel, and extracted using a Nucleospin® Extract II Kit (Example 8). The purified 6.7 kb PCR product was used as template to amplify a FRT-F site containing fragment using primers 1201956 and 1201957. The resulting FRT-F site containing PCR fragment was separated by 0.7% agarose gel electrophoresis in TAE and then excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

Primer 1210956:

5'-TGATTACGAATTGTTTAAACGGATCCGAATGTAGGATTGTTATCCG-3' (SEQ ID NO: 288)

Primer 1201606:

5'-GAAGTTCCTATACTTTCTAGAGAATAGGAACTCGGAATAGGAACTTCAACCTTATGG GACTATCAAGCTGAC-3' (SEQ ID NO: 289)

Primer 1210957:

5'-GTTACATTGACGTACTTATAAGAAGTTCCTATACTTTCTAGAGAATAGGA-3' (SEQ ID NO: 290)

The purified FRT-F site containing PCR fragment was inserted into *Xba* I and *Psi* I digested pDM313 (approximately 5.4 kb) using an IN-FUSION™ HD Cloning Kit. The reaction was composed of 1X IN-FUSION™ HD Enzyme Premix, 323 ng of *Xba* I/*Psi* I digested pDM313, and 200 ng of the 6.7 kb FRT-F site containing the PCR product in a 15 µl reaction volume. The reaction was incubated at 50°C for 15 minutes. After the incubation period, a 2 µl aliquot was transformed into 50 µl of Stellar™ *E. coli* chemically competent cells by addition to a tube containing the competent cells and incubating the cells on ice for 30 minutes. The tube was incubated at 42°C for 45 seconds after which 450 µl of SOC medium were added. The tube was then incubated at 37°C with agitation at 250 rpm for 1 hour. Volumes of 50 µl and 150 µl were transferred to two 150 mm 2XYT plus ampicillin plates. The plates were incubated overnight at 37°C. Plasmid DNA was isolated and

sequenced as described in Example 4. One transformant was identified as containing the insert with no PCR errors and the plasmid was designated pQM45 (Figure 11).

Plasmid pQM45 was digested with *Pme* I and used to test site specific integration at the *T. reesei cbh1* locus in *T. reesei* strain QMJi057-5. The *Pme* I digested pQM45 was transformed into protoplasts of *T. reesei* strain QMJi057-5 as described in Example 1 and the transformation reactions were spread onto COVE plates and incubated at 30°C for 7-10 days. All of the transformations resulted in visible transformants on COVE plates, suggesting that insertion of the FRT-F site into the first intron of the *amdS* gene allows recombination of a functional *amdS* marker and integration at the targeted site.

Genomic DNA was isolated from six transformants according to the procedure described in Example 2. Genomic DNA was digested with *EcoR* I for Southern blot analysis with a 358 bp digoxigenin-labeled *T. reesei cbh1* 5' probe (see below), which was synthesized by incorporation of digoxigenin-11-dUTP by PCR using a PCR DIG Probe Synthesis Kit.

Southern blot analysis of all six transformants resulted in a hybridized signal at approximately 4.2 kb recognized by the *T. reesei cbh1* 5' probe, indicating correct integration at the *cbh1* locus.

T. reesei cbh1 5' probe sequence:

5'-TAGGGTCGGCAACGGCAAAAAAGCACGTGGCTCACCGAAAAGCAAGATGTTTGCGA
TCTAACATCCAGGAACCTGGATACATCCATCATCACGCACGACCACTTTGATCTGCTGT
AAACTCGTATTCGCCCTAAACCGAAGTGCGTGGTAAATCTACACGTGGGCCCCCTTTCG
GTATACTGCGTGTGTCTTCTCTAGGTGCCATTCTTTTCCCTTCCTCTAGTGTTGAATTGT
TTGTGTTGGAGTCCGAGCTGTAACCTCTGAATCTCTGGAGAATGGTGGACTAACGA
CTACCGTGACCTGCATCATGTATATAATAGTGATCCTGAGAAGGGGGGTTTGGAGCAA
TGTGGG-3' (SEQ ID NO: 291)

Example 58: Construction of plasmids pQM37, pQM38 and pQM39 (functional *amdS* marker with FRT-F3 sites in *amdS* introns)

Plasmids pQM37, pQM38 and pQM39 were constructed to test the impact on *amdS* function after inserting a FRT-F3 fragment into one of the three introns of the *amdS* gene. The primers shown below were designed to introduce a FRT-F3 fragment into each of the three introns in the *amdS* gene in plasmid pAllo1 using a QUICKCHANGE® II XL Site-Directed Mutagenesis Kit. Primers 1205503 and 1205504 were used to construct plasmid pQM37, where the FRT-F3 site was inserted into intron 1 of the *amdS* gene. Primers 1205505 and 1205506 were used to construct plasmid pQM38, where the FRT-F3 site was inserted into intron 2 of the *amdS* gene. Primers 1205507 and 1205508 were used to construct pQM39, where the FRT-F3 site was inserted into intron 3 of the *amdS* gene.

Primer 1205503:

5'-GGGAGATGTAACAACGCCTTGAAGTTCCTATTCCGAGTTCCTATTCTTCAAATA
GTATAGGAACCTTCAACCTTATGGGACTATCAAG-3' (SEQ ID NO: 292)

Primer 1205504:

- 5 5'-CTTGATAGTCCCATAAGGTTGAAGTTCCTATACTATTTGAAGAATAGGAACTCG
GAATAGGAACCTTCAAGGCGTTGTTACATCTCCC-3' (SEQ ID NO: 293)

Primer 1205505:

5'-GCCCCTAAGTCGTTAGATGTTTGAAGTTCCTATTCCGAGTTCCTATTCTTCAAATAGT
ATAGGAACCTTCACCCTTTTTGTCAGC-3' (SEQ ID NO: 294)

- 10 Primer 1205506:

5'-GCTGACAAAAAGGGTGAAGTTCCTATACTATTTGAAGAATAGGAACTCGGAATAGGA
ACTTCAAACATCTAACGACTTAGGGGC-3' (SEQ ID NO: 295)

Primer 1205507:

- 15 5'-CTATACCAGGCCTCCACTTGAAGTTCCTATTCCGAGTTCCTATTCTTCAAATAGTATA
GGAACCTTCATGTCCTCCTTTCTTGC-3' (SEQ ID NO: 296)

Primer 1205508:

5'-GCAAGAAAGGAGGACATGAAGTTCCTATACTATTTGAAGAATAGGAACTCGGAATAG
GAACTTCAAGTGGAGGCCTGGTATAG-3' (SEQ ID NO: 297)

- 20 The PCRs were composed of 10 ng of pAllo1, 1 µl of 10 mM dNTPs, 1X Reaction
Buffer, 125 ng of each primer, 1 µl of QUIKSOLUTION® reagent, and 2.5 units of PfuUltra™
HF DNA Polymerase (Agilent Technologies) in a final volume of 50 µl. The reactions were
performed in a thermocycler programmed for 1 cycle at 95°C for 1 minutes; and 18 cycles
each at 95°C for 50 seconds, 60°C for 50 seconds, and 68°C for 7 minutes; and 1 cycle at
68°C for 7 minutes. Ten units of *Dpn* I were added to each reaction and incubated at 37°C
25 for one hour. Two µl of each *Dpn* I treated reaction were transformed into XL10-Gold
Ultracompetent *E. coli* cells. The transformation reactions were spread onto 2XYT plus
ampicillin plates. About 4-6 colonies were picked for each transformation and cultured in 3
ml of LB plus ampicillin medium at 37°C for 15-17 hours with agitation at 250 rpm. Plasmid
DNA was extracted from each colony using a QIAprep® Spin Miniprep Kit and submitted for
30 DNA sequencing. One transformant containing the FRT-F3 site inserted into intron 1 of the
amdS gene was designated pQM37. One transformant containing the FRT-F3 site inserted
into intron 2 of the *amdS* gene was designated pQM38. One transformant containing the
FRT-F3 site inserted into intron 3 of the *amdS* gene was designated pQM39.

- 35 About 30-40 µg of each of pAllo1, pQM37, pQM38, and pQM39 were digested with
Eco RI and *Pac* I. The digested DNAs were purified using a Nucleospin® Extract II Kit
(Example 8). The purified linear DNA fragments of pAllo1, pQM37, pQM38, and pQM39
were each transformed into *T. reesei* RutC30 protoplasts according to Example 1. The

transformation reactions were spread onto COVE plates and incubated at 28°C for 7-10 days. All of the transformations resulted in visible transformants on COVE plates, indicating that *amdS* can be used as a functional selection marker with a FRT-F3 site inserted in any one of the three *amdS* introns.

5

Example 59: Construction of plasmid pDM319

Plasmid pDM319 contains the FRT F13 and FRT F14 sites flanking an expression cassette which contains the *T. reesei eg1* promoter, the *A. fumigatus* cellobiohydrolase II (*cbh2*) coding region, and the *T. reesei eg1* terminator. Plasmid pDM319 also contains the *T.*

10

reesei cbh2 promoter regulating the *S. cerevisiae* flippase gene.

A 1.1 kb fragment containing the FRT F13 site and the *T. reesei eg1* promoter was amplified from *T. reesei* RutC30 genomic DNA using the primers shown below. In the forward primer sequence the FRT F13 site is underlined. The PCR was performed in a manner similar to that described in Example 14.

15 Forward primer:

5'-ACGCGGCCGCGAATTCGGCGCGCCGAAAGTTCCTATTCCGAAGTTCCTATTCTCATAT
AAGTATAGGAACTTCATTAAATTACCATCGTGCTCTG-3' (SEQ ID NO: 298)

Reverse primer:

5'-GGAAGATGCAAGGTGCTTCATTTTGGGACAACAAGAAGGAC-3' (SEQ ID NO: 299)

20

A 1.8 kb PCR fragment containing the *A. fumigatus* cellobiohydrolase II coding region was amplified from plasmid pJfyS144 (WO 2013/028928A1) using the primers shown below. The PCRs were performed in a manner similar to that described in Example 14.

Forward primer:

5'-GTCCTTCTTGTTGTCCCAAATGAAGCACCTTGCATCTTCC-3' (SEQ ID NO: 300)

25 Reverse primer:

5'-ACCAGAGGCAAGTCAACGCTTAATTAAAGGACGGGTTAGCGTTGGT-3' (SEQ ID
 NO: 301)

A 0.32 kb PCR fragment containing the *T. reesei eg1* terminator region was amplified from *T. reesei* RutC30 genomic DNA using the primers shown below.

30 Forward primer:

5'-ACCAACGCTAACCCGTCCTTTTAATTAAGCGTTGACTTGCCTCTGGT-3' (SEQ ID NO:
 302)

Reverse primer:

5'-CCTCGCATACCTAGCCTAGAATTCGCGATCGCGACGATAAGCTTGCCCTGGTGGTG
 TCAACCCTG-3' (SEQ ID NO: 303)

35

The PCRs were performed in a manner similar to that described in Example 14.

The three completed PCR fragments described above were gel purified by 0.8%

agarose gel electrophoresis in TAE buffer. The fragments of 1.1 kb, 1.8 kb, and 0.32 kb were excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

The 1.8 kb and 0.32 kb PCR fragments were joined by SOE PCR using the primers shown below.

5 Forward primer:

5'-GTCCTTCTTGTTGTCCCAAATGAAGCACCTTGCATCTTCC-3' (SEQ ID NO: 304)

Reverse primer:

5'-CCTCGCATACCTAGCCTAGAATTCGCGATCGCGACGATAAGCTTGCCCTGGTGGTG
TCAACCCTG-3' (SEQ ID NO: 305)

10 The PCR was composed of 50 picomoles of each of the primers, 12 ng of the 1.8 kb fragment, 12 ng of the 0.32 kb fragment, 1X PHUSION™ High-Fidelity Hot Start DNA Polymerase buffer, 1 µl of a 10 mM blend of dNTPs, and 1 unit of PHUSION™ High-Fidelity Hot Start DNA Polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; 34 cycles each at 98°C for 15
15 seconds, 59°C for 30 seconds, and 72°C for 1.5 minutes, and 1 cycle at 72°C for 10 minutes. Two SOE PCRs were performed and the reactions were combined. The 2.1 kb SOE PCR fragment was gel purified by 0.8% agarose gel electrophoresis in TAE buffer. The 2.1 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

20 Twenty-five µg of pJfyS156 DNA (Example 8) were digested with *Eco* RI and purified by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 5.0 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

The 2.1 kb SOE PCR fragment, containing the *A. fumigatus* cellobiohydrolase II coding region and the *T. reesei eg1* terminator region, and the 1.1 kb PCR fragment
25 containing the FRT F13 site and the *T. reesei eg1* promoter, were inserted into *Eco* RI digested pJfyS156 using an IN-FUSION® HD Cloning Kit. The cloning and *E. coli* transformation were performed in a similar manner to that described in Example 14. Plasmid DNA was isolated from the transformants according to Example 4. Transformants were screened by restriction digestion with *Asc* I and *Pac* I which produced 2.8 and 5.3 kb
30 fragments. DNA sequencing of one clone verified that the plasmid contained the correct inserts with no PCR errors. The plasmid was designated pDM319a.

A 2.8 kb fragment containing the *amdS* selectable marker was amplified from pMJ09 (U.S. Patent 7,361,495) template DNA using the primers shown below. The PCR was performed in a manner similar to that described in Example 14.

35 Forward primer:

5'-GACACCACCAGGGCAAGCTTTGGAAACGCAACCCTGAAGG-3' (SEQ ID NO: 306)

Reverse primer:

5'-ACCAGAGGCAAGTCAACGCTCGCCGGCGTCTACGCCAGGACCGAGCAA-3' (SEQ ID NO: 307)

A 0.36 kb fragment containing the *T. reesei* *eg1* terminator and FRT F14 site was amplified from *T. reesei* RutC30 genomic DNA using the primers shown below. In the reverse primer sequence the FRT F14 site is underlined. The PCR was performed in a manner similar to that described in Example 14.

Forward primer:

5'-TTGCTCGGTCCTGGCGTAGACGCCGGCGAGCGTTGACTTGCCTCTGGT-3' (SEQ ID NO: 308)

Reverse primer:

5'-CCTAGCCTAGAATTCGCGATCGCGAAGTTCCTATACTTCTGATAGAATAGGAACTTCGGAATAGGAACTTCCCTGCAGGCCCTGGTGGTGTCAAC-3' (SEQ ID NO: 309)

The PCR was performed in a manner similar to that described in Example 14.

The two completed PCR fragments described above were gel purified by 0.8% agarose gel electrophoresis in TAE buffer. The fragments of 2.8 kb and 0.36 kb were excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

Twenty-five µg of pDM319a DNA were digested with *Hind* III and *Asi* SI and purified by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 8.0 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

The 2.8 kb PCR fragment, containing the *amdS* selectable marker and the 0.36 kb PCR fragment containing the *T. reesei* *eg1* terminator and FRT F14 site, were inserted into the *Hind* III and *Asi* SI pDM319a fragment using an IN-FUSION® HD Cloning Kit. The cloning and *E. coli* transformation were performed in a similar manner to that described in Example 14. Plasmid DNA was isolated from the transformants according to Example 4. Transformants were screened by restriction digestion with *Bam* HI and *Spe* I which produced 2.8 and 8.3 kb fragments. DNA sequencing of one clone verified that the plasmid contained the correct inserts with no PCR errors. The plasmid was designated pDM319.

Example 60: Construction of plasmid pDM322

Plasmid pDM322 contains the FRT F13 and FRT F14 sites flanking an expression cassette which contains the *T. reesei* *cbh2* promoter, the *P. emersonii* GH61A coding region, and the *T. reesei* *cbh2* terminator. Plasmid pDM322 also contains the *T. reesei* *cbh2* promoter regulating the *S. cerevisiae* flippase gene.

A 1.9 kb fragment containing the *hpt* marker was amplified using pDM313 template DNA (Example 14) and the primers shown below. PCR conditions were similar to those described in Example 14.

Forward primer:

5'-GTATAACAACCTCCGAGTTAATTAAGGGTTCGAATTCATTTAAACGG-3' (SEQ ID NO: 310)

Reverse primer:

5'-AACTTCGGAATAGGAACTTCCCTGCAGGTGGGAGCGCTCAATATTCATC-3' (SEQ ID NO: 311)

The PCR fragment was purified using a Nucleospin® Extract II Kit. The DNA was eluted with 40 µl of NE buffer supplied with the Nucleospin® Extract II Kit. The eluted DNA was digested with *Pac* I and *Sbf* I and then gel purified by 0.8% agarose gel electrophoresis in TAE buffer. The 1.9 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

Twenty µg of pDM319 DNA were digested with *Pac* I, *Swa* I, and *Sbf* I and purified by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 5.1 kb *Swa* I/*Sbf* I fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

Twenty µg of pQM35 DNA (WO2013028928A1) were digested with *Pme* I and *Pac* I and purified by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 1.8 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8). The 1.8 kb fragment contains the *T. reesei cbh2* promoter, the *P. emersonii* GH61A coding sequence, and *T. reesei cbh2* terminator.

The 1.9 kb PCR fragment and 1.8 kb pQM35 fragment were ligated to the 5.1 kb pDM319 fragment using a QUICK LIGATION™ Kit. The ligation reaction was composed of 1X Quick Ligation buffer, 59 ng of the 5.1 kb *Swa* I/*Sbf* I pDM319 fragment, 64 ng of the 1.8 kb *Pme* I/*Pac* I pQM35 fragment, 57 ng of the 1.9 kb *Pac* I/*Sbf* I PCR fragment, and 1 µl of Quick Ligase in a 20 µl reaction volume. The reaction was incubated at room temperature for 5 minutes and 2 µl were used to transform ONE SHOT® TOP10 *E. coli* chemically competent cells according to Example 4. Plasmid DNA was isolated from the transformants according to Example 4. Transformants were screened by restriction digestion with *Sbf* I and *Nco* I which produced 3.0 and 5.8 kb fragments. DNA sequencing of one clone verified that the plasmid contained the correct inserts with no PCR errors. The plasmid was designated pDM322.

Example 61: Construction of plasmid pDM325

Plasmid pDM325 contains the FRT F13 and FRT F10 sites flanking an expression cassette which contains the *T. reesei cbh2* promoter, the *P. emersonii* GH61A coding sequence, and the *T. reesei cbh2* terminator. Plasmid pDM325 also contains the *T. reesei cbh2* promoter regulating the *S. cerevisiae* flippase gene.

Thirty µg of pDM322 DNA were digested with *Nsi* I and *Eco* RI and purified by 0.8%

agarose gel electrophoresis in TAE buffer where an approximately 2.3 kb *Nsi* I/*Eco* RI fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8). This fragment was used as the template DNA for the 1.8 kb PCR fragment described below.

A 1.8 kb fragment containing the FRT F10 site, the *T. reesei cbh2* promoter, and part of the *S. cerevisiae* flippase coding region was amplified using the primers shown below. In the forward primer sequence the FRT F10 site is underlined.

Forward primer:

5'-GCGCTCCACCTGCAGGGAAGTTCCTATTCCGAAGTTCCTATTCACTAGAATGTATA
GGAACCTTCATTAAATGAATTCTAGGCTAGGTATGC-3' (SEQ ID NO: 312)

Reverse primer:

5'-AGTAGCGGGAGACTAGTGCGAAGT-3' (SEQ ID NO: 313)

Four PCRs were performed in a similar manner as described in Example 14. The four reactions were combined and the PCR fragment was purified using a Nucleospin® Extract II Kit (Example 8).

Thirty µg of pDM322 DNA were digested with *Spe* I and *Sbf* I and purified by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 7.0 kb *Spe* I/*Sbf* I fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

The 1.8 kb PCR fragment was inserted into the 7.0 kb *Spe* I/*Sbf* I pDM322 vector using an IN-FUSION® HD Cloning Kit. The cloning and *E. coli* transformation were performed in a similar manner to that described in Example 14. Plasmid DNA was isolated from the transformants according to Example 4. DNA sequencing of one clone verified that the plasmid contained the correct insert with no PCR errors. The plasmid was designated pDM325 (Figure 12).

Example 62: Construction of plasmid pDM316

Plasmid pDM316 was constructed to introduce FRT F13 and FRT F14 sites at the *T. reesei eg1* locus.

A 1.5 kb fragment containing the *eg1* 5' flanking sequence was amplified using the primers shown below.

Forward primer:

5'-ACATGGTTTAAACGGCGCGCCGGAGCAGATGGCGACACCA-3' (SEQ ID NO: 314)

Reverse primer:

5'-GAATAGGAACTTCGCGGCCCGCCATACCACTTCGTTGATACACTG-3' (SEQ ID NO: 315)

Two PCRs were performed in a similar manner as described in Example 14 using *T.*

reesei RutC30 genomic DNA as the template. The two reactions were combined and gel purified by 0.8% agarose gel electrophoresis in TAE buffer. The 1.5 kb PCR fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

The following oligomers containing the FRT F13 site (underlined) were combined and annealed as described below.

5' -CGGCCGCGAAGTTCCTATTCCGAAGTTCCTATTCTCATATAAGTATAGGAACTTCCG
CGCCCGACAAAACAAGGC-3' (SEQ ID NO: 316)

5' -GCCTTGTTTTGTGCGGCGCGGAAGTTCCTATACTTATATGAGAATAGGAACTTCGGA
ATAGGAACTTCGCGGCCG-3' (SEQ ID NO: 317)

The oligomers were resuspended in sterile distilled water at a concentration of 100 pmoles/μl. Ten μl aliquots of each oligomer were combined along with 2.3 μl of 10X restriction buffer 3 (New England Biolabs). The combined oligomers were heated at 98°C for 2 minutes and then cooled to room temperature. The annealed oligomers were diluted 1:200 using NE buffer from a Nucleospin® Extract II Kit.

Twenty-five μg of pJfyS1579-41-11 DNA (WO 2011/075677) were digested with *Asc* I and purified by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 8.0 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

The 1.5 kb 5' *eg1* flanking PCR fragment and annealed oligomers were inserted into the 8.0 kb *Asc* I pJfyS1579-41-11 fragment using an IN-FUSION® HD Cloning Kit. The cloning and *E. coli* transformation were performed in a similar manner to that described in Example 14. Plasmid DNA was isolated from the transformants according to Example 4. Transformants were screened by restriction digestion with *Pme* I and *Not* I which produced 1.5 and 8.1 kb fragments. DNA sequencing of one clone verified that the plasmid contained the correct insert with no PCR errors. The plasmid was designated pDM316a.

A 1.5 kb fragment containing the *eg1* 3' flanking sequence was amplified using the primers shown below.

Forward primer:

5' -TATAGGAACTTCTTAATTAAGATAATGGCCACTTTCATCTGA-3' (SEQ ID NO: 318)

Reverse primer:

5' -GGCCATATTTAAATCCTGCAGGGTCTGCCGTCCTGATGAGG-3' (SEQ ID NO: 319)

Two PCRs were performed in a similar manner as described in Example 14 using *T. reesei* RutC30 genomic DNA as the template. The two reactions were combined and gel purified by 0.8% agarose gel electrophoresis in TAE buffer. The 1.5 kb PCR fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

The following oligomers containing the FRT F14 site (underlined) were combined and annealed as described above.

5'-CTAGTTGGAGTATTCCTGCAGAAGTTCCTATTCCGAAGTTCCTATTCTATCAGAAGTA
TAGGAACTTCTTAATTAAG-3' (SEQ ID NO: 320)

5'-CTTAATTAAGAAGTTCCTATACTTCTGATAGAATAGGAACTTCGGAATAGGAACTTCT
GCAGGAATACTCCAATA-3' (SEQ ID NO: 321)

5 Twenty µg of pDM316a were digested with *Sbf* I and purified by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 9.6 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

The 1.5 kb *eg1* 3' flanking PCR fragment and annealed oligomers were inserted into the 9.6 kb *Sbf* I pDM316a fragment using an IN-FUSION® HD Cloning Kit. The cloning and
10 *E. coli* transformation were performed in a similar manner to that described in Example 14. Plasmid DNA was isolated from the transformants according to Example 4. DNA sequencing of one clone verified that the plasmid contained the correct insert with no PCR errors. The plasmid was designated pDM316.

15 **Example 63: Construction of plasmid pDM318**

Plasmid pDM318 was constructed to introduce FRT F14 and FRT F15 sites at the *T. reesei eg1* locus.

The 1.5 kb *eg1* 5' flanking sequence was PCR amplified as described in Example 62.

20 The following oligomers containing the FRT F14 site (underlined) were combined and annealed as described in Example 62.

5'-CGGCCGCGAAGTTCCTATTCCGAAGTTCCTATTCTATCAGAAGTATAGGAACTTCCG
CGCCCGACAAAACAAGGC-3' (SEQ ID NO: 322)

5'-GCCTTGTTTTGTCGGGCGCGGAAGTTCCTATACTTCTGATAGAATAGGAACTTCGGA
25 ATAGGAACTTCGCGGCCG-3' (SEQ ID NO: 323)

Twenty-five µg of pJfyS1579-41-11 DNA (WO 2011/075677) were digested with *Asc* I and purified by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 8.0 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

30 The 1.5 kb 5' *eg1* flanking PCR fragment and annealed oligomers were inserted into the 8.0 kb *Asc* I pJfyS1579-41-11 fragment using an IN-FUSION® HD Cloning Kit. The cloning and *E. coli* transformation were performed in a similar manner to that described in Example 14. Plasmid DNA was isolated from the transformants according to Example 4. Transformants were screened by restriction digestion with *Pme* I and *Not* I which produced
35 1.5 and 8.1 kb fragments. DNA sequencing of one clone verified that the plasmid contained the correct insert with no PCR errors. The plasmid was designated pDM318a.

A 1.5 kb 3' *eg1* flanking PCR fragment was generated as described in Example 62.

The following oligomers containing the FRT F15 site (underlined) were combined and annealed as described above.

5'-CTAGTTGGAGTATTCCTGCAGAAGTTCCTATTCCGAAGTTCCTATTCTTATAGGAGTA
TAGGAACTTCTTAATTAAG-3' (SEQ ID NO: 324)

5 5'-CTTAATTAAGAAGTTCCTATACTCCTATAAGAATAGGAACTTCGGAATAGGAACTTCT
GCAGGAATACTCCAACCTAG-3' (SEQ ID NO: 325)

Twenty µg of pDM318a were digested with *Sbf* I and purified by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 9.6 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

10 The 1.5 kb *eg1* 3' flanking PCR fragment and annealed FRT F15 oligomers were inserted into the 9.6 kb *Sbf* I pDM316a fragment using an IN-FUSION® HD Cloning Kit. The cloning and *E. coli* transformation were performed in a similar manner to that described in Example 14. Plasmid DNA was isolated from the transformants according to Example 4. DNA sequencing of one clone verified that the plasmid contained the correct insert with no
15 PCR errors. The plasmid was designated pDM318 (Figure 13).

Example 64: Construction of plasmid pDM320

Plasmid pDM320 contains FRT F14 and FRT F15 sites flanking an expression cassette which contains the *T. reesei eg1* promoter, the *A. fumigatus* cellobiohydrolase II
20 (*cbh2*) coding region, and the *T. reesei eg1 terminator*. Plasmid pDM325 also contains the *T. reesei cbh2* promoter regulating the *S. cerevisiae* flippase gene.

Twenty-five µg of pDM319 (Example 59) were digested with *Asc* I and *Swa* I and purified by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 11 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit
25 (Example 8).

The following oligomers containing the FRT F14 site (underlined) were combined and annealed as described in Example 62.

5'-CGCGAATTCGGCGCGCCCTCGAGGAAGTTCCTATTCCGAAGTTCCTATTCTATCAGA
AGTATAGGAACTTCATTTAAATTACCATCGTGCTCT-3' (SEQ ID NO: 326)

30 5'-AGAGCACGATGGTAATTTAAATGAAGTTCCTATACTTCTGATAGAATAGGAACTTCGG
AATAGGAACTTCCTCGAGGGCGCGCCGAATTCGCG-3' (SEQ ID NO: 327)

The annealed oligomers containing the FRT F14 site were inserted into the 11 kb *Asc* I/*Swa* I pDM319 fragment using an IN-FUSION® HD Cloning Kit. The cloning and *E. coli* transformation were performed in a similar manner to that described in Example 14. Plasmid
35 DNA was isolated from the transformants according to Example 4. DNA sequencing of one clone verified that the plasmid contained the correct insert with no PCR errors. The plasmid was designated pDM320a.

Twenty-five µg of pDM320a were digested with *Sbf* I and *Asi* SI and purified by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 11 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

The following oligomers containing the FRT F15 site (underlined) were combined and annealed as described in Example 62.

5'-GACACCACCAGGGCCTGCAGGGAAGTTCCTATTCCGAAGTTCCTATTCTTATAGGAG
TATAGGAACTTCTACGTAGCGATCGCGAATTCTAGGCT-3' (SEQ ID NO: 328)

5'-AGCCTAGAATTCGCGATCGCTACGTAGAAGTTCCTATACTCCTATAAGAATAGGAAC
TTCGGAATAGGAACTTCCCTGCAGGCCCTGGTGGTGTGTC (SEQ ID NO: 329)

The annealed oligomers containing the FRT F15 site were inserted into the 11 kb *Sbf* I/*Asi* SI pDM320a fragment using an IN-FUSION® HD Cloning Kit. The cloning and *E. coli* transformation were performed in a similar manner to that described in Example 14. Plasmid DNA was isolated from the transformants according to Example 4. DNA sequencing of one clone verified that the plasmid contained the correct insert with no PCR errors. The plasmid was designated pDM320 (Figure 14).

Example 65: Transformation of strain AgJg-FRT2-4B17 to introduce FRT F14 and FRT F15 sites at the *eg1* locus

T. reesei strain AgJg-FRT2-4B17 (Example 12) was transformed with pDM318 (Example 63) to introduce FRT F14 and FRT F15 sites at the *eg1* locus and delete 1 kb of *eg1* promoter sequence, the entire *eg1* coding sequence, and 0.25 kb of the *eg1* terminator sequence. Plasmid pDM318 contains 5' and 3' flanking regions from the *eg1* locus, FRT F14 and FRT F15 sites, and the *E. coli* hygromycin phosphotransferase (*hpt*) gene and the Herpes simplex virus thymidine kinase (*tk*) gene flanked by direct repeats. The direct repeats were used to facilitate the curing out of the *hpt* and *tk* selectable markers.

A total of 125 µg of the transforming plasmid pDM318 was digested with *Pme* I and *Sbf* I. The digestion reaction was purified by 0.8% agarose gel electrophoresis in TAE buffer where an 8 kb DNA band was excised from the gel, and extracted using a Nucleospin® Extract II Kit (Example 8).

Protoplasts of *T. reesei* strain AgJg-FRT2-4B17 were prepared as described in Example 1. Approximately 3 µg of the purified DNA fragment were added to 100 µl of the protoplast solution for hygromycin selection transformation as described in Example 1. Transformants were sub-cultured onto PDA plates to generate spores. Transformants were screened using fungal spore PCR in a manner similar to that described in Example 16.

PCR screen for homologous integration of pDM318: checking 5' end of the *eg1* locus

Forward primer (homologous to 5' flanking sequence of *eg1* locus):

5'-CCCGATGCCACCTCCCAGGATAAGA-3' (SEQ ID NO: 330)

Reverse primer (homologous to *hpt* marker):

5'-TTTGTGAGGCTGCTCAGGGACCCT-3' (SEQ ID NO: 331)

Reverse primer (homologous to *eg1* coding sequence):

5'-TTGTCAACTTGGGATGGACCTCGGGGGTGTGGTACC-3' (SEQ ID NO: 332)

- 5 The three primers above were combined in one PCR. Transformants that had the correct integration of the pDM318 fragment at the *eg1* locus produced a 2.0 kb PCR fragment. Transformants that did not have pDM318 DNA correctly integrated produced a 2.8 kb PCR fragment.

PCR screen for homologous integration of pDM318: checking 3' end of the *eg1* locus

- 10 Forward primer (homologous to *tk* marker):

5'-TACGGTGCGGTATCTGCAGT-3' (SEQ ID NO: 333)

Forward primer (homologous to *eg1* coding sequence):

5'-ACGGAGGAGCTCGACGACTT-3' (SEQ ID NO: 334)

Reverse primer (homologous to 3' flanking sequence of *eg1* locus):

- 15 5'-GCTCTCTCCGCCGATCCAAT-3' (SEQ ID NO: 335)

The three primers were combined in one PCR. Transformants that had the correct integration of the pDM318 fragment at the *eg1* locus produced a 3.0 kb PCR fragment. Transformants that did not have pDM318 DNA correctly integrated produced a 2.2 kb PCR fragment.

- 20 Transformants that produced PCR fragments indicating correct integration at the *eg1* locus were chosen for spore isolation. Spore isolates were generated in a manner similar to that described in Example 15 and plated to PDA medium. The spore isolates were rescreened using the PCR primers and protocol described above. Spores from isolates that yielded the PCR fragments indicative of the correct integration were plated to Trichoderma Minimal medium plates containing 1 μ M FdU (5-fluoro-2'-deoxyuridine) as described in Example 9. Isolates that lost the *tk* marker and looped out both the *hpt* and *tk* markers grow in the presence of FdU. Colonies from the FdU medium were transferred to PDA plates. Spores from the PDA plate cultures were used for screening by fungal spore PCR in a manner similar to that described in Example 16.

30 **PCR screen for loopout of *hpt* and *tk* markers**

Forward primer (homologous to 5' flanking sequence of *eg1* locus):

5'-TACCTCTTTGCGGTCAACTGTGTAAAA-3' (SEQ ID NO: 336)

Reverse primer (homologous to 3' flanking sequence of *eg1* locus):

5'-CTGTCCTTGATTGATGAAAGTGGCC-3' (SEQ ID NO: 337)

- 35 Isolates that had the correct integration of the pDM318 fragment at the *eg1* locus and the correct loop-out of the *hpt* and *tk* markers produced a 2.1 kb PCR fragment. One more round of spore isolation was performed on PDA plates and the resulting isolates were

rescreened by fungal spore PCR using the primers listed above to determine those that produced the 2.1 kb PCR fragment. Genomic DNA from the final spore isolates was prepared in a manner similar to that described in Example 15. For Southern blot analysis approximately 1 µg of each genomic DNA was digested with *Spe* I and *Bam* HI. The digested DNA was subjected to 0.8% agarose gel electrophoresis in TAE buffer and transferred to a NYTRAN® SuperCharge membrane as described in Example 3.

A 1.0 kb probe hybridizing to the 5' flanking sequence of the *eg1* locus was generated using a PCR DIG Probe Synthesis Kit with the primers shown below. The protocol was similar to that described in Example 15 and *T. reesei* RutC30 genomic DNA was used as the template.

Forward primer:

5'-GGAGCAGATGGCGACACCATAGGCGGTGCGAATCGTCCAGAA-3' (SEQ ID NO: 338)

Reverse primer:

5'-TTCCATTTCCCTCCCAGGCCCTGA-3' (SEQ ID NO: 339)

The Southern blot was hybridized, washed and processed as described in Example 3. Southern blot analysis yielded a 3.5 kb hybridizing fragment indicating the correct integration of plasmid pDM318 with the loop-out of the *hpt/tk* markers. The resulting *T. reesei* strain was designated *T. reesei* dlmFRT14FRT15.

Example 66: Construction of plasmid pVCK218

Plasmid pVCK218 contains 5' and 3' flanking regions from the *cbh1* locus, FRT F13 and FRT F14 sites, and the *E. coli* hygromycin phosphotransferase (*hpt*) gene and the Herpes simplex virus thymidine kinase (*tk*) gene flanked by direct repeats. The direct repeats were used to facilitate the curing out of the *hpt* and *tk* selectable markers.

A 1.5 kb fragment containing the 5' *cbh1* region was amplified using the primers shown below and using plasmid pJfyS147 (Example 4) as the template DNA.

Forward primer:

5'-ACATGGTTTAAACGGGGTGAACACCGCCCCCTTCTTG-3' (SEQ ID NO: 340)

Reverse primer:

5'-AGGAACTTCGCGGCCTTCGAACAGCCCCAGTCGGTCAA-3' (SEQ ID NO: 341)

Four PCRs were performed in a similar manner as described in Example 14. The four reactions were combined and the PCR fragment was purified by 0.8% agarose gel electrophoresis in TAE buffer where a 1.5 kb DNA band was excised from the gel, and extracted using a Nucleospin® Extract II Kit (Example 8).

Thirty µg of pDM316 (Example 62) DNA were digested with *Asc* I and *Not* I and purified by 0.8% agarose gel electrophoresis in TBE buffer where an approximately 9.6 kb *Asc* I/*Not* I fragment was excised from the gel and extracted using a Nucleospin® Extract II

Kit (Example 8).

The 1.5 kb PCR fragment was inserted into the 9.6 kb *Asc I/Not I* pDM316 fragment using an IN-FUSION® HD Cloning Kit. The cloning and *E. coli* transformation were performed in a similar manner to that described in Example 14. Plasmid DNA was isolated from the transformants according to Example 4. DNA sequencing of one clone verified that the plasmid contained the correct insert with no PCR errors. The plasmid was designated pVCK218a.

A 1.5 kb fragment containing the 3' *cbh1* region was amplified using the primers shown below and plasmid pJfyS147 (Example 4) as the template DNA.

Forward primer:

5'-TAGGAACTTCTTAATGATAACGGAATAGAAGAAAGAGGAAATTAATA-3' (SEQ ID NO: 342)

Reverse primer:

5'-GCCATATTTAAATCCGTTTAAACAGTCAACACGTCTCCTATGTCTG-3' (SEQ ID NO: 343)

Four PCRs were performed in a similar manner as described in Example 14. The four reactions were combined and the PCR fragment was purified using a Nucleospin® Extract II Kit (Example 8).

Thirty µg of pVCK218a DNA were digested with *Sbf I* and *Pac I* and purified by 0.8% agarose gel electrophoresis in TBE buffer where an approximately 9.6 kb *Sbf I/Pac I* fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

The 1.5 kb 3'*cbh1* PCR fragment was inserted into the 9.6 kb *Sbf I/Pac I* pVCK218a fragment using an IN-FUSION® HD Cloning Kit. The cloning and *E. coli* transformation were performed in a similar manner to that described in Example 14. Plasmid DNA was isolated from the transformants according to Example 4. DNA sequencing of one clone verified that the plasmid contained the correct insert with no PCR errors. The plasmid was designated pVCK218.

Example 67: Construction of plasmid pDM324

Plasmid pDM324 was constructed to introduce FRT F13 and FRT F10 sites at the *T. reesei cbh1* locus. The FRT F14 site in pVCK218 was changed to a FRT F10 site.

Thirty µg of pVCK218 (Example 66) were digested with *Bgl II* and purified by 0.8% agarose gel electrophoresis in TAE buffer where approximately 9.8 kb and 1.4 kb fragments were excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

A 0.33 kb PCR fragment was amplified using the 1.4 kb *Bgl II* pVCK218 fragment as template and the primers shown below. Three PCRs were performed in a similar manner as

described in Example 14. The portion homologous to the FRT F10 site is underlined.

Forward primer (1215840):

5'-TTCTTCCTTGAAGTCTCAGATCTCCCTGCAGGGGGCGACAAAACAAGGCTAC-3'

(SEQ ID NO: 344)

5 Reverse primer:

5'-TTATCATTAAGAAGTTCCTATACATTCTAGTGAATAGGAACTTCGGAATAGGAAC-3'

(SEQ ID NO: 345)

10 The three PCRs were combined and purified by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 0.33 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

A 1.2 kb PCR fragment was amplified using the 1.4 kb *Bgl* II pVCK218 fragment as template and the primers shown below. Three PCRs were performed in a similar manner as described in Example 14. The portion homologous to the FRT F10 site is underlined.

Forward primer:

15 5'-GTTCTATTCCGAAGTTCCTATTCACTAGAAATGTATAGGAACTTCTTAATGATAA-3'

(SEQ ID NO: 346)

Reverse primer (1215843):

5'-CCATGACTGTACGATAGAGAGATCTCCACCATTGCTGTCTGAATT-3' (SEQ ID NO: 347)

20 The three PCRs were combined and purified by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 1.2 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

The 0.33 kb and 1.2 kb PCR fragments were joined by SOE PCR using the primers 1215840 and 1215843 shown above.

25 The PCR was composed of 50 picomoles of each of the primers, 10 ng of the 0.33 kb fragment, 10 ng of the 1.2 kb fragment, 1X PHUSION™ High-Fidelity Hot Start DNA Polymerase buffer, 1 µl of a 10 mM blend of dNTPs, and 1 unit of PHUSION™ High-Fidelity Hot Start DNA Polymerase in a final volume of 50 µl. The reaction was performed in a thermocycler programmed for 1 cycle at 98°C for 2 minutes; 34 cycles each at 98°C for 15
30 seconds, 59°C for 30 seconds, and 72°C for 1 minute; and 1 cycle at 72°C for 10 minutes. Two SOE PCRs were performed and the reactions were combined. The 1.5 kb SOE PCR fragment was gel purified by 0.8% agarose gel electrophoresis in TAE buffer where an approximately 1.5 kb fragment was excised from the gel and extracted using a Nucleospin® Extract II Kit (Example 8).

35 The 1.5 kb SOE PCR fragment was inserted into the 9.8 kb *Bgl* II VCK218 fragment using an IN-FUSION® HD Cloning Kit. The cloning and *E. coli* transformation were performed in a similar manner to that described in Example 14. Plasmid DNA was isolated

from the transformants according to Example 4. Transformants were screened by restriction digestion with *Sbf* I and *Bam* HI which produced 1.6 and 9.7 kb fragments. DNA sequencing of one clone verified that the plasmid contained the correct insert with no PCR errors. The plasmid was designated pDM324 (Figure 15).

5

Example 68: Transformation of strain dlmFRT14FRT15 to introduce FRT F13 and FRT F10 sites at the *cbh1* locus

T. reesei strain dlmFRT14FRT15 (Example 65) was transformed with pDM324 (Example 67) to introduce FRT F13 and FRT F10 sites at the *cbh1* locus. Plasmid pDM324 contains 5' and 3' flanking regions from the *cbh1* locus, FRT F13 and FRT F10 sites, and the *E. coli* hygromycin phosphotransferase (*hpt*) gene and the Herpes simplex virus thymidine kinase (*tk*) gene flanked by direct repeats. The direct repeats were used to facilitate the curing out of the *hpt* and *tk* selectable markers.

A total of 125 µg of the transforming plasmid pDM324 was digested with *Pme* I. The digestion reaction was purified by 0.8% agarose gel electrophoresis in TAE buffer where an 8.1 kb DNA band was excised from the gel, and extracted using a Nucleospin® Extract II Kit (Example 8).

Protoplasts of *T. reesei* strain dlmFRT14FRT15 were prepared as described in Example 1. Approximately 2 µg of the purified DNA fragment was added to 100 µl of the protoplast solution for hygromycin selection transformation as described in Example 1. Transformants were sub-cultured onto PDA plates to generate spores. Transformants were screened using fungal spore PCR in a manner similar to that described in Example 16.

PCR screen for homologous integration of pDM324: checking 5' end of the *cbh1* locus

Forward primer 1209484 (homologous to 5' flanking sequence of *cbh1* locus):

5'-GAGATTCGGACCTCCCAACTG-3' (SEQ ID NO: 348)

Reverse primer 1215169 (homologous to 3' flanking sequence of *cbh1* locus):

5'-AAAAGGCCACCTGTTGAGAG-3' (SEQ ID NO: 349)

Reverse primer 1215168 (homologous to 3' *hpt* marker):

5'-CCCGTGAAGCCGTTTAAATG-3' (SEQ ID NO: 350)

These three primers were combined in one PCR. Transformants that had the correct integration of the pDM324 fragment at the *cbh1* locus produced a 2.1 kb PCR fragment. Transformants that did not have pDM324 DNA correctly integrated produced a 2.7 kb PCR fragment.

PCR screen for homologous integration of pDM324: checking 3' end of the *cbh1* locus

Forward primer (homologous to *tk* marker):

5'-TACGGTGCGGTATCTGCAGT-3' (SEQ ID NO: 351)

Reverse primer (homologous to 3' flanking sequence of *cbh1* locus):

5'-GCGTGCTAATATTCAGTAGAA-3' (SEQ ID NO: 352)

Transformants that had the correct integration of the pDM324 fragment at the *cbh1* locus produced a 2.9 kb PCR fragment. Transformants that produced PCR fragments indicating correct integration at the *cbh1* locus were chosen for spore isolation. Spore isolates were generated in a manner similar to that described in Example 15. The spore isolates were rescreened using the PCR primers and protocol described above. Spores from isolates that yielded the PCR fragments indicative of the correct integration were plated to Trichoderma Minimal medium plates containing 1 μ M FdU (5-fluoro-2'-deoxyuridine) as described in Example 9. Isolates that lost the *tk* marker and looped out both the *hpt* and *tk* markers could grow in the presence of FdU. Colonies from the FdU medium were transferred to PDA plates and incubated at 30°C. Spores from the PDA plates were transferred to PDA plates supplemented with 10 μ g/ml hygromycin and incubated 5 days at 30°C. Isolates that grew on PDA but were unable to grow on PDA plus hygromycin were analyzed further.

Spores from the PDA plate cultures were screened using fungal spore PCR and primers 1215168, 1215169, and 1209484 as described above. Isolates that had pDM324 DNA correctly integrated and had lost the *hpt* and *tk* markers produced a 2.7 kb PCR fragment. Isolates that produced the correct PCR fragment were spore isolated one more time on PDA plates in a manner similar to that described in Example 15.

Genomic DNA was prepared from the final spore isolates in a manner similar to that described in Example 2. Approximately 2 μ g of genomic DNA was digested with *Eco* RI and *Nhe* I. The digested DNA was subjected to 0.7% agarose gel electrophoresis in TAE buffer and transferred to a NYTRAN® SuperCharge membrane as described in Example 3. The DIG labeled probe homologous to the 3' end of the *cbh1* locus was made in a manner similar to that described in Example 12 using the following primers.

Forward primer:

5'-ACCAGTATCGAGGATTGACGGC-3' (SEQ ID NO: 353)

Reverse primer:

5'-AGCAACCATACGATCCAGGAGC-3' (SEQ ID NO: 354)

The Southern blot was hybridized, washed and processed as described in Example 3. Southern blot analysis yielded a 2.9 kb hybridizing fragment indicating the correct integration of pDM324 with subsequent loop-out of the *hpt/tk* markers. The resulting *T. reesei* strain designated *T. reesei* TripleFRT1310-10C12 has FRT F13 and FRT F10 sites at the *cbh1* locus, FRT F and FRT F3 sites at the *cbh2* locus, and FRT F14 and FRT F15 sites at the *eg1* locus.

Example 69: Transformation of *Trichoderma reesei* TripleFRT1310-10C12 to

simultaneously target 3 different expression cassettes to 3 different pairs of FRT sites at 3 loci

T. reesei TripleFRT1310-10C12 (Example 68) has FRT F13 and FRT F10 sites at the *cbh1* locus, FRT F and FRT F3 sites at the *cbh2* locus, and FRT F14 and FRT F15 sites at the *eg1* locus.

Protoplasts of *T. reesei* strain TripleFRT1310-10C12 were prepared as described in Example 1 except that 2% beta-lactose (w/v) was added to the growth medium and protoplast solutions. Approximately 2.2 µg each of plasmids pDM325 (Example 61), pJfyS156 (Example 8) and pDM320 (Example 64) were added per 100 µl of the protoplast solution. The transformation was performed as described in Example 1 and transformation reactions were spread onto COVE plates.

Locus	FRT sites	Targeting plasmid	Marker	Expression cassette
<i>cbh1</i>	FRT F13, FRT F10	pDM325	<i>hpt</i>	<i>cbh2</i> promoter/ <i>P. emersonii</i> GH61A
<i>cbh2</i>	FRT F, FRT F3	pJfyS156	<i>hpt</i>	<i>cbh1</i> promoter/ <i>A. fumigatus</i> BG
<i>eg1</i>	FRT F14, FRT F15	pDM320	<i>amdS</i>	<i>eg1</i> promoter/ <i>A. fumigatus</i> <i>cbh2</i>

The plates were incubated overnight at 30°C. Then 20 ml COVE + 2% beta-lactose overlay containing 2% dextrose and 35 µg/ml hygromycin B were added to each plate. The plates were incubated at 30°C for 5 days. Colonies were transferred to COVE + 2% glucose plates containing 10 µg/ml hygromycin B and were incubated at 30°C for 4 days. Transformants that grew on the COVE + hygromycin medium were transferred to COVE + 2% glucose plates to generate spores. Transformants were screened using fungal spore PCR in a manner similar to that described in Example 16.

PCR screen for targeted integration of pDM325: checking 5' end of the *cbh1* locus

Forward primer (homologous to 5' end of *cbh1* locus):

5'-GGCAACAAGAGGCCAGAGACAATCTATTC-3' (SEQ ID NO: 355)

Reverse primer (homologous to *P. emersonii* GH61A polypeptide coding sequence):

5'-CAGGTAGGTGATGACGGGTCCGTG-3' (SEQ ID NO: 356)

Transformants with pDM325 correctly targeted to the FRT F13 and FRT F10 sites at the *cbh1* locus produced a 1.7 kb PCR fragment from the 5' end of the locus.

PCR screen for targeted integration of pDM325: checking 3' end of the *cbh1* locus

Forward primer (homologous to *P. emersonii* GH61A polypeptide coding sequence):

5'-GACGTATACCATTCCTCGGGGCCGCCTGAGCC-3' (SEQ ID NO: 357)

Reverse primer (homologous to 3' end of *cbh1* locus):

5'-GTTGGCCTCGCAACGGACAAGTT-3' (SEQ ID NO: 358)

Transformants with pDM325 correctly targeted to the FRT F13 and FRT F10 sites at the *cbh1* locus produced a 2.6 kb PCR fragment from the 3' end of the locus.

PCR screen for targeted integration of pJfyS156: checking 5' end of the *cbh2* locus

Forward primer (homologous to 5' end of *cbh2* locus):

5'-AATGGTGAGGACTGAGATAAAAGAATTC-3' (SEQ ID NO: 359)

Reverse primer (homologous to *A. fumigatus* beta-glucosidase coding sequence):

10 5'-CCATGACTATTGTCAACTATATCCGAAACAA-3' (SEQ ID NO: 360)

Transformants with pJfyS156 correctly targeted to the FRT F and FRT F3 sites at the *cbh2* locus produced a 1.4 kb PCR fragment from the 5' end of the locus.

PCR screen for targeted integration of pJfyS156: checking 3' end of the *cbh2* locus

Forward primer (homologous to *hpt* marker):

15 5'-ACCTTCTGGCATGACCTTTTGATGATCG-3' (SEQ ID NO: 361)

Reverse primer (homologous to 3' end of the *cbh2* locus):

5'-CCATCCAAAGAGCTCAACCCAAAGGAGG-3' (SEQ ID NO: 362)

Transformants with pJfyS156 correctly targeted to the FRT F and FRT F3 sites at the *cbh2* locus produced a 0.8 kb PCR fragment from the 3' end of the locus.

20 **PCR screen for targeted integration of pDM320: checking 5' end of the *eg1* locus**

Forward primer (homologous to 5' end of *eg1* locus):

5'-CCCGATGCCACCTCCCAGGATAAGA-3' (SEQ ID NO: 363)

Reverse primer (homologous to *A. fumigatus* cellobiohydrolase II coding region):

5'-GCGATGGAAGATGCAAGGTGCTTCA-3' (SEQ ID NO: 364)

25 Transformants with pDM320 correctly targeted to the FRT F14 and FRT F15 sites at the *eg1* locus produced a 2.7 kb PCR fragment from the 5' end of the locus.

PCR screen for targeted integration of pDM320: checking 3' end of the *eg1* locus

Forward primer (homologous to *amdS* marker):

5'-GTGCCAAGGAAATCTGCGTCGGTTC-3' (SEQ ID NO: 365)

30 Reverse primer (homologous to 3' end of the *eg1* locus):

5'-CGCTCGACGATACGGACGACGATGA-3' (SEQ ID NO: 366)

Transformants with pDM320 correctly targeted to the FRT F14 and FRT F15 sites at the *eg1* locus produced a 1.9 kb PCR fragment from the 3' end of the locus.

Transformants showing correct targeting by the PCR screen were cultured in 25 ml of CIM supplemented with 2% beta-lactose in a 125 ml shake flask at 28°C for 5 days with agitation at 200 rpm. Supernatant from each culture was subjected to SDS-PAGE using a CRITERION® 8-16% TGX Stain-Free gel and PRECISION PLUS® Protein Standards.

Spore isolates were made from transformants that produced all three proteins (*P. emersonii* GH61A polypeptide, *A. fumigatus* beta-glucosidase, and *A. fumigatus* cellobiohydrolase II) as determined by SDS-PAGE. The spore isolates were grown in shake flasks and evaluated by SDS-PAGE. Genomic DNA was made from spore isolates using a method similar to that described in Example 2 and was used for Illumina next-generation sequencing. DNA sequence analysis of the *cbh1*, *cbh2* and *eg1* loci verified simultaneous correct targeting of three expression cassettes to three loci using FRT F and F3, FRT F13 and FRT F10, and FRT F14 and FRT F15 sites.

The present inventions are further described by the following numbered paragraphs:

[1] A method of introducing multiple expression constructs into two or more target loci of a eukaryotic cell, said method comprising. (a) transforming a population of the eukaryotic cell with one or more first constructs and one or more second constructs, wherein the eukaryotic cell comprises (1) one or more first target loci (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprising a pair of a first recombination recognition site and a second recombination recognition site, and (2) one or more second target loci (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprising a first fragment of a first selectable marker lacking a selectable function; wherein the one or more first constructs (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprises one or more first expression cassettes each comprising a first polynucleotide encoding a first protein of interest, wherein in each of the first constructs the one or more first expression cassettes are flanked on one side by the first recombination recognition site and on the other side by the second recombination recognition site corresponding to the same recombination recognition sites of the first target loci; and wherein the one or more second constructs (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprises one or more second expression cassettes each comprising a second polynucleotide encoding a second protein of interest, and wherein in each of the second constructs the one or more second expression cassettes are flanked on one side by a homologous region of the corresponding second target locus and on the other side by a second fragment of the first selectable marker that lacks the selectable function, wherein the second fragment comprises a sequence overlapping homologously the corresponding sequence of the first fragment of the first selectable marker of the second target loci; and (b) selecting a transformant using each of the first selectable markers wherein the first fragment and the second fragment of each of the first selectable markers undergo recombination (e.g., homologous recombination or recombinase-mediated recombination) to become functional and the homologous region of the second constructs undergo homologous recombination with the same corresponding region of the second target loci, wherein each of the second expression cassettes are integrated at the corresponding second target loci, and wherein the

first and second recombination recognition sites of the first constructs undergo recombination at the corresponding first and second recombination recognition sites of the first target loci driven by at least one recombinase integrating each of the first expression cassettes at the corresponding first target loci.

5 [2] The method of paragraph 1, wherein the transforming of the population of the eukaryotic cell with the one or more first constructs and the one or more second constructs is performed sequentially in any order.

[3] The method of paragraph 1, wherein the transforming of the population of the eukaryotic cell with the one or more first constructs and the one or more second constructs
10 is performed as a co-transformation.

[4] The method of any one of paragraphs 1-3, wherein the first and second recombination recognition sites at each of the first target loci are the same recombination recognition sites.

[5] The method of any one of paragraphs 1-3, wherein the first and second
15 recombination recognition sites at each of the first target loci are different recombination recognition sites.

[6] The method of any one of paragraphs 1-3, wherein the first and second recombination recognition sites at each of the first target loci are a combination of the same and different recombination recognition sites.

[7] The method of any one of paragraphs 1-6, wherein the recombination recognition
20 sites are selected from the group consisting of a B2 system from *Zygosaccharomyces bailii*, B3 system from *Zygosaccharomyces bisporus*, beta-recombinase-six system from a 25 *Bacillus subtilis* plasmid, Bxb1 from phage Bxb1, Cre-lox system of bacteriophage P1, Dre from Bacteriophage D6, FLP-FRT of *Saccharomyces cerevisiae*, Delta-gamma-es system from bacterial transposon Tn1000, Gin-gix system from bacteriophage Mu, HK022 from phage HK022, KD system from *Kluyveromyces drosophilum*, Mx9 phage transformation system, *Streptomyces* phage ϕ C31, R-RS system of *Zygosaccharomyces rouxii*, Tn3 from *E. coli*, Vika recombinase from *Vibrio coralliilyticus*, and Xis-att system of temperate lactococcal bacteriophage TP901-1; and combinations thereof.

[8] The method of paragraph 7, wherein the flippase recognition sites of the FLP-FRT
30 system are selected from the group consisting F, F3, F10, F13, F14, F15, Fa, and F3a; and combinations thereof.

[9] The method of paragraph 7, wherein the TP901-1 sites of the Xis-att system are selected from the group consisting *attB* and *attP*, and combinations thereof.

[10] The method of paragraph 7, wherein the Lox sites of the Cre-lox system are
35 selected from the group consisting of LoxP, Lox71, Lox66, 511, Lox 5171, Lox 2272, M2, M3, M7, and M11.

[11] The method of any one of paragraphs 1-10, wherein the eukaryotic cell comprises one or more recombinases, which are native, heterologous, or a combination of native and heterologous to the eukaryotic cell.

[12] The method of paragraph 11, wherein the one or more recombinases are selected from the group consisting of a Bxb1 recombinase, a Cre recombinase, a CinH recombinase, a Flp flippase, a HK022 integrase, a ParA recombinase, a Tn1721 recombinase, a Tn5053 recombinase, a TP901-1 integrase, an U153 recombinase, a λ integrase, and a ϕ C31 recombinase.

[13] The method of any one of paragraphs 1-12, wherein each of the first polynucleotides is the same polynucleotide.

[14] The method of any one of paragraphs 1-12, wherein each of the first polynucleotides is a different polynucleotide.

[15] The method of any one of paragraphs 1-12, wherein each of the first polynucleotides is a combination of the same and different polynucleotides.

[16] The method of any one of paragraphs 1-15, wherein each of the second polynucleotides is the same polynucleotide.

[17] The method of any one of paragraphs 1-15, wherein each of the second polynucleotides is a different polynucleotide.

[18] The method of any one of paragraphs 1-15, wherein each of the second polynucleotides is a combination of the same and different polynucleotides.

[19] The method of any one of paragraphs 1-18, wherein the first selectable marker is the same at each of the second target loci.

[20] The method of any one of paragraphs 1-18, wherein the first selectable marker is different at each of the second target loci.

[21] The method of any one of paragraphs 1-18, wherein the first selectable marker at each of the second target loci is a combination of the same and different selectable markers.

[22] The method of any one of paragraphs 1-21, wherein the first and second fragments of the first selectable markers each further comprise a repeat sequence 5' of the first fragment and a repeat sequence 3' of the second fragment, and wherein the repeat sequences undergo homologous recombination to remove the selectable markers.

[23] The method of any one of paragraphs 1-22, wherein the first and second fragments of the first selectable markers each further comprise repeat sequences allowing removal of a portion of each of the selectable markers by homologous recombination resulting in selectable markers lacking a selectable function.

[24] The method of paragraph 23, wherein one of the repeat sequences is located within an intron of the first selectable markers and the other of the repeat sequences is

located 5' or 3' of the first selectable markers, wherein the repeat sequences undergo homologous recombination to remove a portion of the first selectable markers.

[25] The method of paragraph 23, wherein one of the repeat sequences is located 5' or 3' of the first selectable markers and the other of the repeat sequences is a homologous region of the first selectable markers, wherein the repeat sequences undergo homologous recombination to remove a portion of the first selectable markers.

[26] The method of any one of paragraphs 1-25, wherein the one or more first target loci each further comprises a second selectable marker between the recombination recognition sites.

[27] The method of paragraph 26, wherein the second selectable marker at each of the first target loci is a counter selectable marker for negative selection.

[28] The method of paragraph 27, wherein the counter selectable marker for negative selection is selected from the group consisting of URA3, *amdS*, *fcy1*, *pyrG*, *tk*, and beta-tubulin.

[29] The method of any one of paragraphs 1-28, wherein the one or more second target loci each further comprises a third selectable marker before the non-functional first fragment of the first selectable marker.

[30] The method of paragraph 1-28, wherein the one or more second target loci each further comprises a third selectable marker after the non-functional first fragment of the first selectable marker.

[31] The method of paragraph 30, wherein the third selectable marker at each of the second target loci is a counter selectable marker for negative selection.

[32] The method of paragraph 31, wherein the counter selectable marker is selected from the group consisting of URA3, *amdS*, *fcy1*, *pyrG*, *tk*, and beta-tubulin.

[33] The method of any one of paragraphs 1-32, wherein the one or more first constructs each further comprises a fourth selectable marker between the recombination recognition sites.

[34] The method of paragraph 33, wherein each of the fourth selectable markers is the same selectable marker.

[35] The method of paragraph 33, wherein each of the fourth selectable markers is a different selectable marker.

[36] The method of paragraph 33, wherein each of the fourth selectable markers is a combination of the same and different selectable markers.

[37] The method of any one of paragraphs 33-36, wherein each of the fourth selectable markers is the same as the first selectable markers of the second constructs.

[38] The method of any one of paragraphs 33-36, wherein each of the fourth selectable markers is different from the first selectable markers of the second constructs.

[39] The method of any one of paragraphs 33-36, wherein each of the fourth selectable markers is a combination of the same and different selectable markers from the first selectable markers of the second constructs.

[40] The method of any one of paragraphs 33-39, wherein each of the fourth selectable markers of the one or more first constructs is a counter selectable marker for negative selection.

[41] The method of paragraph 40, wherein the counter selection selectable markers are selected from the group consisting of URA3, *amdS*, *fcy1*, *pyrG*, *tk*, and beta-tubulin.

[42] The method of any one of paragraphs 33-41, wherein each of the fourth selectable markers comprise a repeat sequence 5' and a repeat sequence 3' of each of the selectable markers.

[43] The method of paragraph 42, wherein the repeat sequences undergo homologous recombination to remove the selectable markers.

[44] The method of any one of paragraphs 1-43, wherein the selectable markers are selected from the group consisting of ADE2, ARO4-OPF, FLD1, HIS3, LEU2, LYS2, MET3, TRP1, URA3, *adeA*, *adeB*, *amdS*, *argB*, *bar*, *bleR*, *bsd*, *fcy1*, *hpt*, *hpt-tk*, *nat1*, *niaD*, *ptr1*, *pyrG*, *sC*, *tk*, Tn903kan^r, *trpC*, and beta-tubulin.

[45] The method of any one of paragraphs 1-44, wherein the eukaryotic cell is a yeast cell.

[46] The method of paragraph 45, wherein the yeast cell is selected from the group consisting of a *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, and *Yarrowia* cell.

[47] The method of paragraph 45, wherein the yeast cell is a *Kluyveromyces lactis*, *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, *Saccharomyces oviformis*, or *Yarrowia lipolytica* cell.

[48] The method of any one of paragraphs 1-47, wherein the eukaryotic cell is a filamentous fungal cell.

[49] The method of paragraph 48, wherein the filamentous fungal cell is selected from the group consisting of an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes*, and *Trichoderma* cell.

[50] The method of paragraph 48, wherein the filamentous fungal cell is an *Aspergillus* cell.

[51] The method of paragraph 48, wherein the filamentous fungal cell is *Aspergillus nidulans*.

[52] The method of paragraph 48, wherein the filamentous fungal cell is *Aspergillus niger*.

5 [53] The method of paragraph 48, wherein the filamentous fungal cell is *Aspergillus oryzae*.

[54] The method of paragraph 48, wherein the filamentous fungal cell is a *Fusarium* cell.

10 [55] The method of paragraph 48, wherein the filamentous fungal cell is *Fusarium venenatum*.

[56] The method of paragraph 48, wherein the filamentous fungal cell is a *Myceliophthora* cell.

[57] The method of paragraph 48, wherein the filamentous fungal cell is *Myceliophthora thermophila*.

15 [58] The method of paragraph 48, wherein the filamentous fungal cell is a *Talaromyces* cell.

[59] The method of paragraph 48, wherein the filamentous fungal cell is *Talaromyces emersonii*.

20 [60] The method of paragraph 48, wherein the filamentous fungal cell is a *Trichoderma* cell.

[61] The method of paragraph 48, wherein the filamentous fungal cell is *Trichoderma reesei*.

[62] The method of any one of paragraphs 1-61, wherein the proteins of interest are selected from the group consisting of an antibody, an antigen, an antimicrobial peptide, an enzyme, a growth factor, a hormone, an immunodilator, a neurotransmitter, a receptor, a reporter protein, a structural protein, and a transcription factor.

[63] The method of paragraph 62, wherein the enzyme is selected from the group consisting of a hydrolase, isomerase, ligase, lyase, oxidoreductase, and transferase.

30 [64] The method of any one of paragraphs 1-63, wherein the eukaryotic cell is reusable by repeating steps (a) and (b) with one or more different first constructs, second constructs, or first and second constructs each comprising an expression cassette comprising a polynucleotide encoding a different protein of interest.

[65] A method of constructing a eukaryotic cell having multiple target loci for expressing multiple heterologous proteins of interest, comprising: (a) transforming a population of the eukaryotic cell with a first construct comprising (1) a 5' homologous region of a first target locus, (2) a first recombination recognition site, (3) a first selectable marker conferring a first selectable function, (4) a second recombination recognition site, and (5) a 3'

homologous region of the first target locus; (b) selecting a first transformant having the first construct integrated at the first target locus using the first selectable marker for selection of the first transformant, wherein the 5' homologous region and the 3' homologous region of the first construct undergo homologous recombination with the corresponding regions of the first target locus integrating the first construct at the first target locus, and wherein the first recombination recognition site and the second recombination recognition site are integrated at the first target locus; (c) transforming a population of the first transformant with a second construct comprising (1) a 5' homologous region of a second target locus, (2) a first fragment of a second selectable marker that lacks a second selectable function, (3) a third selectable marker conferring a third selectable function, and (4) a 3' homologous region of the second target locus; (d) selecting a second transformant having the second construct integrated at the second target locus using the third selectable marker for selection of the second transformant, wherein the 5' homologous region and the 3' homologous region of the second construct undergo homologous recombination with the corresponding regions of the second target locus integrating the second construct at the second target locus, and wherein the first fragment of the second selectable marker that lacks a second selectable function is integrated at the second target locus; (e) co-transforming a population of the second transformant with (i) a third construct comprising (1) the first recombination recognition site, (2) one or more first expression cassettes each comprising a first polynucleotide encoding a first protein of interest, and (3) the second recombination recognition site, and (ii) a fourth construct comprising (1) the 5' homologous region of the second target locus, (2) one or more second expression cassettes each comprising a second polynucleotide encoding a second protein of interest, and (3) a second fragment of the second selectable marker that lacks the second selectable function wherein the second fragment comprises a sequence overlapping homologously the corresponding sequence of the first fragment of the first selectable marker; and (f) selecting a third transformant using the second selectable marker wherein the first integrated fragment and the second fragment of the second selectable marker become functional upon recombination (e.g., homologous recombination or recombinase-mediated recombination), wherein the 5' homologous region of the fourth construct undergoes homologous recombination with the same corresponding region of the second target locus, wherein the one or more second expression cassettes are integrated at the second target locus, and wherein the first recombination recognition site and the second recombination recognition site undergo recombination at the first target locus driven by at least one recombinase integrating the one or more first expression cassettes at the first target locus.

[66] The method of paragraph 65, wherein steps (a) and (b) are performed before steps (c) and (d).

[67] The method of paragraph 65, wherein steps (c) and (d) are performed before steps (a) and (b).

[68] The method of any one of paragraphs 65-67, wherein the recombination recognition sites are selected from the group consisting of B2 system from *Zygosaccharomyces bailii*, B3 system from *Zygosaccharomyces bisporus*, beta-recombinase-six system from a 25 *Bacillus subtilis* plasmid, Bxb1 from phage Bxb1, Cre-lox system of bacteriophage P1, Dre from Bacteriophage D6, FLP-FRT of *Saccharomyces cerevisiae*, Delta-gamma-es system from bacterial transposon Tn1000, Gin-gix system from bacteriophage Mu, HK022 from phage HK022, KD system from *Kluyveromyces drosophilum*, Mx9 phage transformation system, *Streptomyces* phage ϕ C31, R-RS system of *Zygosaccharomyces rouxii*, Tn3 from *E. coli*, Vika recombinase from *Vibrio coralliilyticus*, and Xis-att system of temperate lactococcal bacteriophage TP901-1; and combinations thereof.

[69] The method of paragraph 68, wherein the flippase recognition (FRT) sites are selected from the group consisting F, F3, F10, F13, F14, F15, Fa, and F3a; and combinations thereof.

[70] The method of paragraph 68, wherein the TP901 sites are selected from the group consisting *attB* and *attP*, and combinations thereof.

[71] The method of paragraph 68, wherein the Lox sites of the Cre-lox system are selected from the group consisting of LoxP, Lox71, Lox66, 511, Lox 5171, Lox 2272, M2, M3, M7, and M11.

[72] The method of any one of paragraphs 65-71, wherein the eukaryotic cell comprises one or more recombinases.

[73] The method of paragraph 72, wherein the one or more recombinases are selected from the group consisting of a Bxb1 recombinase, a Cre recombinase, a CinH recombinase, a Flp flippase, a HK022 integrase, a ParA recombinase, a Tn1721 recombinase, a Tn5053 recombinase, a TP901-1 integrase, an U153 recombinase, a λ integrase, and a ϕ C31 recombinase.

[74] The method of any one of paragraphs 65-73, wherein the first selectable marker further comprises a first repeat 5' of the first selectable marker and a second repeat 3' of the first selectable marker.

[75] The method of 74, wherein the first and second repeat sequences of the integrated first construct undergo homologous recombination to remove the first selectable marker.

[76] The method of any one of paragraphs 65-75, wherein the third selectable marker further comprises a third repeat 5' of the third selectable marker and a fourth repeat 3' of the third selectable marker.

[77] The method of paragraph 76, wherein the third and fourth repeat sequences of the second construct undergo homologous recombination to remove the third selectable marker.

[78] The method of any one of paragraphs 65-77, wherein the third selectable marker is the same as the first selectable marker.

[79] The method of any one of paragraphs 65-77, wherein the third selectable marker is different than the first selectable marker.

[80] The method of any one of paragraphs 65-77, wherein the second selectable marker is different than the first and third selectable markers.

[81] The method of any one of paragraphs 65-77, wherein the second selectable marker is the same as the first and third selectable markers.

[82] The method of any one of paragraphs 65-77, wherein the second selectable marker is the same as the first selectable marker.

[83] The method of any one of paragraphs 65-82, wherein the first and second non-functional fragments of the second selectable marker each further comprises a recombination recognition site in an intron of the second selectable marker.

[84] The method of paragraph 83, wherein each recombination recognition site in the first and second non-functional fragments is the same, which can undergo recombinase-mediated recombination.

[85] The method of paragraph 83, wherein each recombination recognition site in the first and second non-functional fragments are different but can undergo recombinase-mediated recombination.

[86] The method of any one of paragraphs 65-85, wherein the selectable markers are selected from the group consisting of ADE2, ARO4-OPF, FLD1, HIS3, LEU2, LYS2, MET3, TRP1, URA3, *adeA*, *adeB*, *amdS*, *argB*, *bar*, *bleR*, *bsd*, *fcy1*, *hpt*, *hpt-tk*, *nat1*, *niaD*, *ptr1*, *pyrG*, *sC*, *tk*, Tn903kan^r, *trpC*, and beta-tubulin.

[87] The method of any one of paragraphs 65-77, wherein the first selectable marker is a dual selectable marker system.

[88] The method of paragraph 87, wherein the dual selectable marker system is *hpt-tk*.

[89] The method of any one of paragraphs 65-77, wherein the first selectable marker is a counter selectable marker for negative selection.

[90] The method of any one of paragraphs 65-89, wherein the first construct further comprises a third counter selectable marker for negative selection between the first and the second recombination recognition sites.

[91] The method of any one of paragraphs 65-89, wherein the second construct further comprise a third counter selectable marker for negative selection before or after the non-functional first fragment of the second selectable marker.

[92] The method of any one of paragraphs 65-89, wherein the second transformant comprises the third counter selectable marker at both the first and the second target loci.

[93] The method of any one of paragraphs 65-89, wherein the third transformant comprising no counter selectable marker at both the first and second target loci is selected using the negative selection function of the counter selectable marker.

[94] The method of any one of paragraphs 89-93, wherein the counter selectable marker is selected from the group consisting of URA3, *amdS*, *fcy1*, *pyrG*, and beta-tubulin.

[95] The method of any one of paragraphs 65-94, wherein steps (a)-(f) are repeated at at least two additional target loci with different recombination recognition sites, different polynucleotides encoding proteins of interest, and the same or a different second selectable marker.

[96] The method of any one of paragraphs 65-94, wherein steps (a)-(b) or (c)-(d) are repeated at one additional target locus with different recombination recognition sites, different polynucleotides encoding proteins of interest, and the same or a different selectable marker.

[97] The method of any one of paragraphs 65-96, wherein the eukaryotic cell is a yeast cell.

[98] The method of paragraph 97, wherein the yeast cell is selected from the group consisting of a *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, and *Yarrowia* cell.

[99] The method of paragraph 97, wherein the yeast cell is *Saccharomyces cerevisiae*.

[100] The method of any one of paragraphs 65-96, wherein the eukaryotic cell is a filamentous fungal cell.

[101] The method of paragraph 100, wherein the filamentous fungal cell is selected from the group consisting of an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes*, and *Trichoderma* cell.

[102] The method of paragraph 100, wherein the filamentous fungal cell is an *Aspergillus* cell.

[103] The method of paragraph 100, wherein the filamentous fungal cell is *Aspergillus nidulans*.

[104] The method of paragraph 100, wherein the filamentous fungal cell is *Aspergillus niger*.

[105] The method of paragraph 100, wherein the filamentous fungal cell is *Aspergillus oryzae*.

5 [106] The method of paragraph 100, wherein the filamentous fungal cell is a *Fusarium* cell.

[107] The method of paragraph 100, wherein the filamentous fungal cell is *Fusarium venenatum*.

10 [108] The method of paragraph 100, wherein the filamentous fungal cell is a *Myceliophthora* cell.

[109] The method of paragraph 100, wherein the filamentous fungal cell is *Myceliophthora thermophila*.

[110] The method of paragraph 100, wherein the filamentous fungal cell is a *Talaromyces* cell.

15 [111] The method of paragraph 100, wherein the filamentous fungal cell is *Talaromyces emersonii*.

[112] The method of paragraph 100, wherein the filamentous fungal cell is a *Trichoderma* cell.

20 [113] The method of paragraph 100, wherein the filamentous fungal cell is *Trichoderma reesei*.

[114] The method of any one of paragraphs 65-113, wherein the proteins of interest are selected from the group consisting of an antibody, an antigen, an antimicrobial peptide, an enzyme, a growth factor, a hormone, an immunodilator, a neurotransmitter, a receptor, a reporter protein, a structural protein, and a transcription factor.

25 [115] The method of paragraph 114, wherein the enzyme is selected from the group consisting of a hydrolase, isomerase, ligase, lyase, oxidoreductase, and transferase.

[116] A eukaryotic cell obtained according to the method of any one of paragraphs 1-64.

30 [117] A eukaryotic cell obtained according to the method of any one of paragraphs 65-115.

[118] A eukaryotic cell for expressing multiple heterologous proteins of interest, comprising: (a) one or more first target loci (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprising a pair of a first recombination recognition site and a second recombination recognition site; and (b) one or more second target loci (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprising a first
35 fragment of a first selectable marker lacking a selectable function.

[119] The eukaryotic cell of paragraph 118, wherein the first and second recombination recognition sites are the same recombination recognition sites at each of the first target loci.

[120] The eukaryotic cell of paragraph 118, wherein the first and second recombination recognition sites are different recombination recognition sites at each of the first target loci.

[121] The eukaryotic cell of paragraph 118, wherein the first and second recombination recognition sites are a combination of the same and different recombination recognition sites at each of the first target loci.

[122] The eukaryotic cell of any one of paragraphs 118-121, wherein the recombination recognition sites are selected from the group consisting of a B2 system from *Zygosaccharomyces baillii*, B3 system from *Zygosaccharomyces bisporus*, beta-recombinase-six system from a 25 *Bacillus subtilis* plasmid, Bxb1 from phage Bxb1, Cre-lox system of bacteriophage P1, Dre from Bacteriophage D6, FLP-FRT of *Saccharomyces cerevisiae*, Delta-gamma-es system from bacterial transposon Tn1000, Gin-gix system from bacteriophage Mu, HK022 from phage HK022, KD system from *Kluyveromyces drosophilum*, Mx9 phage transformation system, *Streptomyces* phage ϕ C31, R-RS system of *Zygosaccharomyces rouxii*, Tn3 from *E. coli*, Vika recombinase from *Vibrio coralliilyticus*, and Xis-att system of temperate lactococcal bacteriophage TP901-1; and combinations thereof.

[123] The eukaryotic cell of paragraph 122, wherein the flippase recognition sites of the FLP-FRT system are selected from the group consisting F, F3, F10, F13, F14, F15, Fa, and F3a; and combinations thereof.

[124] The eukaryotic cell of paragraph 122, wherein the TP901-1 sites of the Xis-att system are selected from the group consisting *attB* and *attP*, and combinations thereof.

[125] The eukaryotic cell of paragraph 122, wherein the wherein the Lox sites of the Cre-lox system are selected from the group consisting of LoxP, Lox71, Lox66, 511, Lox 5171, Lox 2272, M2, M3, M7, and M11.

[126] The eukaryotic cell of any one of paragraphs 118-125, which comprises one or more recombinases.

[127] The eukaryotic cell of paragraph 126, wherein the one or more recombinases are selected from the group consisting of a Bxb1 recombinase, a Cre recombinase, a CinH recombinase, a Flp flippase, a HK022 integrase, a ParA recombinase, a Tn1721 recombinase, a Tn5053 recombinase, a TP901-1 integrase, an U153 recombinase, a λ integrase, and a ϕ C31 recombinase.

[128] The eukaryotic cell of any one of paragraphs 118-127, further comprising one or more first expression cassettes each comprising a first polynucleotide encoding a first

protein of interest at each of the first target loci, wherein each of the one or more first expression cassettes are flanked on one side by the first recombination recognition site and on the other side by the second recombination recognition site of the corresponding first target locus.

5 [129] The eukaryotic cell of paragraph 128, wherein each of the first polynucleotides is the same polynucleotide.

[130] The eukaryotic cell of paragraph 128, wherein each of the first polynucleotides is a different polynucleotide.

10 [131] The eukaryotic cell of paragraph 128, wherein each of the first polynucleotides is a combination of the same and different polynucleotides.

[132] The eukaryotic cell of any one of paragraphs 118-131, further comprising one or more second expression cassettes each comprising a second polynucleotide encoding a second protein of interest at each of the second target loci, wherein each of the one or more second expression cassettes are flanked on one side by the first fragment of the selectable
15 marker lacking a selectable function.

[133] The eukaryotic cell of paragraph 132, wherein each of the second polynucleotides is the same polynucleotide.

[134] The eukaryotic cell of paragraph 132, wherein each of the second polynucleotides is a different polynucleotide.

20 [135] The eukaryotic cell of paragraph 132, wherein each of the second polynucleotides is a combination of the same and different polynucleotides.

[136] The eukaryotic cell of any one of paragraphs 118-135, wherein the one or more first target loci each further comprises a second selectable marker between the recombination recognition sites.

25 [137] The eukaryotic cell of paragraph 136, wherein the second selectable marker at each of the first target loci is a counter selectable marker for negative selection.

[138] The eukaryotic cell of paragraph 137, wherein the counter selectable marker for negative selection is selected from the group consisting of URA3, *amdS*, *fcy1*, *pyrG*, *tk*, and beta-tubulin.

30 [139] The eukaryotic cell of any one of paragraphs 118-138, wherein the one or more second target loci each further comprises a third selectable marker before the non-functional first fragment of the first selectable marker.

[140] The eukaryotic cell of paragraph 118-139, wherein the one or more second target loci each further comprises a third selectable marker after the non-functional first
35 fragment of the first selectable marker.

[141] The eukaryotic cell of paragraph 140, wherein the third selectable marker at each of the second target loci is a counter selectable marker for negative selection.

[142] The eukaryotic cell of paragraph 141, wherein the counter selectable marker is selected from the group consisting of URA3, *amdS*, *fcy1*, *pyrG*, *tk*, and beta-tubulin.

[143] The eukaryotic cell of any one of paragraphs 118-142, wherein the first selectable marker is the same at each of the second target loci.

5 [144] The eukaryotic cell of any one of paragraphs 118-142, wherein the first selectable marker is different at each of the second target loci.

[145] The eukaryotic cell of any one of paragraphs 118-142, wherein the first selectable marker is a combination of the same and different selectable markers at each of the second target loci.

10 [146] The eukaryotic cell of any one of paragraphs 118-145, wherein the selectable marker is selected from the group consisting of ADE2, ARO4-OPF, FLD1, HIS3, LEU2, LYS2, MET3, TRP1, URA3, *adeA*, *adeB*, *amdS*, *argB*, *bar*, *bleR*, *bsd*, *fcy1*, *hpt*, *hpt-tk*, *nat1*, *niaD*, *ptr1*, *pyrG*, *sC*, *tk*, Tn903kan^r, *trpC*, and beta-tubulin.

[147] A eukaryotic cell comprising (1) one or more first target loci each comprising
 15 one or more first expression cassettes each comprising a first polynucleotide encoding a first protein of interest, wherein the one or more first expression cassettes are each flanked 5' by a first recombination recognition site and 3' by a second recombination recognition site, and (2) one or more second target loci each comprising one or more second expression cassettes each comprising a second polynucleotide encoding a second protein of interest,
 20 wherein each of the one or more second expression cassettes are flanked on one side by a region of the second target locus and on the other side by a first fragment of a first selectable marker that lacks selectable function, wherein each of the pairs of the first and second recombination recognition sites at the first loci are able to undergo recombination with a first construct comprising one or more third expression cassettes each comprising a
 25 third polynucleotide encoding a third protein of interest, wherein each of the one or more third expression cassettes are flanked on one side by the first recombination recognition site and on the other side by the second recombination recognition site of the corresponding first target locus, and wherein each of the target loci regions and the first fragment of the first selectable marker that lacks selectable function at the second loci are able to undergo
 30 recombination (e.g., homologous recombination or recombinase-mediated recombination) with a second construct comprising one or more fourth expression cassettes each comprising a fourth polynucleotide encoding a fourth protein of interest, wherein each of the one or more fourth expression cassettes are flanked on one side by a homologous region of the corresponding second target locus and on the other side by a second fragment of the
 35 first selectable marker that lacks the selectable function wherein the second fragment comprises a sequence overlapping homologously the corresponding sequence of the first fragment of the first selectable marker.

[148] The eukaryotic cell of paragraph 147, wherein the first and second recombination recognition sites at each of the first target loci are the same recombination recognition sites.

[149] The eukaryotic cell of paragraph 147, wherein the first and second recombination recognition sites at each of the first target loci are different recombination recognition sites.

[150] The eukaryotic cell of paragraph 147, wherein the first and second recombination recognition sites at each of the first target loci are a combination of the same and different recombination recognition sites.

[151] The eukaryotic cell of any one of paragraphs 147-150, wherein each of the first polynucleotides is the same polynucleotide.

[152] The eukaryotic cell of any one of paragraphs 147-150, wherein each of the first polynucleotides is a different polynucleotide.

[153] The eukaryotic cell of any one of paragraphs 147-150, wherein each of the first polynucleotides is a combination of the same and different polynucleotides.

[154] The eukaryotic cell of any one of paragraphs 147-153, wherein each of the second polynucleotides is the same polynucleotide.

[155] The eukaryotic cell of any one of paragraphs 147-153, wherein each of the second polynucleotides is a different polynucleotide.

[156] The eukaryotic cell of any one of paragraphs 147-153, wherein each of the second polynucleotides is a combination of the same and different polynucleotides.

[157] The eukaryotic cell of any one of paragraphs 147-156, wherein the first selectable marker is the same at each of the second target loci.

[158] The eukaryotic cell of any one of paragraphs 147-156, wherein the first selectable marker is different at each of the second target loci.

[159] The eukaryotic cell of any one of paragraphs 147-156, wherein the first selectable marker is a combination of the same and different selectable markers at each of the second target loci.

[160] The eukaryotic cell of any one of paragraphs 116-159, which is a yeast cell.

[161] The eukaryotic cell of paragraph 160, wherein the yeast cell is selected from the group consisting of a *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, and *Yarrowia* cell.

[162] The eukaryotic cell of paragraph 160, wherein the yeast cell is *Saccharomyces cerevisiae*.

[163] The eukaryotic cell of any one of paragraphs 116-159, which is a filamentous fungal cell.

[164] The eukaryotic cell of paragraph 163, wherein the filamentous fungal cell is selected from the group consisting of an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*,
5 *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes*, or *Trichoderma* cell.

[165] The eukaryotic cell of paragraph 163, wherein the filamentous fungal cell is an *Aspergillus* cell.

[166] The eukaryotic cell of paragraph 163, wherein the filamentous fungal cell is
10 *Aspergillus nidulans*.

[167] The eukaryotic cell of paragraph 163, wherein the filamentous fungal cell is *Aspergillus niger*.

[168] The eukaryotic cell of paragraph 163, wherein the filamentous fungal cell is *Aspergillus oryzae*.

[169] The eukaryotic cell of paragraph 163, wherein the filamentous fungal cell is a
15 *Fusarium* cell.

[170] The eukaryotic cell of paragraph 163, wherein the filamentous fungal cell is *Fusarium venenatum*.

[171] The eukaryotic cell of paragraph 163, wherein the filamentous fungal cell is a
20 *Myceliophthora* cell.

[172] The eukaryotic cell of paragraph 163, wherein the filamentous fungal cell is *Myceliophthora thermophila*.

[173] The eukaryotic cell of paragraph 163, wherein the filamentous fungal cell is a *Talaromyces* cell.

[174] The eukaryotic cell of paragraph 163, wherein the filamentous fungal cell is
25 *Talaromyces emersonii*.

[175] The eukaryotic cell of paragraph 163, wherein the filamentous fungal cell is a *Trichoderma* cell.

[176] The eukaryotic cell of paragraph 163, wherein the filamentous fungal cell is
30 *Trichoderma reesei*.

[177] The eukaryotic cell of any one of paragraphs 147-176, wherein the selectable markers are selected from the group consisting of ADE2, ARO4-OFP, FLD1, HIS3, LEU2, LYS2, MET3, TRP1, URA3, *adeA*, *adeB*, *amdS*, *argB*, *bar*, *bleR*, *bsd*, *fcy1*, *hpt*, *hpt-tk*, *nat1*, *niaD*, *ptr1*, *pyrG*, *sC*, *tk*, Tn903kan^r, *trpC*, and beta-tubulin.

[178] A method for producing proteins of interest, comprising cultivating the
35 eukaryotic cell of any one of paragraphs 116-177 under conditions conducive for production of the proteins.

[179] The method of paragraph 178, further comprising recovering the proteins.

[180] A nucleic acid construct selected from the group consisting of: (a) a first nucleic acid construct comprising (i) a 5' homologous region of a first target locus of a eukaryotic cell, (ii) a first recombination recognition site, (iii) a first repeat sequence, (iv) a first selectable marker conferring a first selectable function, (v) a second repeat sequence, (vi) a second recombination recognition site, and (vii) a 3' homologous region of the first target locus of the eukaryotic cell; (b) a second nucleic acid construct comprising: (1) a 5' homologous region of a second target locus of a eukaryotic cell, (2) a first fragment of a second selectable marker that lacks a second selectable function, (3) a third repeat sequence, (4) a third selectable marker conferring a third selectable function, (5) a fourth repeat sequence, and (6) a 3' homologous region of the second target locus of the eukaryotic cell; (c) a third nucleic acid construct comprising: (1) a first recombination recognition site, (2) one or more first expression cassettes each comprising a first polynucleotide encoding a first protein of interest, and (3) a second recombination recognition site; and (d) a fourth nucleic acid construct comprising: (1) a 5' homologous region of the second target locus, (2) one or more second expression cassettes each comprising a second polynucleotide encoding a second protein of interest, and (3) a second fragment of a second selectable marker that lacks a second selectable function.

[181] A eukaryotic cell comprising one or more of the nucleic acid constructs of paragraph 180.

[182] A method of introducing multiple expression constructs into a eukaryotic cell, said method comprising. (a) transforming a population of the eukaryotic cell with one or more first constructs and one or more second constructs, wherein the eukaryotic cell comprises (1) one or more first target loci (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprising a pair of a first recombination recognition site and a second recombination recognition site, wherein the first recombination recognition site and a second recombination recognition site are TP901-1 sites of the Xis-att system, and (2) one or more second target loci (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprising a pair of a third recombination recognition site and a fourth recombination recognition site, wherein the third recombination recognition site and a fourth recombination recognition site are flippase recognition sites of the FLP-FRT system; wherein the one or more first constructs (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprises one or more first expression cassettes each comprising a first polynucleotide encoding a first protein of interest, wherein in each of the first constructs the one or more first expression cassettes are flanked on one side by the first recombination recognition site and on the other side by the second recombination recognition site corresponding to the same recombination recognition sites of the first target loci; wherein the one or more second constructs (e.g., 1, 2, 3, 4, 5, 6, 7, 8, etc.) each comprises one or more second expression cassettes each comprising a

second polynucleotide encoding a second protein of interest, and wherein in each of the second constructs the one or more second expression cassettes are flanked on one side by the third recombination recognition site and on the other side by the fourth recombination recognition site corresponding to the same recombination recognition sites of the second target loci; and wherein one or more of the first constructs and second constructs comprise one or more first selectable markers; (b) selecting a transformant using the one or more first selectable markers, wherein each of the second expression cassettes are integrated at the corresponding second target loci, and wherein the first and second recombination recognition sites of the first constructs undergo recombination at the corresponding first and second recombination recognition sites of the first target loci driven by at least one recombinase integrating each of the first expression cassettes at the corresponding first target loci.

[183] The method of paragraph 182, wherein the TP901-1 sites of the Xis-att system are selected from the group consisting of *attB* and *attP*, and combinations thereof.

[184] The method of paragraph 182, wherein the flippase recognition sites of the FLP-FRT system are selected from the group consisting of F, F3, F10, F13, F14, F15, Fa, and F3a; and combinations thereof.

[185] The method of any one of paragraphs 182-184, wherein the one or more first selectable markers are the same selectable markers.

[186] The method of any one of paragraphs 182-184, wherein the one or more first selectable markers are different selectable markers.

[187] The method of any one of paragraphs 182-186, wherein the one or more first target loci each further comprises a second selectable marker between the recombination recognition sites.

[188] The method of any one of paragraphs 182-187, wherein the selectable markers are selected from the group consisting of ADE2, ARO4-OFP, FLD1, HIS3, LEU2, LYS2, MET3, TRP1, URA3, *adeA*, *adeB*, *amdS*, *argB*, *bar*, *bleR*, *bsd*, *fcy1*, *hpt*, *hpt-tk*, *nat1*, *niaD*, *ptr1*, *pyrG*, *sC*, *tk*, Tn903kan^r, *trpC*, and beta-tubulin.

[189] The method of paragraph 187, wherein the second selectable marker at each of the first target loci is a counter selectable marker for negative selection.

[190] The method of paragraph 189, wherein the counter selectable marker for negative selection is selected from the group consisting of URA3, *amdS*, *fcy1*, *pyrG*, *tk*, and beta-tubulin.

[191] The method of any one of paragraphs 182-190, wherein the one or more second target loci each further comprises a third selectable marker between the recombination recognition sites.

[192] The method of paragraph 191, wherein the third selectable marker at each of the second target loci is a counter selectable marker for negative selection.

[193] The method of paragraph 192, wherein the counter selectable marker is selected from the group consisting of URA3, *amdS*, *fcy1*, *pyrG*, *tk*, and beta-tubulin.

5 [194] The method of any one of paragraphs 182-193, wherein the one or more first constructs, the one or more second constructs, or the one or more first constructs and the one or more second constructs each further comprises a fourth selectable marker between the recombination recognition sites.

10 [195] The method of paragraph 194, wherein each of the fourth selectable markers is the same selectable marker.

[196] The method of paragraph 194, wherein each of the fourth selectable markers is a different selectable marker.

[197] The method of paragraph 194, wherein each of the fourth selectable markers is a combination of the same and different selectable markers.

15 [198] The method of paragraph 194, wherein each of the fourth selectable markers is the same as the first selectable markers of the second constructs, different from the first selectable markers of the second constructs, or a combination of the same and different selectable markers from the first selectable markers of the second constructs.

20 [199] The method of any one of paragraphs 194-198, wherein each of the fourth selectable markers of the one or more first constructs, the one or more second constructs, and the one or more first constructs and the one or more second constructs is a counter selectable marker for negative selection.

25 [199] The method of paragraph 199, wherein the counter selection selectable markers are selected from the group consisting of URA3, *amdS*, *fcy1*, *pyrG*, *tk*, and beta-tubulin.

[200] A eukaryotic cell obtained according to the method of any one of paragraphs 182-199.

30 [201] A eukaryotic cell for expressing multiple heterologous proteins of interest, comprising: (1) one or more first target loci each comprising a pair of a first recombination recognition site and a second recombination recognition site, wherein the first recombination recognition site and a second recombination recognition site are TP901-1 sites of the Xis-att system, and (2) one or more second target loci each comprising a pair of a third recombination recognition site and a fourth recombination recognition site, wherein the third recombination recognition site and a fourth recombination recognition site are flippase
35 recognition sites of the FLP-FRT system.

Claims

What is claimed is:

- 5 1. A method of introducing multiple expression constructs into two or more target loci of a eukaryotic cell, said method comprising.
- (a) transforming a population of the eukaryotic cell with one or more first constructs and one or more second constructs,
- wherein the eukaryotic cell comprises (1) one or more first target loci each
10 comprising a pair of a first recombination recognition site and a second recombination recognition site, and (2) one or more second target loci each comprising a first fragment of a first selectable marker lacking a selectable function;
- wherein the one or more first constructs each comprises one or more first expression cassettes each comprising a first polynucleotide encoding a first protein of interest, wherein
15 in each of the first constructs the one or more first expression cassettes are flanked on one side by the first recombination recognition site and on the other side by the second recombination recognition site corresponding to the same recombination recognition sites of the first target loci; and
- wherein the one or more second constructs each comprises one or more second
20 expression cassettes each comprising a second polynucleotide encoding a second protein of interest, and wherein in each of the second constructs the one or more second expression cassettes are flanked on one side by a homologous region of the corresponding second target locus and on the other side by a second fragment of the first selectable marker that lacks the selectable function, wherein the second fragment comprises a sequence
25 overlapping homologously the corresponding sequence of the first fragment of the first selectable marker of the second target loci; and
- (b) selecting a transformant using each of the first selectable markers wherein the first fragment and the second fragment of each of the first selectable markers undergo recombination to become functional and the homologous region of the second constructs
30 undergo homologous recombination with the same corresponding region of the second target loci, wherein each of the second expression cassettes are integrated at the corresponding second target loci, and wherein the first and second recombination recognition sites of the first constructs undergo recombination at the corresponding first and second recombination recognition sites of the first target loci driven by at least one
35 recombinase integrating each of the first expression cassettes at the corresponding first target loci.

2. The method of claim 1, wherein the transforming of the population of the eukaryotic cell with the one or more first constructs and the one or more second constructs is performed sequentially in any order or as a co-transformation.

3. The method of claim 1 or 2, wherein the first and second fragments of the first selectable markers each further comprise a repeat sequence 5' of the first fragment and a repeat sequence 3' of the second fragment, and wherein the repeat sequences undergo homologous recombination to remove the selectable markers; or wherein the first and second fragments of the first selectable markers each further comprise repeat sequences allowing removal of a portion of each of the selectable markers by homologous recombination resulting in selectable markers lacking a selectable function.

4. The method of any one of claims 1-3, wherein the eukaryotic cell is reusable by repeating steps (a) and (b) with one or more different first constructs, second constructs, or first and second constructs each comprising an expression cassette comprising a polynucleotide encoding a different protein of interest.

5. A method of constructing a eukaryotic cell having multiple target loci for expressing multiple heterologous proteins of interest, comprising:

(a) transforming a population of the eukaryotic cell with a first construct comprising (1) a 5' homologous region of a first target locus, (2) a first recombination recognition site, (3) a first selectable marker conferring a first selectable function, (4) a second recombination recognition site, and (5) a 3' homologous region of the first target locus;

(b) selecting a first transformant having the first construct integrated at the first target locus using the first selectable marker for selection of the first transformant, wherein the 5' homologous region and the 3' homologous region of the first construct undergo homologous recombination with the corresponding regions of the first target locus integrating the first construct at the first target locus, and wherein the first recombination recognition site and the second recombination recognition site are integrated at the first target locus;

(c) transforming a population of the first transformant with a second construct comprising (1) a 5' homologous region of a second target locus, (2) a first fragment of a second selectable marker that lacks a second selectable function, (3) a third selectable marker conferring a third selectable function, and (4) a 3' homologous region of the second target locus;

(d) selecting a second transformant having the second construct integrated at the second target locus using the third selectable marker for selection of the second

transformant, wherein the 5' homologous region and the 3' homologous region of the second construct undergo homologous recombination with the corresponding regions of the second target locus integrating the second construct at the second target locus, and wherein the first fragment of the second selectable marker that lacks a second selectable function is integrated at the second target locus;

(e) co-transforming a population of the second transformant with (i) a third construct comprising (1) the first recombination recognition site, (2) one or more first expression cassettes each comprising a first polynucleotide encoding a first protein of interest, and (3) the second recombination recognition site, and (ii) a fourth construct comprising (1) the 5' homologous region of the second target locus, (2) one or more second expression cassettes each comprising a second polynucleotide encoding a second protein of interest, and (3) a second fragment of the second selectable marker that lacks the second selectable function wherein the second fragment comprises a sequence overlapping homologously the corresponding sequence of the first fragment of the first selectable marker; and

(f) selecting a third transformant using the second selectable marker wherein the first integrated fragment and the second fragment of the second selectable marker become functional upon recombination (e.g., homologous recombination or recombinase-mediated recombination), wherein the 5' homologous region of the fourth construct undergoes homologous recombination with the same corresponding region of the second target locus, wherein the one or more second expression cassettes are integrated at the second target locus, and wherein the first recombination recognition site and the second recombination recognition site undergo recombination at the first target locus driven by at least one recombinase integrating the one or more first expression cassettes at the first target locus.

6. The method of claim 5, wherein steps (a) and (b) are performed before or after steps (c) and (d).

7. The method of claim 5 or 6, wherein the first selectable marker further comprises a first repeat 5' of the first selectable marker and a second repeat 3' of the first selectable marker, wherein the first and second repeat sequences of the integrated first construct undergo homologous recombination to remove the first selectable marker.

8. The method of any one of claims 5-7, wherein the third selectable marker further comprises a third repeat 5' of the third selectable marker and a fourth repeat 3' of the third selectable marker, wherein the third and fourth repeat sequences of the second construct undergo homologous recombination to remove the third selectable marker.

9. The method of any one of claims 5-8, wherein steps (a)-(f) are repeated at at least two additional target loci with different recombination recognition sites, different polynucleotides encoding proteins of interest, and the same or a different second selectable marker; or wherein steps (a)-(b) or (c)-(d) are repeated at one additional target locus with different recombination recognition sites, different polynucleotides encoding proteins of interest, and the same or a different selectable marker.

10. The method of any one of claims 1-9, wherein the recombination recognition sites are selected from the group consisting of B2 system from *Zygosaccharomyces bailii*, B3 system from *Zygosaccharomyces bisporus*, beta-recombinase-six system from a 25 *Bacillus subtilis* plasmid, Bxb1 from phage Bxb1, Cre-lox system of bacteriophage P1, Dre from Bacteriophage D6, FLP-FRT of *Saccharomyces cerevisiae*, Delta-gamma-es system from bacterial transposon Tn1000, Gin-gix system from bacteriophage Mu, HK022 from phage HK022, KD system from *Kluyveromyces drosophilum*, Mx9 phage transformation system, *Streptomyces* phage ϕ C31, R-RS system of *Zygosaccharomyces rouxii*, Tn3 from *E. coli*, Vika recombinase from *Vibrio coralliilyticus*, and Xis-att system of temperate lactococcal bacteriophage TP901-1; and combinations thereof.

11. A method of introducing multiple expression constructs into a eukaryotic cell, said method comprising.

(a) transforming a population of the eukaryotic cell with one or more first constructs and one or more second constructs,

wherein the eukaryotic cell comprises (1) one or more first target loci each comprising a pair of a first recombination recognition site and a second recombination recognition site, wherein the first recombination recognition site and a second recombination recognition site are TP901-1 sites of the Xis-att system, and (2) one or more second target loci each comprising a pair of a third recombination recognition site and a fourth recombination recognition site, wherein the third recombination recognition site and a fourth recombination recognition site are flippase recognition sites of the FLP-FRT system;

wherein the one or more first constructs each comprises one or more first expression cassettes each comprising a first polynucleotide encoding a first protein of interest, wherein in each of the first constructs the one or more first expression cassettes are flanked on one side by the first recombination recognition site and on the other side by the second recombination recognition site corresponding to the same recombination recognition sites of the first target loci;

wherein the one or more second constructs each comprises one or more second expression cassettes each comprising a second polynucleotide encoding a second protein of interest, and wherein in each of the second constructs the one or more second expression cassettes are flanked on one side by the third recombination recognition site and on the other side by the fourth recombination recognition site corresponding to the same recombination recognition sites of the second target loci; and

wherein one or more of the first constructs and second constructs comprise one or more first selectable markers;

(b) selecting a transformant using the one or more first selectable markers, wherein each of the second expression cassettes are integrated at the corresponding second target loci, and wherein the first and second recombination recognition sites of the first constructs undergo recombination at the corresponding first and second recombination recognition sites of the first target loci driven by at least one recombinase integrating each of the first expression cassettes at the corresponding first target loci.

12. The method of claim 11, wherein the one or more first target loci each further comprises a second selectable marker between the recombination recognition sites, and/or wherein the one or more second target loci each further comprises a third selectable marker between the recombination recognition sites.

13. The method of any one of claims 1-12, wherein the eukaryotic cell comprises one or more recombinases, which are native, heterologous, or a combination of native and heterologous to the eukaryotic cell (e.g., a Bxb1 recombinase, a Cre recombinase, a CinH recombinase, a Flp flippase, a HK022 integrase, a ParA recombinase, a Tn1721 recombinase, a Tn5053 recombinase, a TP901-1 integrase, an U153 recombinase, a λ integrase, or a ϕ C31 recombinase).

14. The method of any one of claims 1-13, wherein the selectable markers are selected from the group consisting of ADE2, ARO4-OPF, FLD1, HIS3, LEU2, LYS2, MET3, TRP1, URA3, *adeA*, *adeB*, *amdS*, *argB*, *bar*, *bleR*, *bsd*, *fcy1*, *hpt*, *hpt-tk*, *nat1*, *niaD*, *ptr1*, *pyrG*, *sC*, *tk*, Tn903kan^r, *trpC*, and beta-tubulin.

15. A eukaryotic cell for expressing multiple heterologous proteins of interest, comprising:

(a) one or more first target loci each comprising a pair of a first recombination recognition site and a second recombination recognition site; and

(b) one or more second target loci each comprising a first fragment of a first selectable marker lacking a selectable function.

16. The eukaryotic cell of claim 15, further comprising one or more first expression
5 cassettes each comprising a first polynucleotide encoding a first protein of interest at each of
the first target loci, wherein each of the one or more first expression cassettes are flanked on
one side by the first recombination recognition site and on the other side by the second
recombination recognition site of the corresponding first target locus; and/or further
10 comprising one or more second expression cassettes each comprising a second
polynucleotide encoding a second protein of interest at each of the second target loci,
wherein each of the one or more second expression cassettes are flanked on one side by
the first fragment of the selectable marker lacking a selectable function.

17. A eukaryotic cell comprising (1) one or more first target loci each comprising one or
15 more first expression cassettes each comprising a first polynucleotide encoding a first
protein of interest, wherein the one or more first expression cassettes are each flanked 5' by
a first recombination recognition site and 3' by a second recombination recognition site, and
(2) one or more second target loci each comprising one or more second expression
cassettes each comprising a second polynucleotide encoding a second protein of interest,
20 wherein each of the one or more second expression cassettes are flanked on one side by a
region of the second target locus and on the other side by a first fragment of a first
selectable marker that lacks selectable function,

wherein each of the pairs of the first and second recombination recognition sites at
the first loci are able to undergo recombination with a first construct comprising one or more
25 third expression cassettes each comprising a third polynucleotide encoding a third protein of
interest, wherein each of the one or more third expression cassettes are flanked on one side
by the first recombination recognition site and on the other side by the second recombination
recognition site of the corresponding first target locus, and

wherein each of the target loci regions and the first fragment of the first selectable
30 marker that lacks selectable function at the second loci are able to undergo recombination
(e.g., homologous recombination or recombinase-mediated recombination) with a second
construct comprising one or more fourth expression cassettes each comprising a fourth
polynucleotide encoding a fourth protein of interest, wherein each of the one or more fourth
expression cassettes are flanked on one side by a homologous region of the corresponding
35 second target locus and on the other side by a second fragment of the first selectable
marker that lacks the selectable function wherein the second fragment comprises a

sequence overlapping homologously the corresponding sequence of the first fragment of the first selectable marker.

18. The eukaryotic cell of any one of claims 15-17, wherein the recombination recognition sites are selected from the group consisting of a B2 system from *Zygosaccharomyces bailii*, B3 system from *Zygosaccharomyces bisporus*, beta-recombinase-six system from a 25 *Bacillus subtilis* plasmid, Bxb1 from phage Bxb1, Cre-lox system of bacteriophage P1, Dre from Bacteriophage D6, FLP-FRT of *Saccharomyces cerevisiae*, Delta-gamma-es system from bacterial transposon Tn1000, Gin-gix system from bacteriophage Mu, HK022 from phage HK022, KD system from *Kluyveromyces drosophilum*, Mx9 phage transformation system, *Streptomyces* phage ϕ C31, R-RS system of *Zygosaccharomyces rouxii*, Tn3 from *E. coli*, Vika recombinase from *Vibrio coralliilyticus*, and Xis-att system of temperate lactococcal bacteriophage TP901-1; and combinations thereof.

19. The eukaryotic cell of any one of claims 15-18, wherein the selectable markers are selected from the group consisting of ADE2, ARO4-OFD, FLD1, HIS3, LEU2, LYS2, MET3, TRP1, URA3, *adeA*, *adeB*, *amdS*, *argB*, *bar*, *bleR*, *bsd*, *fcy1*, *hpt*, *hpt-tk*, *nat1*, *niaD*, *ptr1*, *pyrG*, *sC*, *tk*, Tn903kan^r, *trpC*, and beta-tubulin.

20. A eukaryotic cell for expressing multiple heterologous proteins of interest, comprising: (1) one or more first target loci each comprising a pair of a first recombination recognition site and a second recombination recognition site, wherein the first recombination recognition site and a second recombination recognition site are TP901-1 sites of the Xis-att system, and (2) one or more second target loci each comprising a pair of a third recombination recognition site and a fourth recombination recognition site, wherein the third recombination recognition site and a fourth recombination recognition site are flippase recognition sites of the FLP-FRT system.

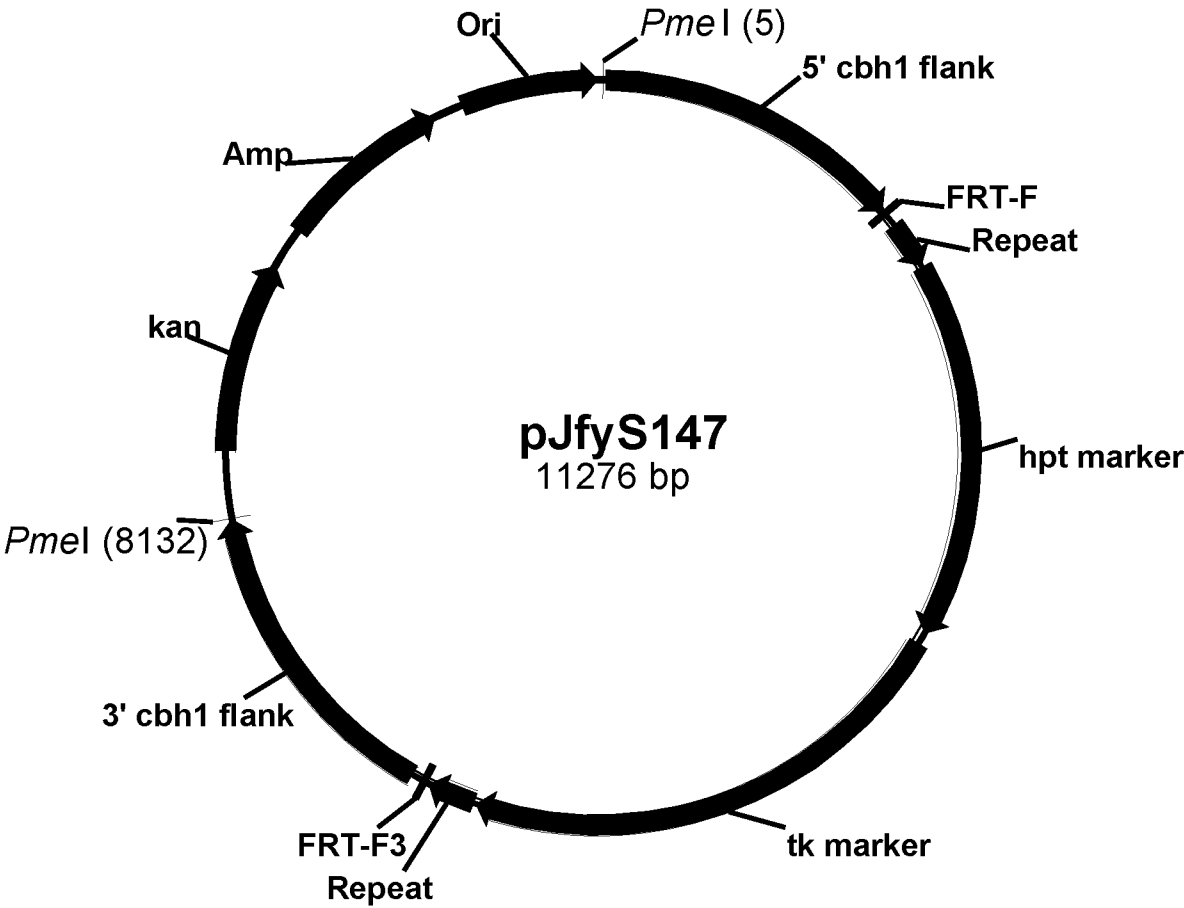


Fig. 1

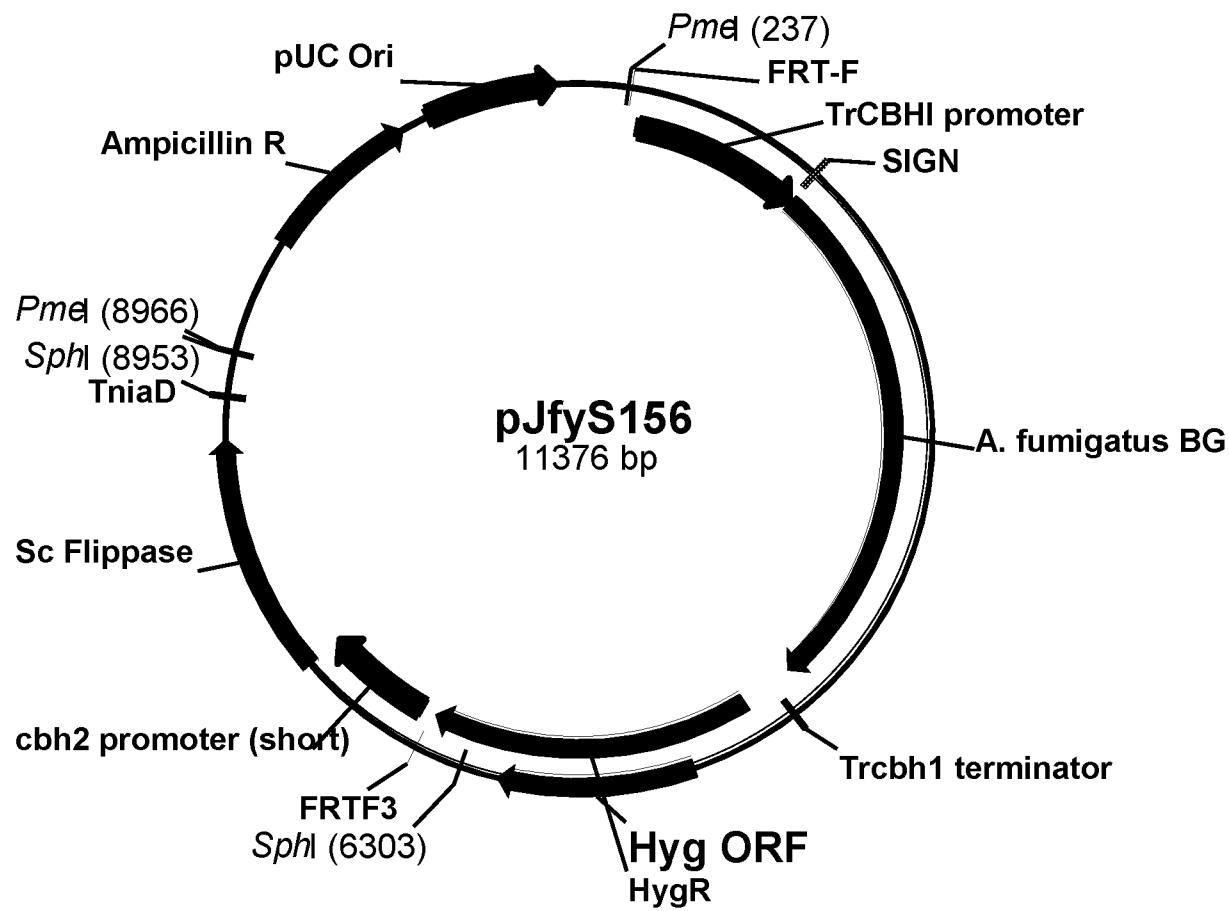


Fig. 2

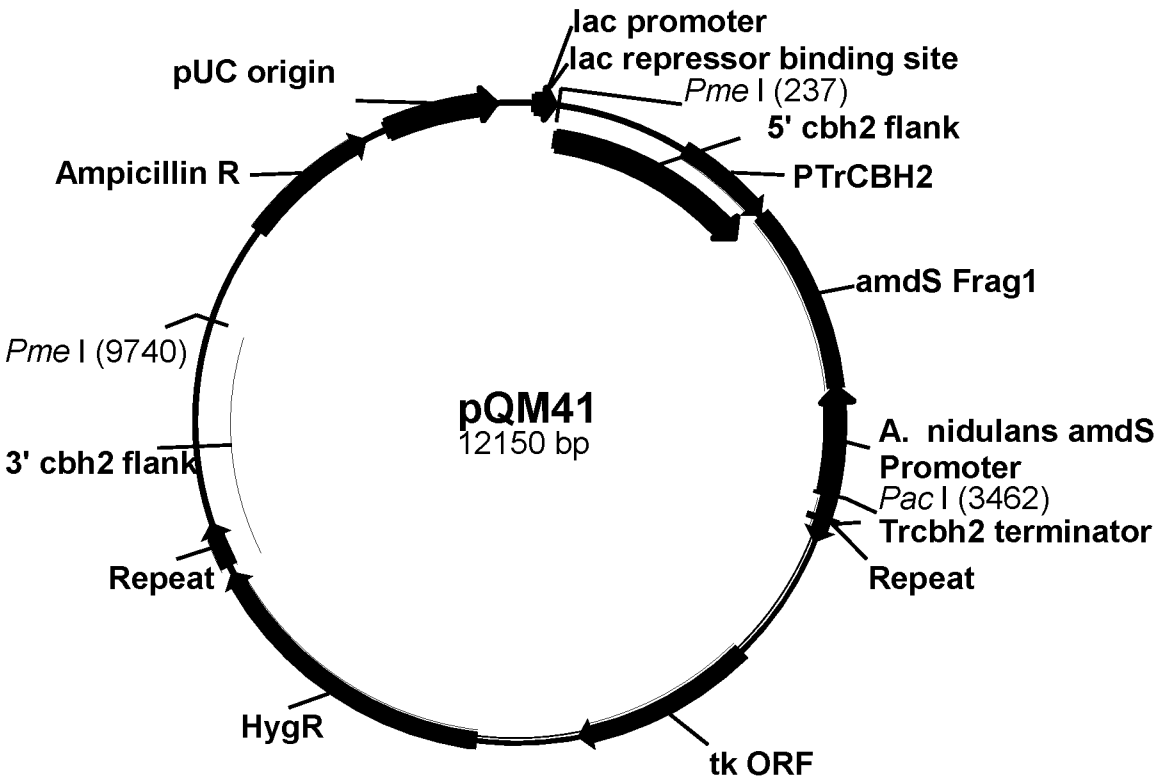
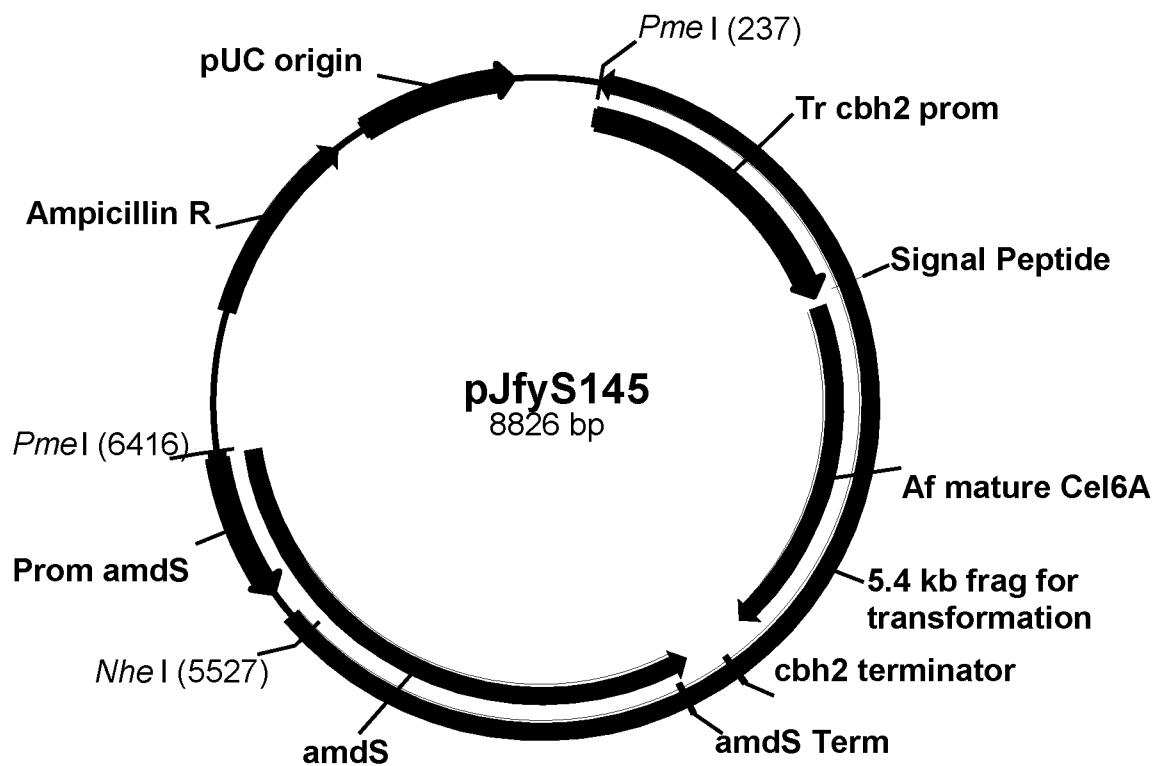
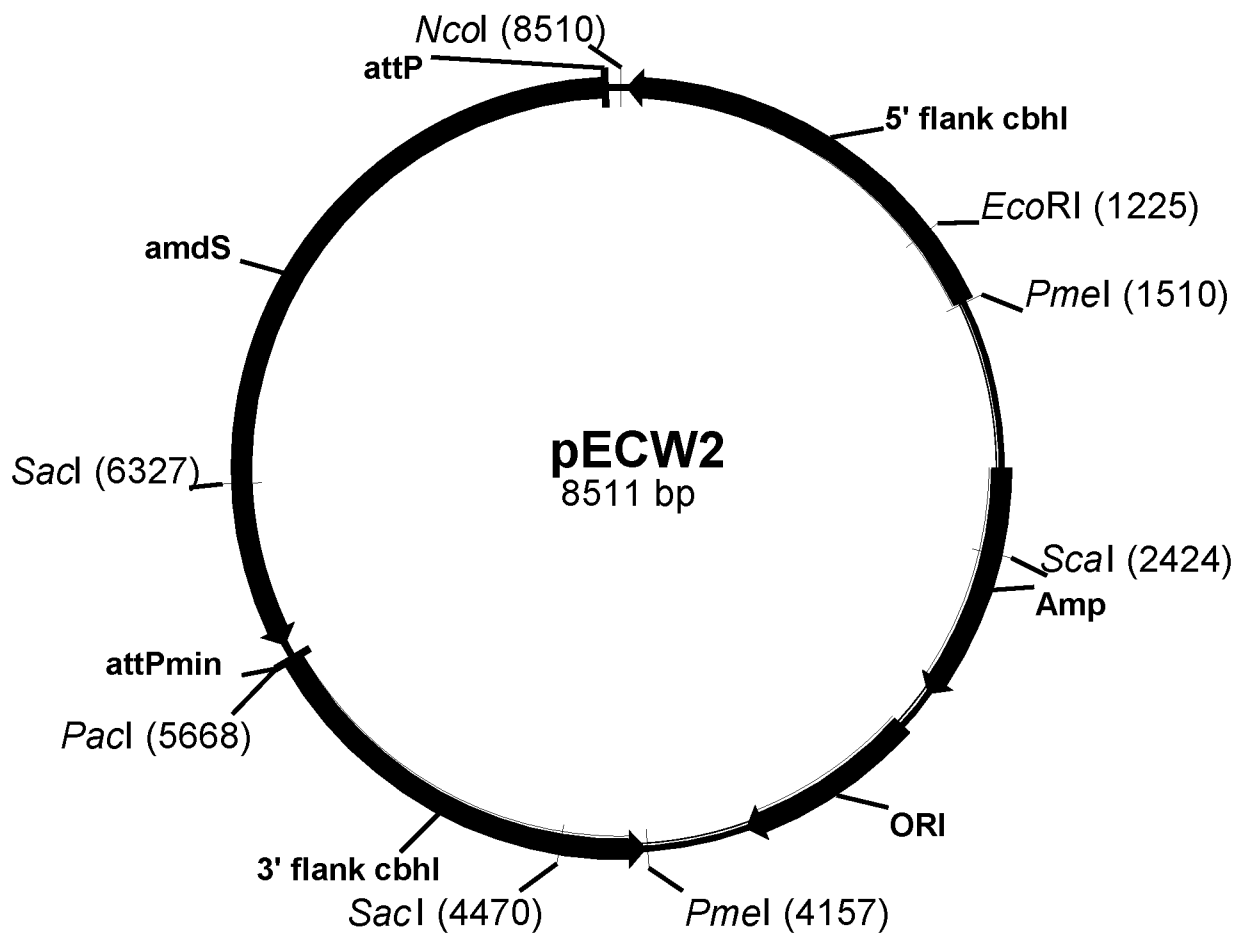


Fig. 3

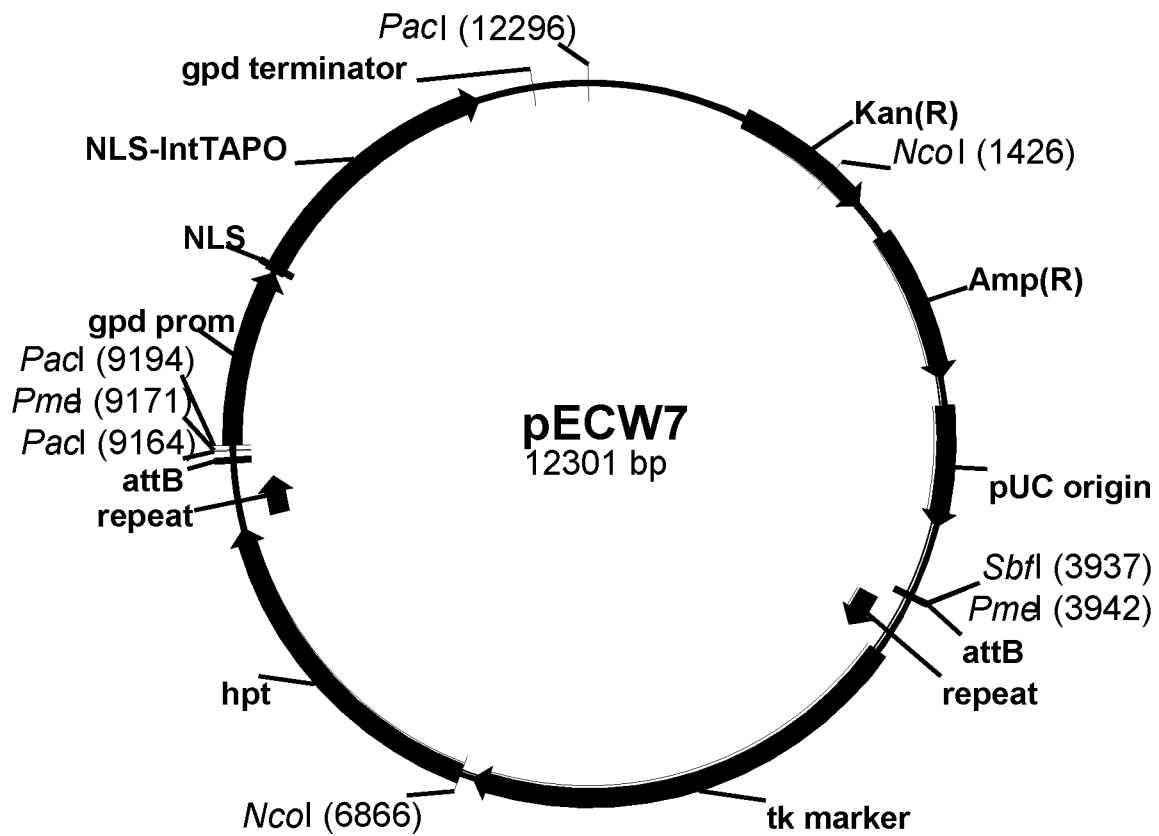
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**Fig. 4**

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**Fig. 5**

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**Fig. 6**

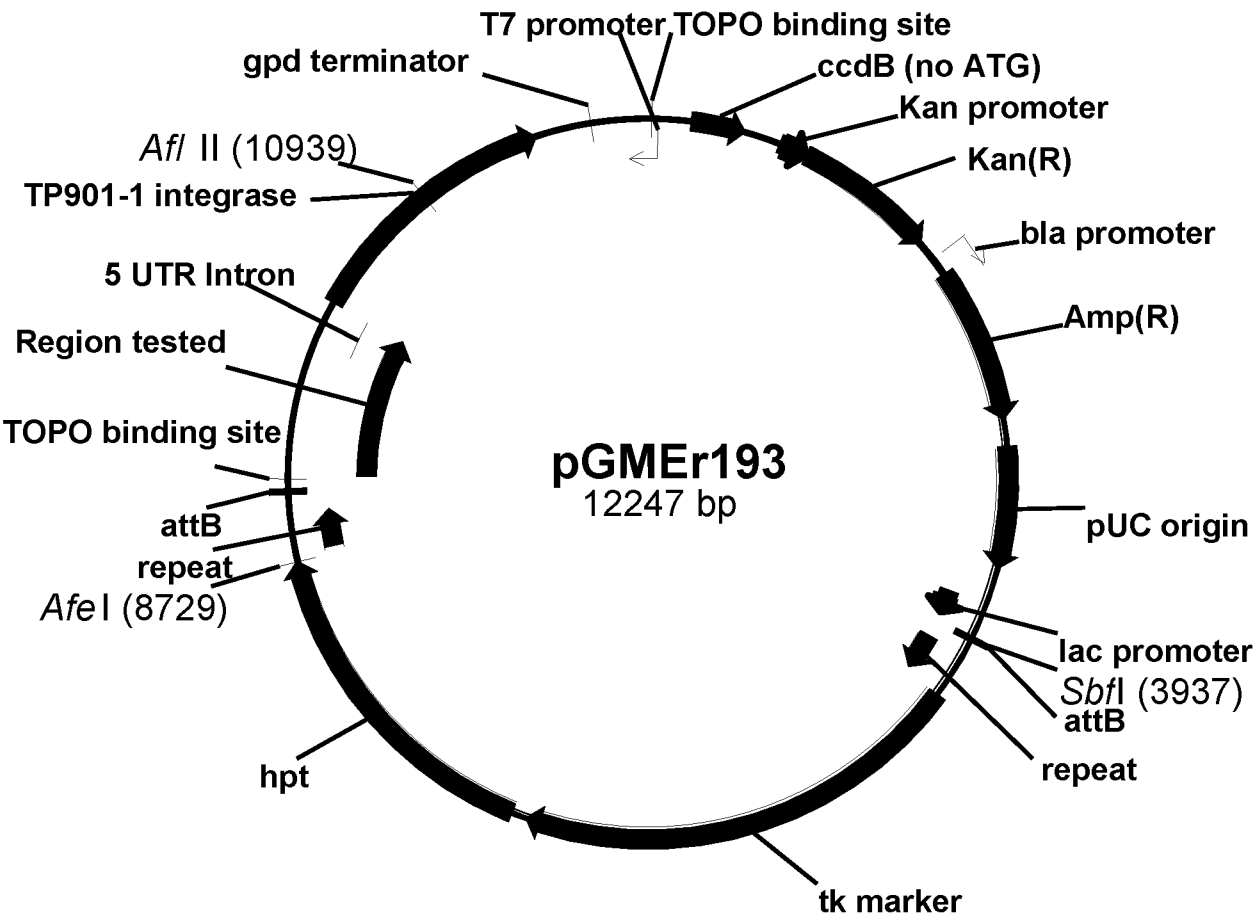


Fig. 7

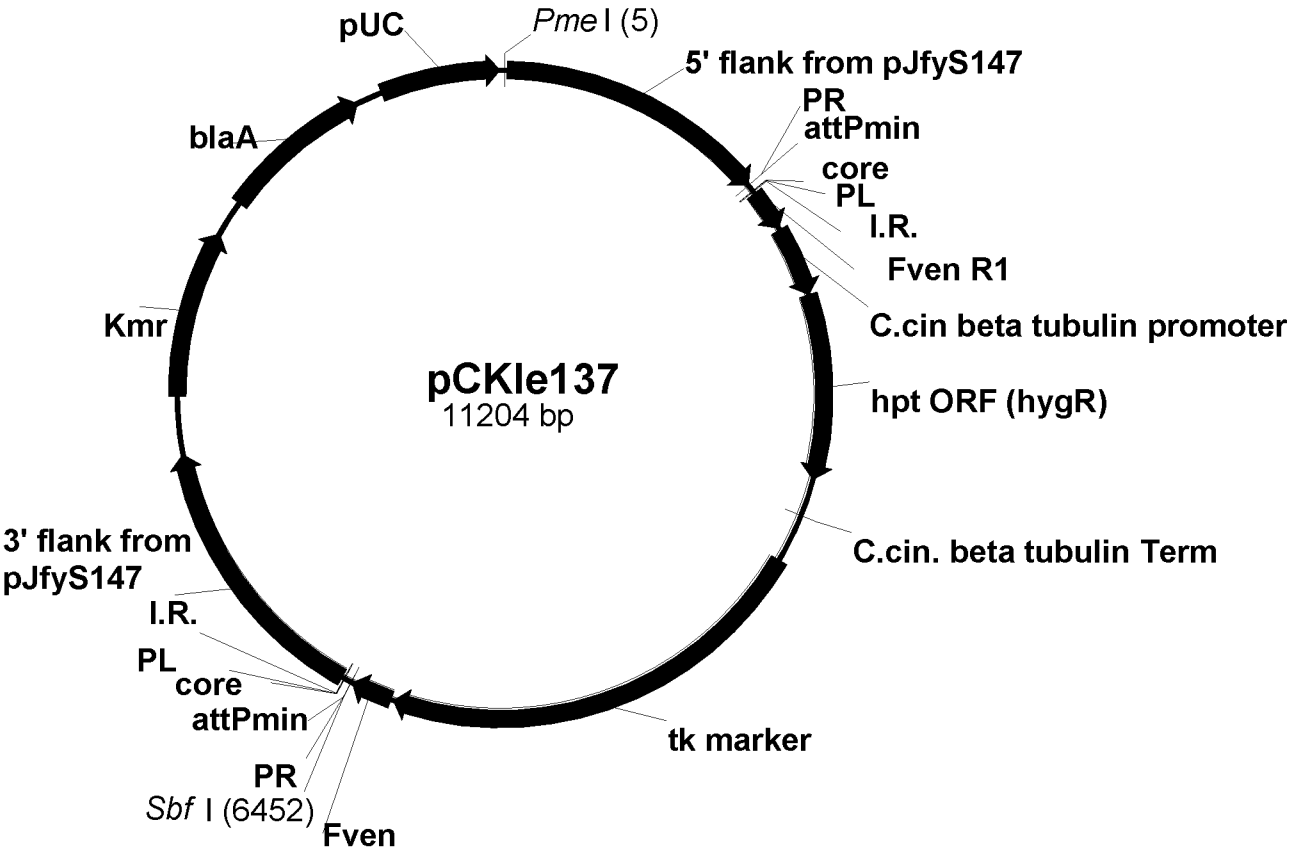
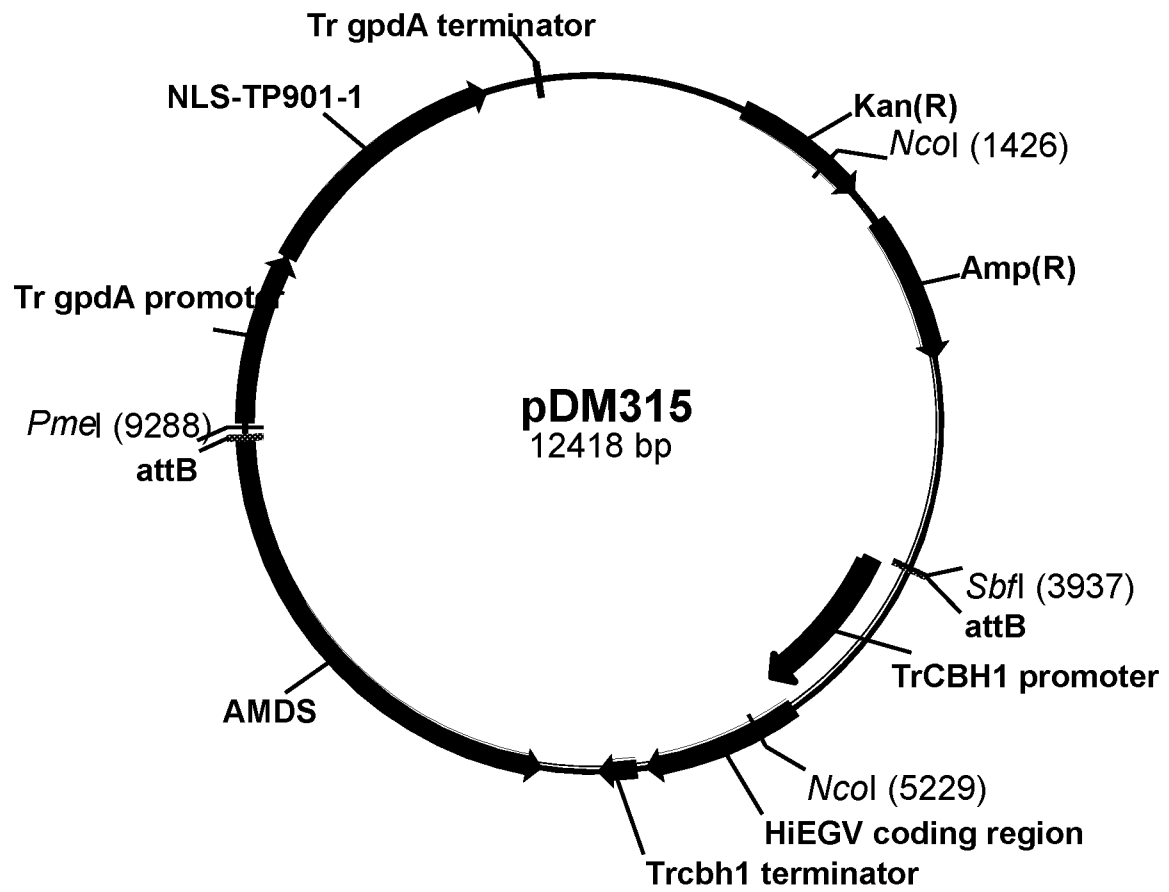


Fig. 8

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**Fig. 9**

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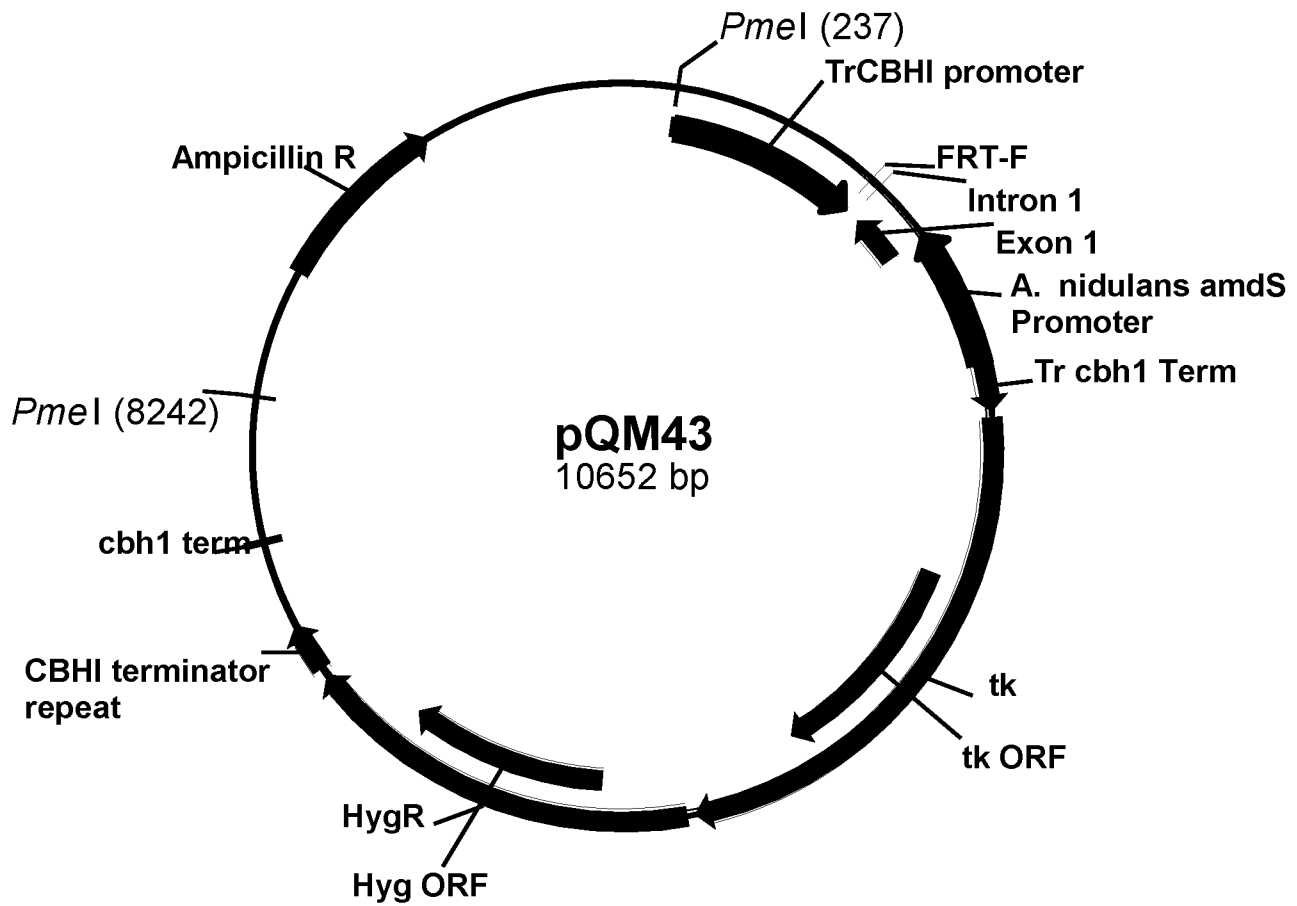


Fig. 10

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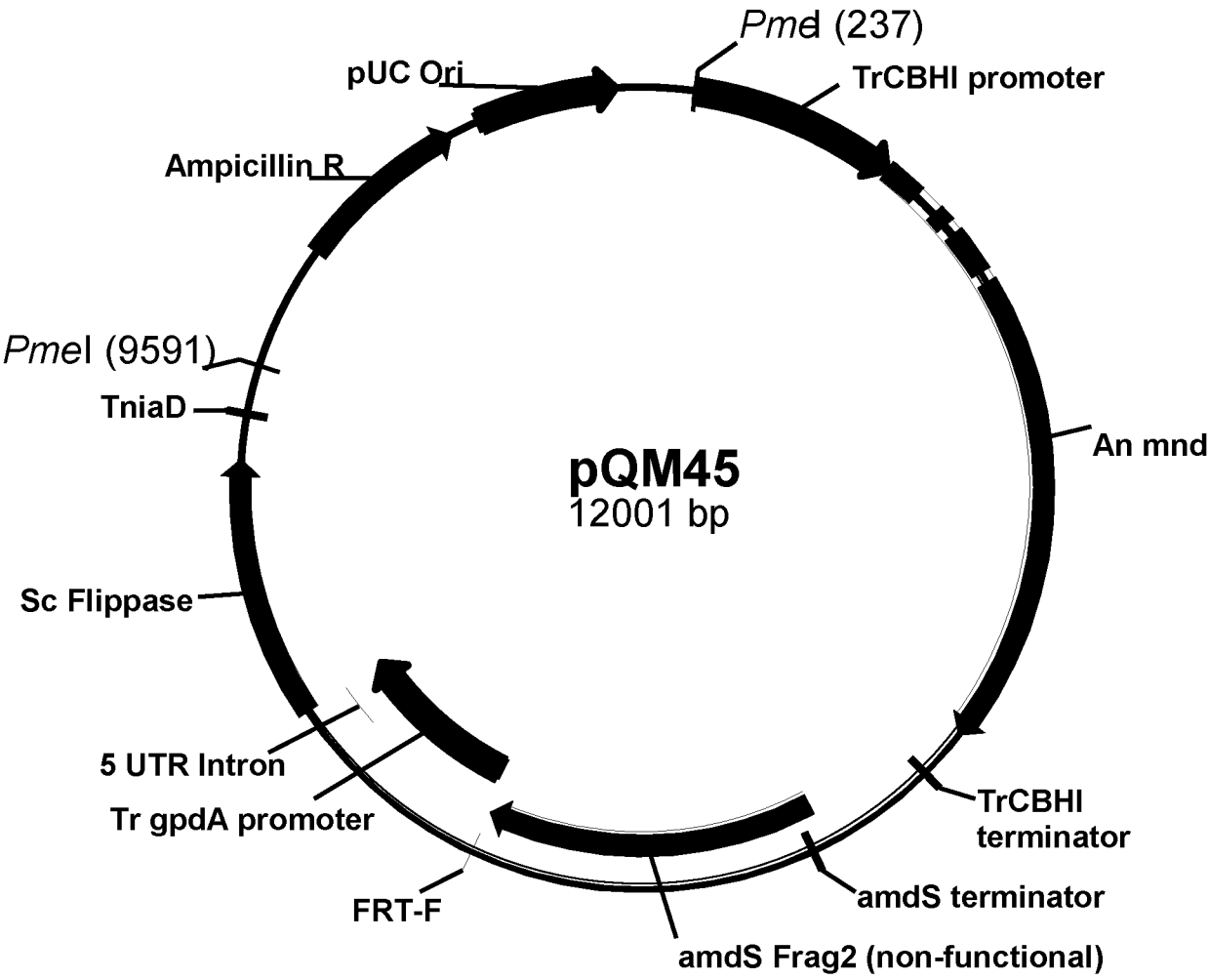


Fig. 11

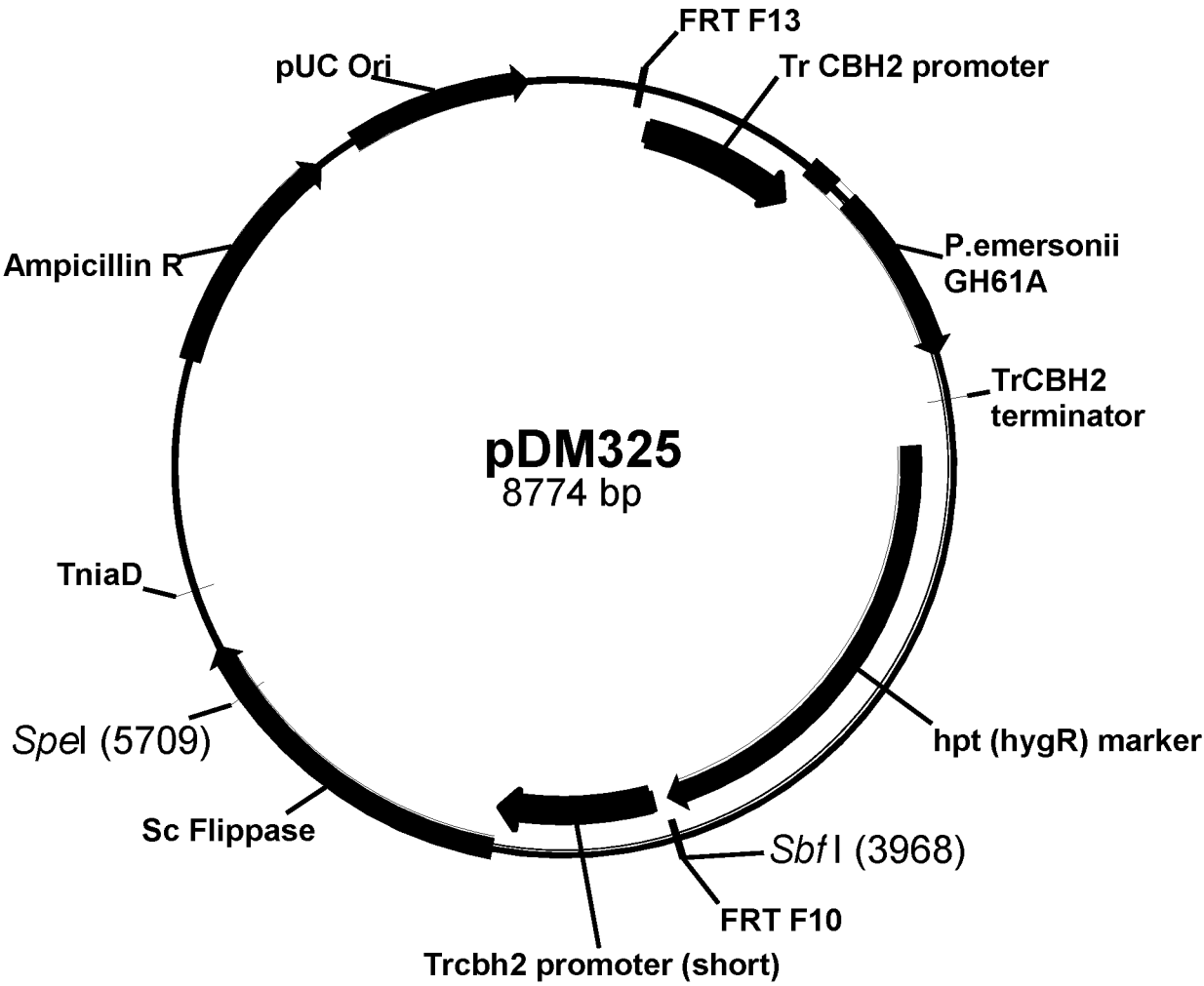


Fig. 12

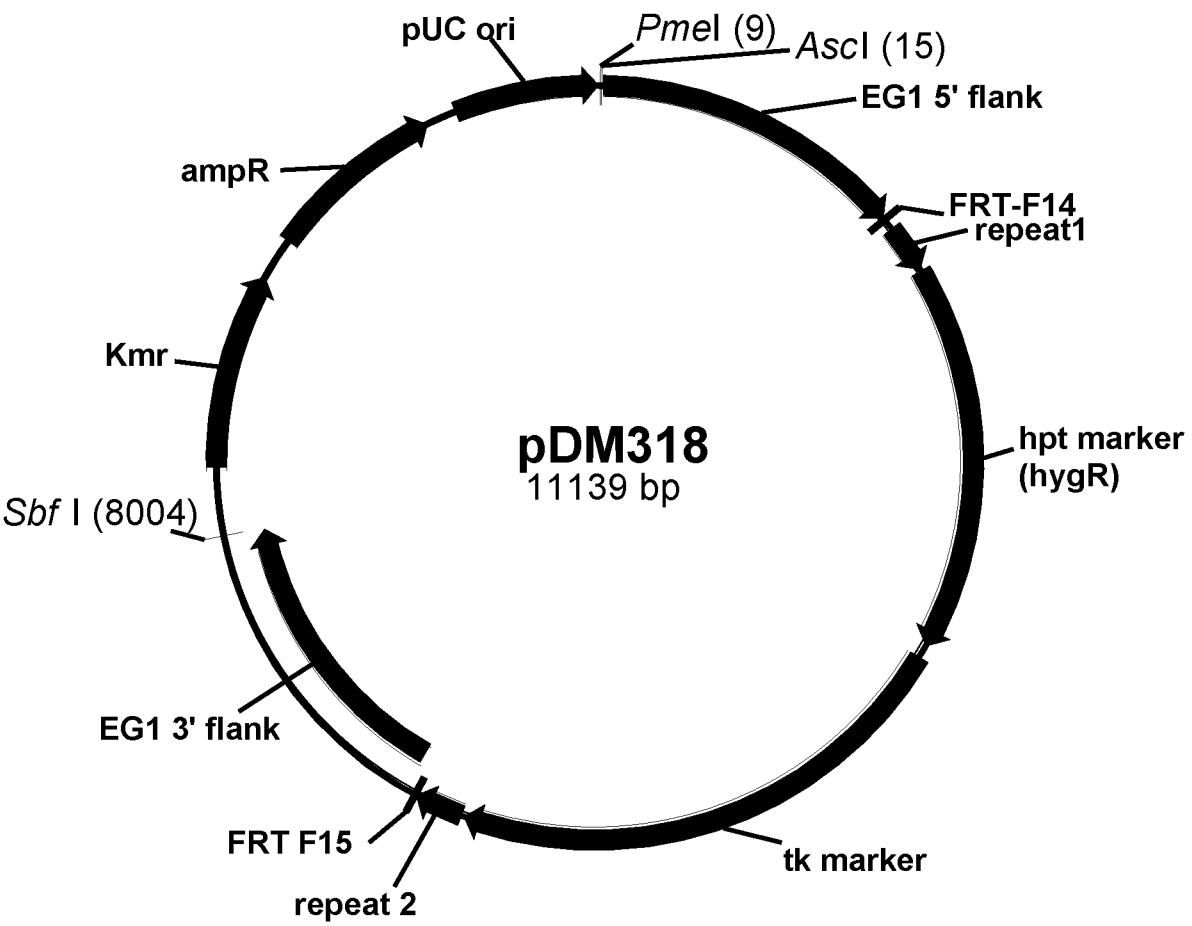


Fig. 13

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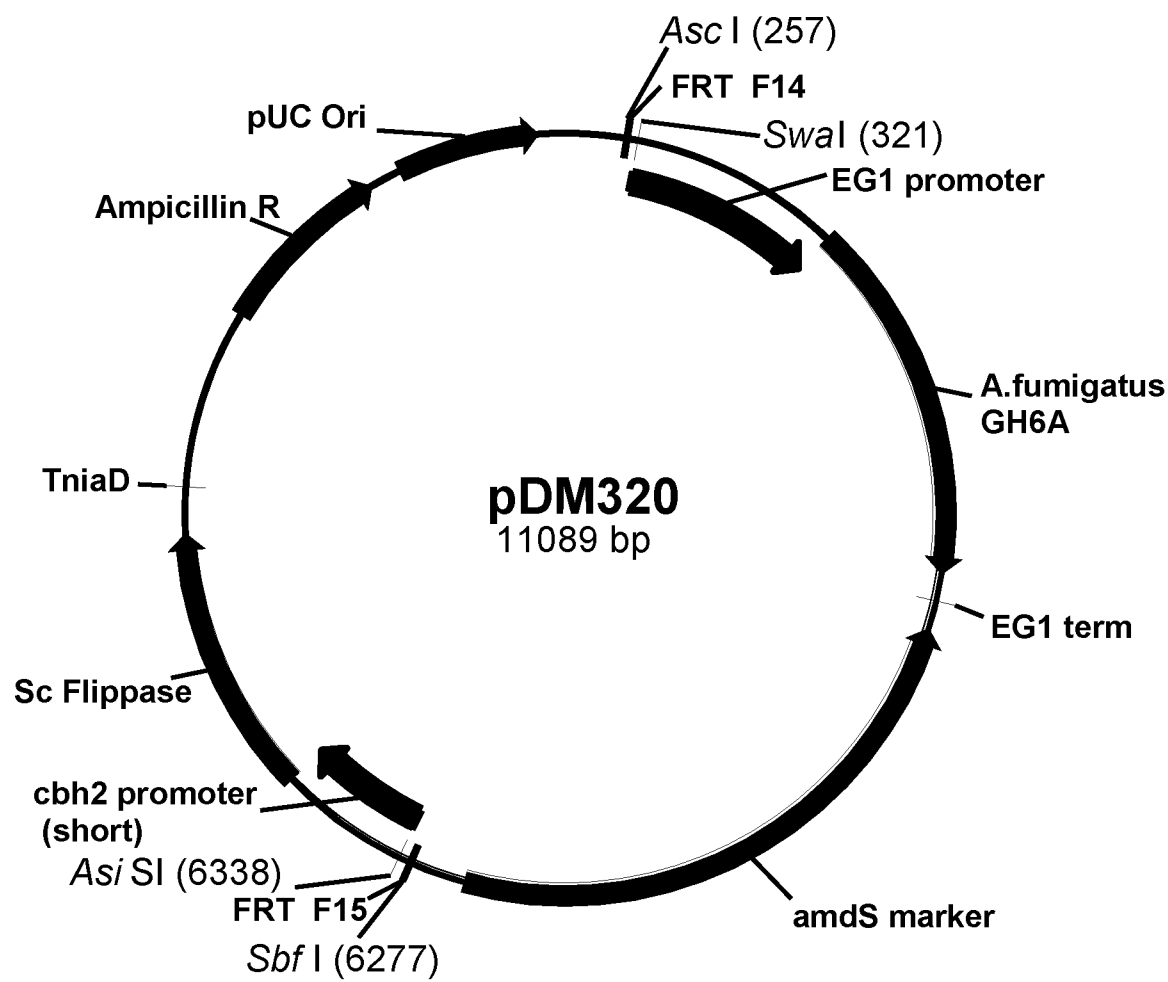


Fig. 14

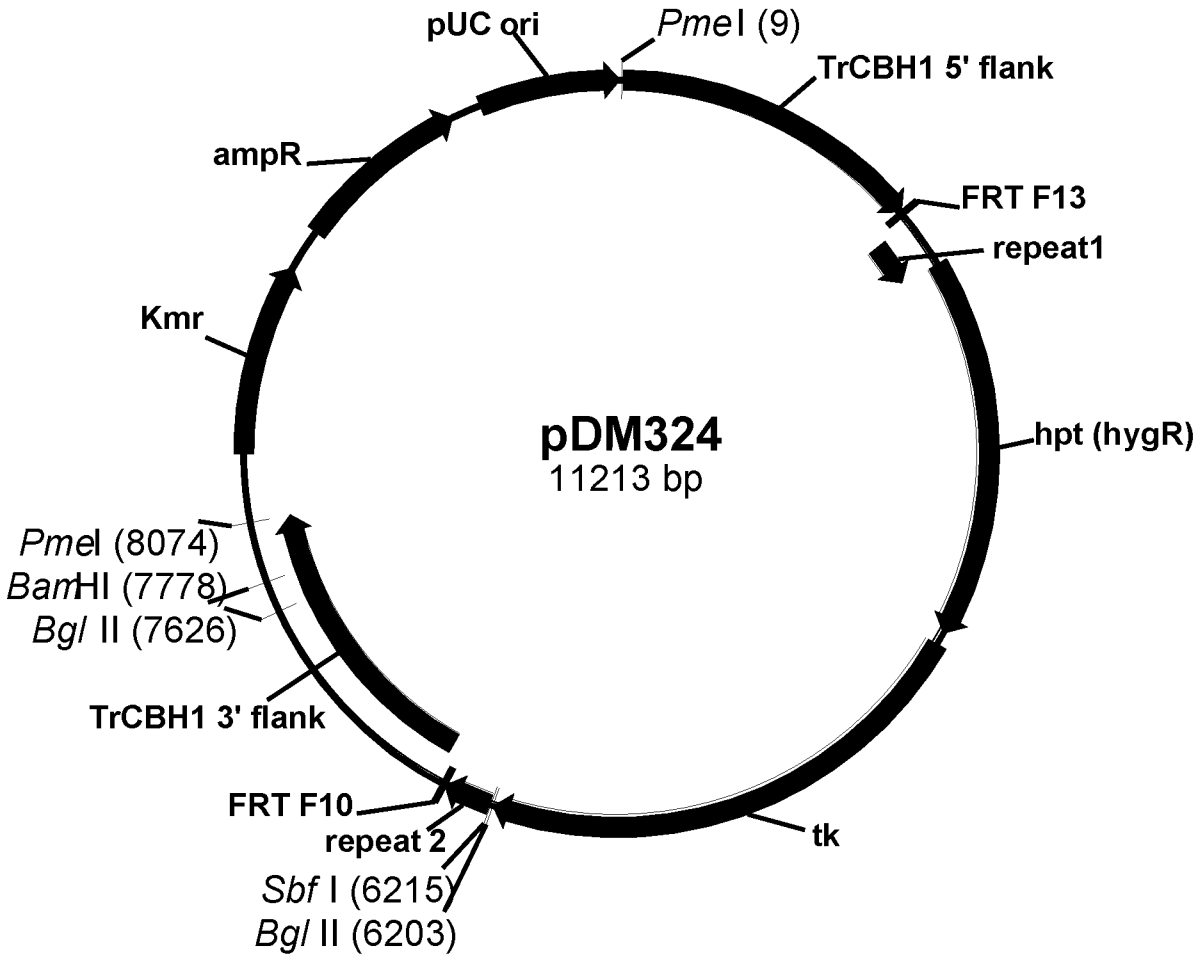


Fig. 15

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
- a. ☒ forming part of the international application as filed:
- ☒ in the form of an Annex C/ST.25 text file.
- ☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
- ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
- ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/021568

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/90 C12N15/79
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, COMPENDEX, EMBASE, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/028912 A2 (NOVOZYMES INC [US]; YAYER DEBBIE [US]; JIN QIMING [US]) 28 February 2013 (2013-02-28) cited in the application the whole document, in particular the claims -----	15-19
X	WO 2010/039889 A2 (NOVOZYMES INC [US]; YODER WENDY [US]; SHASKY JEFFREY [US]) 8 April 2010 (2010-04-08) cited in the application the whole document, in particular the claims -----	15,16
X	EP 2 527 448 A1 (NOVOZYMES AS [DK]) 28 November 2012 (2012-11-28) the whole document, in particular claims and figures ----- -/--	15,16



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 May 2016

Date of mailing of the international search report

31/05/2016

Name and mailing address of the ISA/

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Authorized officer

Bassias, Ioannis

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2016/021568

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LUBERTOZZI D ET AL: "Developing Aspergillus as a host for heterologous expression", BIOTECHNOLOGY ADVANCES, ELSEVIER PUBLISHING, BARKING, GB, vol. 27, no. 1, 1 January 2009 (2009-01-01), pages 53-75, XP025781148, ISSN: 0734-9750, DOI: 10.1016/J.BIOTECHADV.2008.09.001 [retrieved on 2008-09-23] -----	1-20

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2016/021568

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