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[Continued on next page]

(54) Title: COMPOSITIONS AND METHODS FOR HELPER STRAIN-MEDIATED FUNGAL GENOME MODIFICATION

(57) Abstract: Compositions and methods are provided employing a helper strain system for promoting genetic alterations in a fungal host cell, e.g., a filamentous fungal host cell.

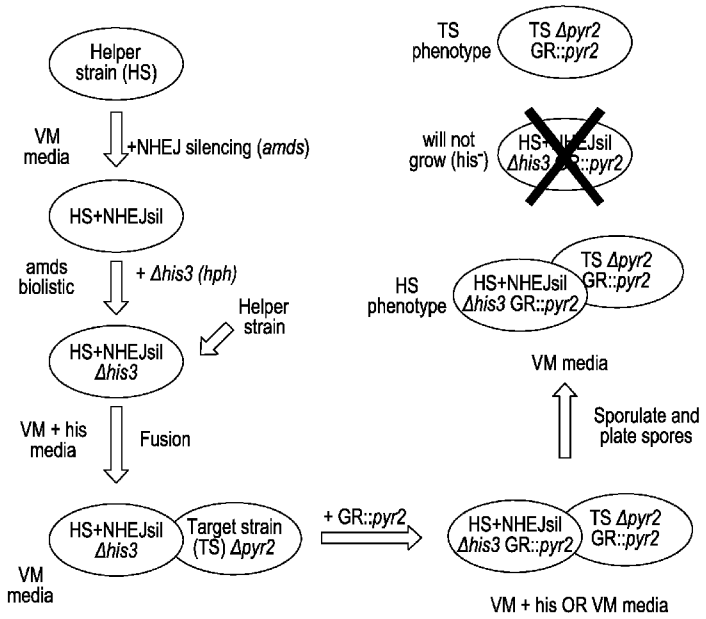

FIG. 1

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COMPOSITIONS AND METHODS FOR HELPER STRAIN-MEDIATED FUNGAL GENOME MODIFICATION

CROSS-REFERENCE TO RELATED APPLICATIONS

[01] The present application claims priority to U. S. Provisional Application Ser. No. 62/092,444, filed December 16, 2014, which is hereby incorporated by reference in its entirety.

SEQUENCE LISTING

[02] The sequence listing submitted via EFS, in compliance with 37 C.F.R. §1.52(e), is incorporated herein by reference. The sequence listing text file submitted via EFS contains the file "40692-WO-PCT_2015-579 final_ST25.txt" created on December 11, 2015, which is 100 kilobytes in size.

BACKGROUND

[03] A pre-requisite for applying the helper strain concept for fungal genome modification is the ability to form a heterokaryon by fusing two different cell types: the helper strain (HS) and the target strain (TS). In some fungal cells (e.g., N. crassa), this fusion can be accomplished by simply mixing the spores of the HS and TS and allowing them to germinate together on selective medium. In other fungal cells (e.g., T. reesei), cell fusion does not occur so readily, and it is necessary to make protoplasts to facilitate fusion. Once the cells from the HS and TS are fused, the shared cytoplasm allows the benefit that the HS provides to be available to the TS. In N. crassa, the cells are connected into a syncytium which allows the cytoplasm as well as organelles to migrate between cell compartments (Roper, M. Simonin, A., Hickey, P. C., Leeder, A., and Glass, N. L. 2013. Nuclear dynamics in fungal chimera. Proc. Nat. Acad. Sci. 110 (32), 12875-12880). The beneficial components produced by the HS nuclei are, therefore, freely distributed throughout the mycelium. The compartments of T. reesei, on the other hand, are separated by septae that limit the migration of cell components.

[04] Once the benefit from the heterokaryon is utilized, the component strains need to be separated. This is achieved by sporulating the heterokaryon, plating the individual conidiospores, and verifying strains that derived from single uninucleate

spores. In N. crassa microconidia, containing a single nucleus, can be produced on media containing iodoacetate (Ebbole, D. and Sachs, M. S. 1990. A rapid and simple method for isolation of Neurospora crassa homokaryons using microconidia. Fungal Genet. Newslett. 37), while some T. reesei strains have predominantly multinucleate conidiospores. In T. reesei, if a strain that produces multinucleate conidiospores cannot be substituted with one that produces uninucleate conidiospores, additional rounds of spore-purification need to be performed, lengthening the procedure.

[05] As a result of these and other differences, the helper strain concept has not been incorporated into methods for routine manipulation and strain improvement in T. reesei.

[06] Thus, there still remains a need for developing efficient and effective helper strain-mediated genome engineering methods and compositions for many fungal host cells, including *T. reesei*.

BRIEF SUMMARY

[07] Compositions and methods are provided employing a helper strain system for promoting genetic alterations in a fungal host cell, e.g., a filamentous fungal host cell.

[08] Aspects of the disclosure are drawn to compositions and methods for homologous recombination of a donor DNA with a genomic locus in a fungal cell, e.g., a filamentous fungal cell.

[09] Therefore, aspect of the disclosure include methods for homologous recombination of a donor DNA with a genomic locus in a fungal cell, the method comprising: (a) generating a heterokaryon between a helper fungal strain and a target fungal strain, wherein the helper fungal strain comprises an expression construct that silences the non-homologous end joining (NHEJ) mechanism; (b) introducing a donor DNA into the heterokaryon, wherein the donor DNA comprises a region of homology to a genomic locus in the target strain sufficient for homologous recombination at the genomic locus; (c) generating and plating spores from the heterokaryon cells of (b); and (d) identifying cells from the plated spores in which (i) the donor DNA has integrated into the genome by homologous recombination at the

genomic locus, and (ii) the expression construct that silences the non-homologous end joining (NHEJ) mechanism is not present.

[010] In certain embodiments, the expression construct silences one or more of: ku80, ku70, rad50, mre11, xrs2, lig4, and xrs. In certain embodiments, the expression construct silences ku80, ku70, or both.

[011] In certain embodiments, the method further comprises introducing a functional Cas/guide RNA complex into the heterokaryon, wherein the Cas/guide RNA complex has a target site within the genomic locus. In certain embodiments, the Cas is a Cas nickase. In some instances, the Cas endonuclease is operably linked to one or more nuclear targeting signal (also referred to as a nuclear localization signal/sequence; NLS). SEQ ID NO:1 and SEQ ID NO:2 provide an example of a filamentous fungal cell optimized Cas9 gene with NLS sequences at the N- and C-termini and the encoded amino acid sequence, respectively. Many different NLSs are known in eukaryotes. They include monopartite, bipartite and tripartite types. Any convenient NLS can be used, the monopartite type being somewhat more convenient with examples including the SV40 NLS (SEQ ID NO:9), a NLS derived from the T. reesei blr2 (blue light regulator 2; SEQ ID NO:10) gene, or a combination of both.

[012] In certain embodiments, the donor DNA comprises a polynucleotide sequence of interest, wherein homologous recombination at the genomic locus results in insertion of the polynucleotide sequence of interest in the genomic locus.

[013] In certain embodiments, the Cas endonuclease is a Cas9 endonuclease or variant thereof. In certain embodiments, the Cas9 endonuclease or variant thereof comprises a full length Cas9 or a functional fragment thereof from a species selected from the group consisting of: Streptococcus sp., S. pyogenes, S. mutans, S. thermophilus, Campylobacter sp., C. jejuni, Neisseria sp., N. meningitides, Francisella sp., F. novicida, and Pasteurella sp., P. multocida.

[014] In certain embodiments, introducing the functional Cas/guide RNA complex into the heterokaryon comprises introducing a DNA construct comprising an expression cassette for the Cas endonuclease into the fungal cells.

[015] In certain embodiments, introducing the functional Cas/guide RNA complex into the heterokaryon comprises introducing a DNA construct comprising an expression cassette for the guide RNA into the fungal cells.

[016] In certain embodiments, the introducing step comprises directly introducing the Cas endonuclease into the fungal cells.

[017] In certain embodiments, the introducing step comprises directly introducing the guide RNA into the fungal cells.

[018] In certain embodiments, the fungal cell is a filamentous fungal cell. In certain embodiments, the fungal cell is a Eumycotina or Pezizomycotina fungal cell. In certain embodiments, the fungal cell is selected from the group consisting of Trichoderma, Penicillium, Aspergillus, Humicola, Chrysosporium, Fusarium, Myceliophthora, Neurospora, Hypocrea, and Emericella. In certain embodiments, the fungal cell is a Trichoderma sp. cell. In certain embodiments, the fungal cell is a Trichoderma sp., e.g., Trichoderma reesei.

[019] In some embodiments, the donor DNA has partially integrated into the genome at the genomic locus of the fungal cell. In some embodiments, the donor DNA has completely integrated into the genome at the genomic locus of the fungal cell.

[020] In certain embodiments, integration of the donor DNA results in a modification of the genomic locus. In specific embodiments, the modification is selected from the group consisting of a deletion of one or more nucleotides, an insertion of one or more nucleotides, insertion of an expression cassette encoding a protein of interest, a substitution of one or more nucleotides, and any combination thereof.

[021] In certain embodiments, the identifying step comprises culturing cells grown from the spores from step (c) under conditions to select for or screen for the integration of the donor DNA at the genomic locus or the modification of the genomic locus.

[022] Aspects of the present disclosure are drawn to recombinant fungal cells produced by the methods described above as well as those for use as parental host cells in performing the methods.

[023] Additional embodiments of the methods and compositions of the present disclosure are shown herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[024] The disclosure can be more fully understood from the following detailed description and the accompanying drawings, which form a part of this application.

[025] **FIG. 1**. Schematic presentation of the strategy to use a helper strain with silenced NHEJ to improve homologous integration of targeted DNA cassettes. NHEJsil = Non-Homologous End Joining silencing cassette, GR = Gene Replacement sequence, amdS = gene encoding acetamidase from Aspergillus nidulans, his 3 = multidomain structural gene encoding histidinol dehydrogenase (EC 1.1.1.23), phosphoribosyl-ATP-pyrophosphohydrolase (EC 3.6.1.31) from T. reesei, and phosphoribosyl-AMP cyclohydrolase (EC 3.5.4.19), hph = gene encoding hygromycin phosphotransferase gene from E. coli, pyr2 = gene encoding orotidine 5'-monophosphate pyrophosphorylase.

[026] **FIGS. 2A-2C**. Illustrations of Ku80 silencing constructs: pAVTrku80sil (FIG. 2A), pAVTrku70ku80sil (FIG. 2B), and pAVTrku70lig4ku80sil (FIG. 2C).

[027] **FIG. 3**. Illustration of a his3 deletion construct pAVdelta-his3. hph = hygromycin resistance gene encoding hygromycin phosphotransferase from E. coli, kan(R) = kanamycin resistance gene encoding phosphotransferase.

[028] **FIG. 4**. Gene replacement (GR) construct pAV GR pyr2. pyr2 = gene encoding orotidine 5'-monophosphate pyrophosphorylase, Apr = ampicillin resistance gene encoding β-lactamase, F1 ORI = F1 origin of replication, URA3 = Saccharomyces cerevisiae gene encoding orotidine 5'-phosphate decarboxylase, 2 MICRON = S. cerevisiae 2-micron plasmid-originating sequence.

[029] **FIG. 5**. Heterokaryon transformation where multiple strains are simultaneously transformed with a construct.

[030] **FIG. 6**. Telomeric vector containing the *cre* recombinase gene (Expression Clone/pTrex-Tel-pyrG13/pDONR221/0927853cre).

[031] **FIG. 7**. Use of helper strain for complementing colonial growth and determining allele dominance.

[032] Abbreviations in FIGS. 5 and 7:

his 3 = multidomain structural gene encoding histidinol dehydrogenase (EC 1.1.1.23), phosphoribosyl-ATP-pyrophosphohydrolase (EC 3.6.1.31) from *T. reesei*, and phosphoribosyl-AMP cyclohydrolase (EC 3.5.4.19)

hph = gene encoding hygromycin phosphotransferase gene from *E. coli*,

ad3A = gene encoding phosphoribosylaminoimidazole-succinocarboxamide synthase (EC 6.3.2.6) from *T. reesei*.

pyr2 = orotidine 5'-monophosphate pyrophosphorylase gene

[033] **FIG. 8**. Illustration of a his3 deletion construct pAVdelta-his3.

DETAILED DESCRIPTION

[034] Compositions and methods are provided employing a helper strain system for promoting genetic alterations in a fungal host cell, e.g., a filamentous fungal host cell.

[035] Before the present compositions and methods are described in greater detail, it is to be understood that the present compositions and methods are not limited to particular embodiments described, and as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present compositions and methods will be limited only by the appended claims.

[036] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the present compositions and methods. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges and are also encompassed within the present compositions and methods, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the present compositions and methods.

[037] Certain ranges are presented herein with numerical values being preceded by the term "about." The term "about" is used herein to provide literal support for the exact number that it precedes, as well as a number that is near to or approximately the number that the term precedes. In determining whether a number is near to or approximately a specifically recited number, the near or approximating unrecited number may be a number which, in the context in which it is presented, provides the substantial equivalent of the specifically recited number. For example, in connection

with a numerical value, the term "about" refers to a range of -10% to +10% of the numerical value, unless the term is otherwise specifically defined in context. In another example, the phrase "a pH value of about 6" refers to pH values of from 5.4 to 6.6, unless the pH value is specifically defined otherwise.

[038] The headings provided herein are not limitations of the various aspects or embodiments of the present compositions and methods which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification as a whole.

[039] The present document is organized into a number of sections for ease of reading; however, the reader will appreciate that statements made in one section may apply to other sections. In this manner, the headings used for different sections of the disclosure should not be construed as limiting.

[040] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the present compositions and methods belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present compositions and methods, representative illustrative methods and materials are now described.

[041] All publications and patents cited in this specification are herein incorporated by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference and are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present compositions and methods are not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

[042] In accordance with this detailed description, the following abbreviations and definitions apply. Note that the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an enzyme" includes a plurality of such enzymes, and reference to "the

dosage" includes reference to one or more dosages and equivalents thereof known to those skilled in the art, and so forth.

[043] It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely," "only" and the like in connection with the recitation of claim elements, or use of a "negative" limitation.

[044] As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present compositions and methods described herein. Any recited method can be carried out in the order of events recited or in any other order which is logically possible.

Definitions

[045] The terms "functional fragment", "fragment that is functionally equivalent", "functionally equivalent fragment", and the like, are used interchangeably and refer to a portion or subsequence of a parent polypeptide that retains the qualitative enzymatic activity of the parent polypeptide. It is noted here that a functional fragment may have altered quantitative enzymatic activity as compared to the parent polypeptide.

[046] The terms "functional variant ", "variant that is functionally equivalent", "functionally equivalent variant", and the like are used interchangeably and refer to a variant of a parent polypeptide that retains the qualitative enzymatic activity of the parent polypeptide. It is noted here that a functional variant may have altered quantitative enzymatic activity as compared to the parent polypeptide.

[047] Fragments and variants can be obtained via any convenient method, including site-directed mutagenesis and synthetic construction.

[048] The term "genome" as it applies to a fungal cell cells encompasses not only chromosomal DNA found within the nucleus, but organelle DNA found within subcellular components (e.g., mitochondria) of the cell.

[049] A "codon-modified gene" or "codon-preferred gene" or "codon-optimized gene" is a gene having its frequency of codon usage designed to mimic the frequency of preferred codon usage of the host cell. The nucleic acid changes made to codon-optimize a gene are "synonymous", meaning that they do not alter the amino acid sequence of the encoded polypeptide of the parent gene. However, both native and variant genes can be codon-optimized for a particular host cell, and as such no limitation in this regard is intended.

[050] "Coding sequence" refers to a polynucleotide sequence which codes for a specific amino acid sequence. "Regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include, but are not limited to: promoters, translation leader sequences, 5' untranslated sequences, 3' untranslated sequences, introns, polyadenylation target sequences, RNA processing sites, effector binding sites, and stem-loop structures.

[051] "Promoter" refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. The promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. An "enhancer" is a DNA sequence that can stimulate promoter activity, and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, and/or comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of some variation may have identical promoter activity. As is well-known in the art, promoters can be categorized according to their strength and/or the conditions under which they are active, e.g., constitutive promoters, strong promoters, weak promoters, inducible/repressible promoters, tissue-specific/developmentally regulated promoters, cell-cycle dependent promoters, etc.

[052] "RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. "Messenger RNA" or "mRNA" refers to the RNA that is without introns and that can be translated into protein by the cell. "cDNA" refers to a DNA that is complementary to, and synthesized from, a mRNA template using the enzyme reverse transcriptase. "Sense" RNA refers to RNA transcript that includes the mRNA and can be translated into protein within a cell or in vitro. "Antisense RNA" refers to an RNA transcript that is complementary to all or part of a target primary transcript or mRNA, and that, under certain conditions, blocks the expression of a target gene (see, e.g., U.S. Patent No. 5,107,065). The complementarity of an antisense RNA may be with any part of the specific gene transcript, i.e., at the 5' non-coding sequence, 3' non-coding sequence, introns, or the coding sequence. "Functional RNA" refers to antisense RNA, ribozyme RNA, or other RNA that may not be translated into a polypeptide but yet has an effect on cellular processes. The terms "complement" and "reverse complement" are used interchangeably herein with respect to mRNA transcripts, and are meant to define the antisense RNA of the message.

[053] As used herein, "functionally attached" or "operably linked" means that a regulatory region or functional domain of a polypeptide or polynucleotide sequence having a known or desired activity, such as a promoter, enhancer region, terminator, signal sequence, epitope tag, etc., is attached to or linked to a target (e.g., a gene or polypeptide) in such a manner as to allow the regulatory region or functional domain to control the expression, secretion or function of that target according to its known or desired activity. For example, a promoter is operably linked with a coding sequence when it is capable of regulating the expression of that coding sequence (i.e., the coding sequence is under the transcriptional control of the promoter).

[054] The term "recombinant," when used in reference to a biological component or composition (e.g., a cell, nucleic acid, polypeptide/enzyme, vector, etc.) indicates that the biological component or composition is in a state that is not found in nature. In other words, the biological component or composition has been modified by human intervention from its natural state. For example, a recombinant cell encompass a cell that expresses one or more genes that are not found in its native parent (i.e., non-recombinant) cell, a cell that expresses one or more native genes in an amount that is different than its native parent cell, and/or a cell that expresses one or more native genes under different conditions than its native parent cell.

Recombinant nucleic acids may differ from a native sequence by one or more nucleotides, be operably linked to heterologous sequences (e.g., a heterologous promoter, a sequence encoding a non-native or variant signal sequence, etc.), be devoid of intronic sequences, and/or be in an isolated form. Recombinant polypeptides/enzymes may differ from a native sequence by one or more amino acids, may be fused with heterologous sequences, may be truncated or have internal deletions of amino acids, may be expressed in a manner not found in a native cell (e.g., from a recombinant cell that over-expresses the polypeptide due to the presence in the cell of an expression vector encoding the polypeptide), and/or be in an isolated form. It is emphasized that in some embodiments, a recombinant polynucleotide or polypeptide/enzyme has a sequence that is identical to its wild-type counterpart but is in a non-native form (e.g., in an isolated or enriched form).

[055] The terms "plasmid", "vector" and "cassette" refer to an extra chromosomal element that carries a polynucleotide sequence of interest, e.g., a gene of interest to be expressed in a cell (an "expression vector" or "expression cassette"). Such elements are generally in the form of double-stranded DNA and may be autonomously replicating sequences, genome integrating sequences, phage, or nucleotide sequences, in linear or circular form, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a polynucleotide of interest into a cell. The polynucleotide sequence of interest may be a gene encoding a polypeptide or functional RNA that is to be expressed in the target cell. Expression cassettes/vectors generally contain a gene with operably linked elements that allow for expression of that gene in a host cell.

[056] As used herein, a polypeptide referred to as a "Cas endonuclease" or having "Cas endonuclease activity" relates to a CRISPR associated (Cas) polypeptide encoded by a Cas gene where the Cas protein is capable of cutting a target DNA sequence when functionally coupled with one or more guide polynucleotides (see, e.g., US Patent 8697359 entitled "CRISPR-Cas systems and methods for altering expression of gene products"). Variants of Cas endonucleases that retain guide polynucleotide directed endonuclease activity are also included in this definition, including Cas variants that have nicking endonuclease activity, i.e., they introduce single strand nick at a double-stranded DNA target site (see definition below). (It is noted that wild-type Cas endonucleases identified to date introduce double-strand

breaks at the target site.) A Cas endonuclease is guided by the guide polynucleotide to recognize and cleave a specific target site in double stranded DNA, e.g., at a target site in the genome of a cell. Several different types of CRISPR-Cas systems have been described and can be classified as Type I, Type II, and Type III CRISPR-Cas systems (see, e.g., the description in Liu and Fan, CRISPR-Cas system: a powerful tool for genome editing. Plant Mol Biol (2014) 85:209-218). In certain aspects, the CRISPR-Cas system is a Type II CRISPR-Cas system employing a Cas9 endonuclease or variant thereof (including, e.g., a Cas nickase). The Cas9 endonuclease may be any convenient Cas9 endonuclease, including but not limited to Cas9 endonucleases, and functional fragments thereof, from the following bacterial species: Streptococcus sp. (e.g., S. pyogenes, S. mutans, and S. thermophilus), Campylobacter sp. (e.g., C. jejuni), Neisseria sp. (e.g., N. meningitides), Francisella sp. (e.g., F. novicida), and Pasteurella sp. (e.g., P. multocida). Numerous other species of Cas9 can be used. For example, functional Cas9 endonucleases or variants thereof containing an amino acid sequence that has at least 70% identity to any one of SEQ ID NOs:2, 8, and 11-16 may be employed, e.g., at least 80% identity, at least 90% identity, at least 95% identity, at least 96% identity, at least 97% identity, at least 98% identity, at least 99% identity, and including up to 100% identity to any one of SEQ ID NOs: 2, 8, and 11-16. In other embodiments, the Cas endonuclease or variant thereof is a Cpf1 endonuclease of the Type II CRISPR-Cas system. Cpf1 mediates robust DNA interference with features distinct from Cas9. Cpf1 lacks tracrRNA and utilizes a T-rich protospacer-adjacent motif. It cleaves DNA via a staggered DNA double-stranded break. See, e.g., Zetsche etal., Cell (2015) 163:759–771.

[057] As used herein, a “Cas nickase” is a Cas endonuclease that, when functionally coupled with one or more guide polynucleotides, is capable of introducing a single-strand nick into a target double stranded DNA sequence. Cas nickases can be generated recombinantly by inactivating one of the two nuclease domains in a parent Cas endonuclease (e.g., by site-directed mutagenesis). One non-limiting example of a Cas nickase is the Cas9 nickase described in Sander and Joung (Nature Biotechnology, 2013, 1-9) in which the RuvC domain is inactivated by a D10A mutation. As mentioned above, the general term “Cas endonuclease” encompasses both double-strand cutting and nicking Cas polypeptides. For example, if a guide RNA is described as being capable of directing a Cas

endonuclease to a desired target site, it would do so for both a double-strand cutting Cas endonuclease and a nicking Cas polypeptide (as defined below).

[058] As used herein, the term "guide polynucleotide" relates to a polynucleotide sequence that can form a complex with a Cas endonuclease and enables the Cas endonuclease to recognize and cleave a DNA target site. The guide polynucleotide can be a single molecule or a double molecule. The guide polynucleotide sequence can be a RNA sequence, a DNA sequence, or a combination thereof (a RNA-DNA combination sequence). A guide polynucleotide that solely comprises ribonucleic acids is also referred to as a "guide RNA".

[059] The guide polynucleotide can be a double molecule (also referred to as duplex guide polynucleotide) comprising a first nucleotide sequence domain (referred to as Variable Targeting domain or VT domain) that is complementary to a nucleotide sequence in a target DNA (also called the "protospacer" or "target site" below) and a second nucleotide sequence domain (referred to as Cas endonuclease recognition domain or CER domain) that interacts with a Cas endonuclease polypeptide. The CER domain of the double molecule guide polynucleotide comprises two separate molecules that are hybridized along a region of complementarity. The two separate molecules can be RNA, DNA, and/or RNA-DNA- combination sequences. In some embodiments, the first molecule of the duplex guide polynucleotide comprising a VT domain linked to a CER domain is referred to as "crDNA" (when composed of a contiguous stretch of DNA nucleotides) or "crRNA" (when composed of a contiguous stretch of RNA nucleotides), or "crDNA-RNA" (when composed of a combination of DNA and RNA nucleotides). The crNucleotide can comprise a fragment of the crRNA naturally occurring in Bacteria and Archaea. In one embodiment, the size of the fragment of the crRNA naturally occurring in Bacteria and Archaea that is present in a crNucleotide disclosed herein can range from, but is not limited to, 2, 3, 4, 5, 6, 7, 8, 9,10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more nucleotides. In some embodiments the second molecule of the duplex guide polynucleotide comprising a CER domain is referred to as "tracrRNA" (when composed of a contiguous stretch of RNA nucleotides) or "tracrDNA" (when composed of a contiguous stretch of DNA nucleotides) or "tracrDNA-RNA" (when composed of a combination of DNA and RNA nucleotides). In certain embodiments, the RNA that guides the RNA/Cas9 endonuclease complex is a duplexed RNA comprising a duplex crRNA-tracrRNA.

[060] The guide polynucleotide can also be a single molecule comprising a first nucleotide sequence domain (referred to as Variable Targeting domain or VT domain) that is complementary to a nucleotide sequence in a target DNA and a second nucleotide domain (referred to as Cas endonuclease recognition domain or CER domain) that interacts with a Cas endonuclease polypeptide. By "domain" it is meant a contiguous stretch of nucleotides that can be RNA, DNA, and/or RNA-DNA-combination sequence. The VT domain and / or the CER domain of a single guide polynucleotide can comprise a RNA sequence, a DNA sequence, or a RNA-DNA-combination sequence. In some embodiments the single guide polynucleotide comprises a crNucleotide (comprising a VT domain linked to a CER domain) linked to a tracrNucleotide (comprising a CER domain), wherein the linkage is a nucleotide sequence comprising a RNA sequence, a DNA sequence, or a RNA-DNA combination sequence. The single guide polynucleotide being comprised of sequences from the crNucleotide and tracrNucleotide may be referred to as "single guide RNA" (when composed of a contiguous stretch of RNA nucleotides) or "single guide DNA" (when composed of a contiguous stretch of DNA nucleotides) or "single guide RNA-DNA" (when composed of a combination of RNA and DNA nucleotides). In one embodiment of the disclosure, the single guide RNA comprises a crRNA or crRNA fragment and a tracrRNA or tracrRNA fragment of the type II CRISPR/Cas system that can form a complex with a type II Cas endonuclease, wherein the guide RNA/Cas endonuclease complex can direct the Cas endonuclease to a fungal cell genomic target site, enabling the Cas endonuclease to introduce a double strand break into the genomic target site.

[061] One aspect of using a single guide polynucleotide versus a duplex guide polynucleotide is that only one expression cassette needs to be made to express the single guide polynucleotide in a target cell.

[062] The term "variable targeting domain" or "VT domain" is used interchangeably herein and includes a nucleotide sequence that is complementary to one strand (nucleotide sequence) of a double strand DNA target site. The % complementation between the first nucleotide sequence domain (VT domain) and the target sequence is at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 63%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or is

100% complementary. The VT domain can be at least 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 nucleotides in length. In some embodiments, the VT domain comprises a contiguous stretch of 12 to 30 nucleotides. The VT domain can be composed of a DNA sequence, a RNA sequence, a modified DNA sequence, a modified RNA sequence, or any combination thereof.

[063] The term "Cas endonuclease recognition domain" or "CER domain" of a guide polynucleotide is used interchangeably herein and includes a nucleotide sequence (such as a second nucleotide sequence domain of a guide polynucleotide), that interacts with a Cas endonuclease polypeptide. The CER domain can be composed of a DNA sequence, a RNA sequence, a modified DNA sequence, a modified RNA sequence (see for example modifications described herein), or any combination thereof.

[064] The nucleotide sequence linking the crNucleotide and the tracrNucleotide of a single guide polynucleotide can comprise a RNA sequence, a DNA sequence, or a RNA-DNA combination sequence. In one embodiment, the nucleotide sequence linking the crNucleotide and the tracrNucleotide of a single guide polynucleotide can be at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 nucleotides in length. In another embodiment, the nucleotide sequence linking the crNucleotide and the tracrNucleotide of a single guide polynucleotide can comprise a tetraloop sequence, such as, but not limiting to a GAAA tetraloop sequence.

[065] As used herein, the tem "guide polynucleotide/Cas endonuclease system" (and equivalents) includes a complex of a Cas endonuclease and a guide polynucleotide (single or double) that is capable of introducing a double strand break at a DNA target site. The Cas endonuclease unwinds the DNA duplex in close proximity of the DNA target site and cleaves both DNA strands upon recognition of a target sequence by a guide RNA , but only if the correct protospacer-adjacent motif (PAM) is appropriately oriented at the 3' end of the target sequence.

[066] The term "expression", as used herein, refers to the production of a functional end-product (e.g., an mRNA, guide RNA, or a protein) in either precursor or mature form.

[067] "Introduced" in the context of inserting a polynucleotide or polypeptide into a cell (e.g., a recombinant DNA construct/expression construct) refers to any method for performing such a task, and includes any means of "transfection", "transformation", "transduction", physical means, or the like, to achieve introduction of the desired biomolecule.

[068] "Mature" protein refers to a post-translationally processed polypeptide (i.e., one from which any pre- or propeptides present in the primary translation product have been removed). "Precursor" protein refers to the primary product of translation of mRNA (i.e., with pre- and propeptides still present). Pre- and propeptides may be but are not limited to intracellular localization signals.

[069] "Stable transformation" refers to the transfer of a nucleic acid fragment into a genome of a host organism, including both nuclear and organellar genomes, resulting in genetically stable inheritance. In contrast, "transient transformation" refers to the transfer of a nucleic acid fragment into the nucleus, or other DNA-containing organelle, of a host organism resulting in gene expression without integration or stable inheritance (sometimes referred to herein as "unstable transformation"). Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" organisms.

[070] "Fungal cell", "fungi", "fungal host cell", and the like, as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota (as defined by Hawksworth et al., In, Ainsworth and Bisby's Dictionary of The Fungi, 8th edition, 1995, CAB International, University Press, Cambridge, UK) as well as the Oomycota (as cited in Hawksworth et al., supra) and all mitosporic fungi (Hawksworth et al., supra). In certain embodiments, the fungal host cell is a yeast cell, where by "yeast" is meant ascosporogenous yeast (Endomycetales), basidiosporogenous yeast, and yeast belonging to the Fungi Imperfecti (Blastomycetes). As such, a yeast host cell includes a Candida, Hansenula, Kluyveromyces, Pichia, Saccharomyces, Schizosaccharomyces, or Yarrowia cell. Species of yeast include, but are not limited to, the following: Saccharomyces carlsbergensis, Saccharomyces cerevisiae, Saccharomyces diastaticus,

Saccharomyces douglasii, Saccharomyces kluyveri, Saccharomyces norbensis, Saccharomyces oviformis, Kluyveromyces lactis, and Yarrowia lipolytica cell.

[071] The term "filamentous fungal cell" includes all filamentous forms of the subdivision Eumycotina. Suitable cells of filamentous fungal genera include, but are not limited to, cells of Acremonium, Aspergillus, Aureobasidium, Bjerkandera, Ceriporiopsis, Chrysosporium, Coprinus, Coriolus, Corynascus, Chaertomium, Cryptococcus, Filobasidium, Fusarium, Gibberella, Humicola, Magnaporthe, Mucor, Myceliophthora, Mucor, Neocallimastix, Neurospora, Paecilomyces, Penicillium, Phanerochaete, Phlebia, Piromyces, Pleurotus, Scytaldium, Schizophyllum, Sporotrichum, Talaromyces, Thermoascus, Thielavia, Tolypocladium, Trametes, and Trichoderma. Filamentous fungal species include, but are not limited to, cells of Aspergillus awamori, Aspergillus fumigatus, Aspergillus foetidus, Aspergillus japonicus, Aspergillus nidulans, Aspergillus niger, Aspergillus oryzae, Chrysosporium lucknowense, 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, Bjerkandera adusta, Ceriporiopsis aneirina, Ceriporiopsis aneirina, Ceriporiopsis caregiea, Ceriporiopsis gilvescens, Ceriporiopsis pannocinta, Ceriporiopsis rivulosa, Ceriporiopsis subrufa, Ceriporiopsis subvermispora, Coprinus cinereus, Coriolus hirsutus, Humicola insolens, Humicola lanuginosa, Mucor miehei, Myceliophthora thermophila, Neurospora crassa, Neurospora intermedia, Penicillium purpurogenum, Penicillium canescens, Penicillium solitum, Penicillium funiculosum Phanerochaete chrysosporium, Phlebia radiate, Pleurotus eryngii, Talaromyces flavus, Thielavia terrestris, Trametes villosa, Trametes versicolor, Trichoderma harzianum, Trichoderma koningii, Trichoderma longibrachiatum, Trichoderma reesei, and Trichoderma viride.

[072] When used in the context of a Cas/guide polynucleotide system, the terms "target site", "target sequence", "genomic target site", "genomic target sequence" (and equivalents) are used interchangeably herein and refer to a polynucleotide sequence in the genome of a fungal cell at which a Cas endonuclease cleavage is desired. The context in which this term is used, however, can slightly alter its

meaning. For example, the target site for a Cas endonuclease is generally very specific and can often be defined to the exact nucleotide sequence/position, whereas in some cases the target site for a desired genome modification can be defined more broadly than merely the site at which DNA cleavage occurs, e.g., a genomic locus or region where homologous recombination is desired. Thus, in certain cases, the genome modification that occurs via the activity of Cas/guide RNA DNA cleavage is described as occurring "at or near" the target site. The target site can be an endogenous site in the fungal cell genome, or alternatively, the target site can be heterologous to the fungal cell and thereby not be naturally occurring in the genome, or the target site can be found in a heterologous genomic location compared to where it occurs in nature.

[073] As used herein, "nucleic acid" means a polynucleotide and includes a single or a double-stranded polymer of deoxyribonucleotide or ribonucleotide bases. Nucleic acids may also include fragments and modified nucleotides. Thus, the terms "polynucleotide", "nucleic acid sequence", "nucleotide sequence" and "nucleic acid fragment" are used interchangeably to denote a polymer of RNA and/or DNA that is single- or double-stranded, optionally containing synthetic, non-natural, or altered nucleotide bases. Nucleotides (usually found in their 5'-monophosphate form) are referred to by their single letter designation as follows: "A" for adenosine or deoxyadenosine (for RNA or DNA, respectively), "C" for cytosine or deoxycytosine, "G" for guanosine or deoxyguanosine, "U" for uridine, "T" for deoxythymidine, "R" for purines (A or G), "Y" for pyrimidines (C or T), "K" for G or T, "H" for A or C or T, "I" for inosine, and "N" for any nucleotide.

[074] The term "derived from" encompasses the terms "originated from," "obtained from," "obtainable from," "isolated from," and "created from," and generally indicates that one specified material find its origin in another specified material or has features that can be described with reference to the another specified material.

[075] As used herein, the term "hybridization conditions" refers to the conditions under which hybridization reactions are conducted. These conditions are typically classified by degree of "stringency" of the conditions under which hybridization is measured. The degree of stringency can be based, for example, on the melting temperature (Tm) of the nucleic acid binding complex or probe. For example, "maximum stringency" typically occurs at about Tm - 5°C (5°C below the Tm of the probe); "high stringency" at about 5-10°C below the Tm; "intermediate stringency" at

about 10-20°C below the Tm of the probe; and "low stringency" at about 20-25°C below the Tm. Alternatively, or in addition, hybridization conditions can be based upon the salt or ionic strength conditions of hybridization, and/or upon one or more stringency washes, e.g.: 6X SSC = very low stringency; 3X SSC = low to medium stringency; 1X SSC = medium stringency; and 0.5X SSC = high stringency. Functionally, maximum stringency conditions may be used to identify nucleic acid sequences having strict identity or near-strict identity with the hybridization probe; while high stringency conditions are used to identify nucleic acid sequences having about 80% or more sequence identity with the probe. For applications requiring high selectivity, it is typically desirable to use relatively stringent conditions to form the hybrids (e.g., relatively low salt and/or high temperature conditions are used).

[076] As used herein, the term "hybridization" refers to the process by which a strand of nucleic acid joins with a complementary strand through base pairing, as known in the art. More specifically, "hybridization" refers to the process by which one strand of nucleic acid forms a duplex with, i.e., base pairs with, a complementary strand, as occurs during blot hybridization techniques and PCR techniques. A nucleic acid sequence is considered to be "selectively hybridizable" to a reference nucleic acid sequence if the two sequences specifically hybridize to one another under moderate to high stringency hybridization and wash conditions. Hybridization conditions are based on the melting temperature (Tm) of the nucleic acid binding complex or probe. For example, "maximum stringency" typically occurs at about Tm -5°C (5° below the Tm of the probe); "high stringency" at about 5-10°C below the Tm; "intermediate stringency" at about 10-20°C below the Tm of the probe; and "low stringency" at about 20-25°C below the Tm. Functionally, maximum stringency conditions may be used to identify sequences having strict identity or near-strict identity with the hybridization probe; while intermediate or low stringency hybridization can be used to identify or detect polynucleotide sequence homologs.

[077] Intermediate and high stringency hybridization conditions are well known in the art. For example, intermediate stringency hybridizations may be carried out with an overnight incubation at 37°C in a solution comprising 20% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 x Denhardt's solution, 10% dextran sulfate and 20 mg/mL denatured sheared salmon sperm DNA, followed by washing the filters in 1x SSC at about 37 - 50°C. High stringency hybridization conditions may be hybridization at 65°C and 0.1X SSC

(where 1X SSC = 0.15 M NaCl, 0.015 M Na citrate, pH 7.0). Alternatively, high stringency hybridization conditions can be carried out at about 42°C in 50% formamide, 5X SSC, 5X Denhardt's solution, 0.5% SDS and 100 μg/mL denatured carrier DNA followed by washing two times in 2X SSC and 0.5% SDS at room temperature and two additional times in 0.1X SSC and 0.5% SDS at 42°C. And very high stringent hybridization conditions may be hybridization at 68°C and 0.1X SSC. Those of skill in the art know how to adjust the temperature, ionic strength, etc. as necessary to accommodate factors such as probe length and the like.

[078] The phrase "substantially similar" or "substantially identical," in the context of at least two nucleic acids or polypeptides, means that a polynucleotide or polypeptide comprises a sequence that has 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 even at least 99% identical to a parent or reference sequence, or does not include amino acid substitutions, insertions, deletions, or modifications made only to circumvent the present description without adding functionality.

[079] "Sequence identity" or "identity" in the context of nucleic acid or polypeptide sequences refers to the nucleic acid bases or amino acid residues in two sequences that are the same when aligned for maximum correspondence over a specified comparison window.

[080] The term "percentage of sequence identity" refers to the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the results by 100 to yield the percentage of sequence identity. Useful examples of percent sequence identities include, but are not limited to, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95%, or any integer percentage from 50% to 100%. These identities can be determined using any of the programs described herein.

[081] Sequence alignments and percent identity or similarity calculations may be determined using a variety of comparison methods designed to detect homologous sequences including, but not limited to, the MegAlign™ program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Within the context of this application it will be understood that where sequence analysis software is used for analysis, that the results of the analysis will be based on the "default values" of the program referenced, unless otherwise specified. As used herein "default values" will mean any set of values or parameters that originally load with the software when first initialized.

[082] The "Clustal V method of alignment" corresponds to the alignment method labeled Clustal V (described by Higgins and Sharp, (1989) CABIOS 5:151-153; Higgins et al., (1992) Comput Appl Biosci 8:189-191) and found in the MegAlign™ program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). For multiple alignments, the default values correspond to GAP PENALTY=10 and GAP LENGTH PENALTY=10. Default parameters for pairwise alignments and calculation of percent identity of protein sequences using the Clustal method are KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. For nucleic acids these parameters are KTUPLE=2, GAP PENALTY=5, WINDOW=4 and DIAGONALS SAVED=4. After alignment of the sequences using the Clustal V program, it is possible to obtain a "percent identity" by viewing the "sequence distances" table in the same program.

[083] The "Clustal W method of alignment" corresponds to the alignment method labeled Clustal W (described by Higgins and Sharp, (1989) CABIOS 5:151-153; Higgins et al., (1992) Comput Appl Biosci 8:189-191) and found in the MegAlign™ v6.1 program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Default parameters for multiple alignment (GAP PENALTY=10, GAP LENGTH PENALTY=0.2, Delay Divergen Seqs (%)=30, DNA Transition Weight=0.5, Protein Weight Matrix=Gonnet Series, DNA Weight Matrix=IUB). After alignment of the sequences using the Clustal W program, it is possible to obtain a "percent identity" by viewing the "sequence distances" table in the same program.

[084] Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using GAP Version 10 (GCG, Accelrys, San Diego, CA) using the following parameters: % identity and % similarity for a nucleotide

sequence using a gap creation penalty weight of 50 and a gap length extension penalty weight of 3, and the nwsgapdna.cmp scoring matrix; % identity and % similarity for an amino acid sequence using a GAP creation penalty weight of 8 and a gap length extension penalty of 2, and the BLOSUM62 scoring matrix (Henikoff and Henikoff, (1989) *Proc. Natl. Acad. Sci. USA* 89:10915). GAP uses the algorithm of Needleman and Wunsch, (1970) *J Mol Biol* 48:443-53, to find an alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap positions and creates the alignment with the largest number of matched bases and the fewest gaps, using a gap creation penalty and a gap extension penalty in units of matched bases.

[085] It is well understood by one skilled in the art that many levels of sequence identity are useful in identifying polypeptides from other species or modified naturally or synthetically wherein such polypeptides have the same or similar function or activity. Useful examples of percent identities include, but are not limited to, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95%, or any integer percentage from 50% to 100%. Indeed, any integer amino acid identity from 50% to 100% may be useful in describing the present disclosure, such as 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%.

[086] "Gene" includes a nucleic acid fragment that encodes and is capable to express a functional molecule such as, but not limited to, a specific polypeptide (e.g., an enzyme) or a functional RNA molecule (e.g., a guide RNA, an anti-sense RNA, ribozyme, etc.), and includes regulatory sequences preceding (5' non-coding sequences) and/or following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. A recombinant gene refers to a gene that is regulated by a different gene's regulatory sequences which could be from a different organism or the same organism.

[087] A "mutated gene" is a gene that has been altered through human intervention. Such a "mutated gene" has a sequence that differs from the sequence of the corresponding non-mutated gene by at least one nucleotide addition, deletion,

or substitution. In certain embodiments of the disclosure, the mutated gene comprises an alteration that results from a guide polynucleotide/Cas endonuclease system as disclosed herein. A mutated fungal cell is a fungal cell comprising a mutated gene.

[088] As used herein, a "targeted mutation" is a mutation in a native gene that was made by altering a target sequence within the native gene using a method involving a double-strand-break-inducing agent that is capable of inducing a double-strand break in the DNA of the target sequence as disclosed herein or known in the art.

[089] The term "donor DNA" or "donor nucleic acid sequence" or "donor polynucleotide" refers to a polynucleotide that contains a polynucleotide sequence of interest that is to be inserted into a target cell genome. As such, the polynucleotide sequence of interest in the donor DNA may include a novel region to be inserted at or near a target site and/or a modified polynucleotide sequence when compared to the nucleotide sequence to be replaced/edited at or near a target site. In certain embodiments, the donor DNA construct further comprises a first and a second region of homology that flank the polynucleotide sequence of interest. The first and second regions of homology of the donor DNA share homology to a first and a second genomic region, respectively, present in the fungal cell genome. By "homology" is meant DNA sequences that are similar. For example, a "region of homology to a genomic region" that is found on the donor DNA is a region of DNA that has a similar sequence to a given "genomic region" in the fungal cell genome. A region of homology can be of any length that is sufficient to promote homologous recombination at the cleaved target site. For example, the region of homology can comprise at least 5-10, 5-15, 5-20, 5-25, 5-30, 5-35, 5-40, 5-45, 5- 50, 5-55, 5-60, 5-65, 5- 70, 5-75, 5-80, 5-85, 5-90, 5-95, 5-100, 5-200, 5-300, 5-400, 5-500, 5-600, 5-700, 5-800, 5-900, 5-1000, 5-1100, 5-1200, 5-1300, 5-1400, 5-1500, 5-1600, 5-1700, 5-1800, 5-1900, 5-2000, 5-2100, 5-2200, 5-2300, 5-2400, 5-2500, 5-2600, 5-2700, 5-2800. 5-2900, 5-3000, 5-3100 or more bases in length such that the region of homology has sufficient homology to undergo homologous recombination with the corresponding genomic region. "Sufficient homology" indicates that two polynucleotide sequences have sufficient structural similarity to act as substrates for a homologous recombination reaction. The structural similarity includes overall length of each polynucleotide fragment, as well as the sequence similarity of the polynucleotides. Sequence similarity can be described by the percent sequence

identity over the whole length of the sequences, and/or by conserved regions comprising localized similarities such as contiguous nucleotides having 100% sequence identity, and percent sequence identity over a portion of the length of the sequences.

[090] The amount of homology or sequence identity shared by a target and a donor polynucleotide can vary and includes total lengths and/or regions having unit integral values in the ranges of about 1-20 bp, 20-50 bp, 50-100 bp, 75-150 bp, 100-250 bp, 150-300 bp, 200-400 bp, 250-500 bp, 300-600 bp, 350-750 bp, 400-800 bp, 450-900 bp, 500-1000 bp, 600-1250 bp, 700-1500 bp, 800-1750 bp, 900-2000 bp, 1-2.5 kb, 1.5–3 kb, 2-4 kb, 2.5-5 kb, 3-6 kb, 3.5-7 kb, 4-8 kb, 5-10 kb, or up to and including the total length of the target site. These ranges include every integer within the range, for example, the range of 1-20 bp includes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 bp. The amount of homology can also described by percent sequence identity over the full aligned length of the two polynucleotides which includes percent sequence identity of about at least 50%, 55%, 60%, 65%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%. Sufficient homology includes any combination of polynucleotide length, global percent sequence identity, and optionally conserved regions of contiguous nucleotides or local percent sequence identity, for example sufficient homology can be described as a region of 75-150 bp having at least 80% sequence identity to a region of the target locus. Sufficient homology can also be described by the predicted ability of two polynucleotides to specifically hybridize under high stringency conditions, see, for example, Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, NY); Current Protocols in Molecular Biology, Ausubel et al., Eds (1994) Current Protocols, (Greene Publishing Associates, Inc. and John Wiley & Sons, Inc); and, Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes, (Elsevier, New York).

[091] As used herein, a “genomic region” is a segment of a chromosome in the genome of a fungal cell (e.g., a target cell). The genomic region can comprise at least 5-10, 5-15, 5-20, 5-25, 5-30, 5-35, 5-40, 5-45, 5- 50, 5-55, 5-60, 5-65, 5- 70, 5-75, 5-80, 5-85, 5-90, 5-95, 5-100, 5-200, 5-300, 5-400, 5-500, 5-600, 5-700, 5-800, 5-900, 5-1000, 5-1100, 5-1200, 5-1300, 5-1400, 5-1500, 5-1600, 5-1700, 5-1800, 5-

1900, 5-2000, 5-2100, 5-2200, 5-2300, 5-2400, 5-2500, 5-2600, 5-2700, 5-2800. 5-2900, 5-3000, 5-3100 or more bases such that the genomic region has sufficient homology to undergo homologous recombination with the corresponding region of homology. A genomic region can include the regions that surround a Cas/guide RNA target site in the genome.

[092] As used herein, "homologous recombination" includes the exchange of DNA fragments between two DNA molecules at the sites of homology and is well described in the art.

[093] A phenotypic marker is a screenable or selectable marker that includes visual markers and selectable markers whether it is a positive or negative selectable marker. Any phenotypic marker can be used. Specifically, a selectable or screenable marker comprises a DNA segment that allows one to identify, select for, or screen for or against a molecule or a cell that contains it, often under particular conditions. These markers can encode an activity, such as, but not limited to, production of RNA, peptide, or protein, or can provide a binding site for RNA, peptides, proteins, inorganic and organic compounds or compositions and the like.

[094] Examples of selectable markers include, but are not limited to, DNA segments that comprise restriction enzyme sites; DNA segments that encode products which provide resistance against otherwise toxic compounds and antibiotics, such as, chlorimuron ethyl, benomyl, Basta, and hygromycin phosphotransferase (HPT); DNA segments that encode products which are otherwise lacking in the recipient cell (e.g., tRNA genes, auxotrophic markers, dominant heterologous marker-amdS); DNA segments that encode products which can be readily identified (e.g., phenotypic markers such as β-galactosidase, GUS; fluorescent proteins such as green fluorescent protein (GFP), cyan (CFP), yellow (YFP), red (RFP), and cell surface proteins); the generation of new primer sites for PCR (e.g., the juxtaposition of two DNA sequence not previously juxtaposed), the inclusion of DNA sequences not acted upon or acted upon by a restriction endonuclease or other DNA modifying enzyme, chemical, etc.; and, the inclusion of a DNA sequences required for a specific modification (e.g., methylation) that allows its identification.

Methods and Compositions for Modifying a Fungal Cell Genome

[095] As summarized above, the present disclosure provides compositions and methods for employing a helper strain system for promoting genetic alterations in a fungal host cell, e.g., a filamentous fungal host cell.

[096] Before describing the aspects of the present disclosure, it is important to note that a multitude of cellular processes are executed or controlled in different ways between different fungal cell species, e.g., between T. reesei and N. crassa, e.g., the molecular processes that regulate senescence. In N. crassa, introduction of mutations into mus-10, an F-box protein that is a part of the ubiquitin ligase complex (Kato, et al., 2010, Genetics 185, 1257-1269), or fzo-1, a mitofuzin (Kurashima, et al., 2013, Eukaryot. Cell 12 (2), 233-243), cause senescence ending in cell death. When equivalent mutations are introduced into T. reesei mus-10 and fzo-1 homologues, cell death is not observed. As such, processes demonstrated in one species (e.g., N. crassa) cannot automatically be extrapolated to all others (e.g., T. reesei).

[097] T. reesei has two main modes by which it incorporates DNA fragments into its genome: homologous integration and ectopic integration. The non-homologous end-joining (NHEJ) machinery is important for ectopic integration, and if a component of this machinery is deleted, the rate of homologous integration increases significantly (Guangtao, et al., 2009, J. Biotechnol. 139, 146-151).

[098] Therefore, an alternative way to increase homologous recombination of DNA fragments in a target strain is to use a helper strain in which one or more NHEJ components are silenced (FIG. 1). First, a helper strain (HS) is produced containing an NHEJ gene silencing construct (with amdS selection marker; acetamidase) and a first nutritional marker (Δhis; strain lacks histidinol dehydrogenase). This strain is fused to a target strain (TS) containing a second, different nutritional marker (Δpyr2). For convenience and easier differentiation of target and helper strain, a recessive feature can be added to the target strain (such as altered colony morphology or spore color), but this addition is optional. On minimal medium the shared cytoplasm of the forced heterokaryon allows NHEJ silencing in both strains. This heterokaryon is then transformed with a DNA fragment containing sequences homologous to the target strain genomic DNA (GR) and a selection marker that complements the nutritional marker deficiency of the target strain (pyr2; the construct is indicated as

GR::pyr2). Transformants are plated on selective medium, and the predominantly uninucleate conidiospores are harvested. Conidiospores are plated on selective minimal medium without any supplements. Colonies derived from spores that have the target strain nuclei with the homologously integrated gene replacement (GR::pyr2) will grow on selective minimal medium because they are supplemented for the second nutritional marker deficiency (Δpyr). They will also have the recessive phenotype (such as altered colony morphology or spore color). The spores that only have the helper strain nuclei will not grow, since the minimal medium does not contain the first nutritional marker supplement (in this case, L-histidine). Colonies derived from heterokaryotic spores will grow on minimal medium, but their phenotype will be dominant (such as wild type colony morphology or wild type spore color), and they will be eliminated.

[099] By the use of a helper strain silenced for the NHEJ machinery, an increase in homologous recombination can be achieved without modifying the genome of the target strain, reducing the number of laborious and time-consuming steps required for strain development.

[0100] Aspects of the disclosure are drawn to compositions and methods for homologous recombination of a donor DNA with a genomic locus in a fungal cell, e.g., a filamentous fungal cell.

[0101] Therefore, aspect of the disclosure include methods for homologous recombination of a donor DNA with a genomic locus in a fungal cell, the method comprising: (a) generating a heterokaryon between a helper fungal strain and a target fungal strain, wherein the helper fungal strain comprises an expression construct that silences the non-homologous end joining (NHEJ) mechanism; (b) introducing a donor DNA into the heterokaryon, wherein the donor DNA comprises a region of homology to a genomic locus in the target strain sufficient for homologous recombination at the genomic locus; (c) generating and plating spores from the heterokaryon cells of (b); and (d) identifying cells from the plated spores in which (i) the donor DNA has integrated into the genome by homologous recombination at the genomic locus, and (ii) the expression construct that silences the non-homologous end joining (NHEJ) mechanism is not present.

[0102] In certain embodiments, the expression construct silences one or more of: ku80, ku70, rad50, mre11, xrs2, lig4, and xrs. In certain embodiments, the expression construct silences ku80, ku70, or both.

[0103] In certain embodiments, the Cas endonuclease is a Cas9 endonuclease or variant thereof. In certain embodiments, the Cas9 endonuclease or variant thereof comprises a full length Cas9 or a functional fragment thereof from a species selected from the group consisting of: Streptococcus sp., S. pyogenes, S. mutans, S. thermophilus, Campylobacter sp., C. jejuni, Neisseria sp., N. meningitides, Francisella sp., F. novicida, and Pasteurella sp., P. multocida.

[0104] In certain embodiments, introducing the functional Cas/guide RNA complex into the heterokaryon comprises introducing a DNA construct comprising an expression cassette for the Cas endonuclease into the fungal cells.

[0105] In certain embodiments, introducing the functional Cas/guide RNA complex into the heterokaryon comprises introducing a DNA construct comprising an expression cassette for the guide RNA into the fungal cells.

[0106] In certain embodiments, the introducing step comprises directly introducing the Cas endonuclease into the fungal cells.

[0107] In certain embodiments, the introducing step comprises directly introducing the guide RNA into the fungal cells.

[0108] In certain embodiments, the fungal cell is a filamentous fungal cell. In certain embodiments, the fungal cell is a Eumycotina or Pezizomycotina fungal cell. In certain embodiments, the fungal cell is selected from the group consisting of Trichoderma, Penicillium, Aspergillus, Humicola, Chrysosporium, Fusarium, Myceliophthora, Neurospora, Hypocrea, and Emericella. In certain embodiments, the fungal cell is a Trichoderma sp. cell. In certain embodiments, the fungal cell is a Trichoderma sp., e.g., Trichoderma reesei.

[0109] In some embodiments, the donor DNA has partially integrated into the genome at the genomic locus of the fungal cell. In some embodiments, the donor DNA has completely integrated into the genome at the genomic locus of the fungal cell. By “partially integrated”, it may include scenarios where a part of the donor DNA recombined with the genome of the fungal cell, e.g., 20-50 bp, 50-100 bp, 75-150 bp, 100-250 bp, 150-300 bp, 200-400 bp, 250-500 bp, 300-600 bp, 350-750 bp,

400-800 bp, 450-900 bp, 500-1000 bp, 600-1250 bp, 700-1500 bp, 800-1750 bp, 900-2000 bp, 1-2.5 kb, 1.5–3 kb, 2-4 kb, 2.5-5 kb, 3-6 kb, 3.5-7 kb, 4-8 kb, 5-10 kb, or more of the donor DNA got integrated into the genome of the fungal cell.

[0110] In certain embodiments, integration of the donor DNA results in a modification of the genomic locus. In specific embodiments, the modification is selected from the group consisting of a deletion of one or more nucleotides, an insertion of one or more nucleotides, insertion of an expression cassette encoding a protein of interest, a substitution of one or more nucleotides, and any combination thereof. In some embodiments, the modification is originally present in the donor DNA. In certain embodiments, the protein of interest encoded by the expression cassette is an enzyme. In particular embodiments, the protein of interest is a hemicellulase, a peroxidase, a protease, a cellulase, a xylanase, a lipase, a phospholipase, an esterase, a cutinase, a pectinase, a keratinase, a reductase, an oxidase, a phenol oxidase, a lipoxygenase, a ligninase, a pullulanase, a tannase, a pentosanase, a mannanase, a beta-glucanase, an arabinosidase, a hyaluronidase, a chondroitinase, a laccase, an amylase, a glucoamylase, a variant thereof, a functional fragment thereof, or a hybrid or mixture of two or more thereof. In yet other particular embodiments, the protein of interest is a peptide hormone, a growth factor, a clotting factor, a chemokine, a cytokine, a lymphokine, an antibody, a receptor, an adhesion molecule, a microbial antigen, a variant thereof, a functional fragment thereof, or a hybrid or mixture of two or more thereof.

[0111] In certain embodiments, the identifying step comprises culturing cells grown from the spores from step (c) under conditions to select for or screen for the integration of the donor DNA at the genomic locus or the modification of the genomic locus. Further detail for each of these aspects is provided below.

[0112] Introduction of the Cas endonuclease, guide polynucleotide, and/or the donor DNA can be done in any convenient manner, including transfection, transduction, transformation, electroporation, particle bombardment, cell fusion techniques, etc. Each of these components can be introduced simultaneously or sequentially as desired by the user. For example, a fungal cell can first be stably transfected with a Cas expression DNA construct followed by introduction of a guide polynucleotide into the stable transfectant (either directly or using a guide polynucleotide expressing DNA construct)with. This set up may even be advantageous as the user can generate a population of stable Cas transfectant fungal cells into which

different guide polynucleotides can be introduced independently (in some cases, more than one guide polynucleotide can be introduced into the same cells should this be desired). In some embodiments, a Cas expressing fungal cell is obtained by the user, and thus the user does not need to introduce a recombinant DNA construct capable of expressing a Cas endonuclease into the cell, but rather only need introduce a guide polynucleotide into the Cas expressing cell.

[0113] In certain embodiments, a guide polynucleotide is introduced into a fungal cell/heterokaryon by introducing a recombinant DNA construct that includes an expression cassette (or gene) encoding the guide polynucleotide. In some embodiments, the expression cassette is operably linked to a eukaryotic RNA pol III promoter. These promoters are of particular interest as transcription by RNA pol III does not lead to the addition of a 5' cap structure or polyadenylation that occurs upon transcription by RNA polymerase II from an RNA pol II dependent promoter. In certain embodiments, the RNA pol III promoter is a filamentous fungal cell U6 polymerase III promoter (e.g., SEQ ID NO:3 and functional variants thereof, e.g., SEQ ID NO:4; described in further detail below). In certain embodiments, the gene controlled by the U6 polymerase III promoter includes the U6 gene intron (SEQ ID NO:5) and/or the U6 gene transcriptional terminator (SEQ ID NO:6).

[0114] We have found in filamentous fungi that non-homologous insertion of transformed DNA at the double-strand break is highly favored over simple end-joining between the two ends of the chromosomal DNA at a double-strand break. Therefore, in cases where the Cas endonuclease or guide RNA is provided by transformation with an expression cassette containing DNA construct or constructs, those DNA constructs, or fragments thereof, are inserted at the double-strand break at high frequency. This insertion occurs in the absence of homology between DNA sequences on the Cas endonuclease or guide RNA expression constructs and the sequences around the double-strand break. This process is also problematic when homologous recombination between a donor DNA and a genomic locus is desired, as insertion of the entire donor DNA is favored over homologous recombination. We have found that undesirable insertion of transformed DNA occurs even when it is in the form of a vector including telomere sequences that is expected to be maintained autonomously in the fungal cell.

[0115] Certain embodiments of the present disclosure include integrating a Cas endonuclease expression cassette (and optionally a selectable marker) in the

genome of a helper strain to produce a Cas endonuclease expressing helper strain. These helper cells can be employed in numerous ways to obtain a genetic modification of interest in a target strain, e.g., including homologous recombination of a donor DNA with the genome of a target strain.

[0116] For example, a Cas endonuclease expressing host cell can be used to create a "helper strain" that can provide, in trans, the Cas endonuclease to a "target strain". In brief, a heterokaryon can be created between the helper strain and the target strain, e.g., by fusion of protoplasts from each strain or by anastomosis of hyphae depending on the species of filamentous fungus. Maintenance of the heterokaryon will depend on appropriate nutritional or other marker genes or mutations in each parental strain and growth on suitable selective medium such that the parental strains are unable to grow whereas the heterokaryon, due to complementation, is able to grow. Either at the time of heterokaryon formation or subsequently, a guide RNA is introduced by transfection (and optionally a donor DNA). The guide RNA may be directly introduced or introduced via a DNA construct having a Cas endonuclease expression cassette and a selectable marker gene. Cas endonuclease is expressed from the gene in the helper strain nucleus and is present in the cytoplasm of the heterokaryon. The Cas endonuclease associates with the guide RNA to create an active complex that is targeted to the desired target site(s) in the genome. Subsequently, spores are recovered from the heterokaryon and subjected to selection or screening to recover the target strain with a modification at or near the target site (e.g., homologous recombination with the donor DNA at a genomic locus). In cases in which an expression cassette is used to introduce the guide RNA, heterokaryons are chosen in which the guide RNA expression construct is not stably maintained.

[0117] As noted above, methods of the present disclosure include introducing a DNA construct into the cell (or donor DNA) that has DNA sequence homology with regions of the chromosomal DNA (e.g., on each side of the target site of the Cas/guide RNA complex). The intent is for the DNA fragment (e.g., a linear DNA fragment) to integrate by homologous integration/recombination into the target cell genome.

[0118] With respect to DNA repair in fungal cells, we have found that in the presence of a functioning NHEJ pathway, error-prone repair is highly favored over homologous recombination at a double strand break site. In other words, with

respect to DNA repair of a double strand break (e.g., those introduced at a target site by a Cas/guide RNA complex) in filamentous fungal cells, we have found that in the presence of a functioning NHEJ pathway, non-homologous insertion of DNA at the break is highly favored over (1) non-homologous end joining without DNA insertion and (2) homologous recombination at the double strand break site with a donor DNA. Therefore, in certain aspects of the present invention, the functioning of the non-homologous end joining (NHEJ) pathway at the target site in the fungal cell in the population is inhibited, not activated, non-functional, or reduced. This may be achieved in any convenient manner, some of which are described below.

[0119] In some instances, the donor DNA includes a first region and a second region that are homologous to corresponding first and second regions in the genome of the fungal cell. For example, the regions of homology can include or surround the target site at which the genomic DNA is cleaved by a Cas endonuclease. These regions of homology promote homologous recombination with their corresponding genomic regions of homology resulting in exchange of DNA between the donor DNA and the genome. As such, the provided methods result in the integration of the polynucleotide of interest of the donor DNA at a homologous region in the target cell genome of a heterokaryon, which, upon applying appropriate sporulation/selection criteria to the heterokaryon, generates an altered genome in the target cell.

[0120] The structural similarity between a given genomic region and the corresponding region of homology found on the donor DNA can be any degree of sequence identity that allows for homologous recombination to occur. For example, the amount of homology or sequence identity shared by the "region of homology" of the donor DNA and the "genomic region" of the fungal cell genome can be at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or even 100% sequence identity, such that the sequences undergo homologous recombination.

[0121] The donor DNA may be introduced by any convenient means (as discussed elsewhere herein).

[0122] In certain embodiments in which a Cas/guide RNA system is employed, the Cas endonuclease is a Cas9 endonuclease (see, e.g., WO 2013141680 entitled "RNA-directed DNA Cleavage by the Cas9-crRNA Complex"). Examples of Cas9 endonucleases include those from Streptococcus sp. (e.g., S. pyogenes, S. mutans,

and S. thermophilus), Campylobacter sp. (e.g., C. jejuni), Neisseria sp. (e.g., N. meningitides), Francisella sp. (e.g., F. novicida), and Pasteurella sp. (e.g., P. multocida) (e.g., SEQ ID NOs:8 and 11 to 16, functional fragments thereof, and sequences having at least 80% sequence identity to any one of these sequences that retain functional activity) (see, e.g., Cas9 endonucleases described in Fonfara et al., Nucleic Acids Res., 2013, pages 1-14: incorporated herein by reference). In some embodiments, the Cas endonuclease is encoded by an optimized Cas9 endonuclease gene, e.g., optimized for expression in a fungal cell (e.g., Cas9 encoding genes containing SEQ ID NO:7, e.g., SEQ ID NO:1, as described below).

[0123] In certain instances, the Cas endonuclease gene is operably linked to one or more polynucleotides encoding nuclear localization signals such that the Cas endonuclease/guide polynucleotide complex that is expressed in the cell is efficiently transported to the nucleus. Any convenient nuclear localization signal may be used, e.g., a polynucleotide encoding an SV40 nuclear localization signal present upstream of and in-frame with the Cas codon region and a polynucleotide encoding a nuclear localization signal derived from the T. reesei blr2 (blue light regulator 2) gene present downstream and in frame with the Cas codon region. Other nuclear localization signals can be employed. For example, the Cas endonuclease can be operably linked to one or more nuclear targeting signal (also referred to as a nuclear localization signal/sequence; NLS). SEQ ID NO:1 and SEQ ID NO:2 provide an example of a filamentous fungal cell optimized Cas9 gene with NLS sequences at the N- and C-termini and the encoded amino acid sequence, respectively. Many different NLSs are known in eukaryotes. They include monopartite, bipartite and tripartite types. Any convenient NLS can be used, the monopartite type being somewhat more convenient with examples including the SV40 NLS, a NLS derived from the T. reesei blr2 (blue light regulator 2) gene, or a combination of both.

[0124] In certain embodiments of the disclosure, the guide polynucleotide is a guide RNA that includes a crRNA region (or crRNA fragment) and a tracrRNA region (or tracrRNA fragment) of the type II CRISPR/Cas system that can form a complex with a type II Cas endonuclease. As indicated above, the guide RNA/Cas endonuclease complex can direct the Cas endonuclease to a fungal cell genomic target site, enabling the Cas endonuclease to introduce a double strand break into the genomic target site. In some cases, the RNA that guides the RNA/ Cas9 endonuclease

complex is a duplex that includes a crRNA and a separate tracrRNA. In other instances, the guide RNA is a single RNA molecule that includes both a crRNA region and a tracrRNA region (sometimes referred to herein as a fused guide RNA). One advantage of using a fused guide RNA versus a duplexed crRNA-tracrRNA is that only one expression cassette needs to be made to express the fused guide RNA.

[0125] Virtually any site in a fungal cell genome may be targeted by a Cas endonuclease using the disclosed methods, so long as the target site includes the required protospacer adjacent motif (PAM). In the case of the S. pyogenes Cas9, the PAM has the sequence NGG (5' to 3'; where N is A, G, C or T), and thus does not impose significant restrictions on the selection of a target site in the genome. Other known Cas9 endonucleases have different PAM sites (see, e.g., Cas9 endonuclease PAM sites described in Fonfara et al., Nucleic Acids Res., 2013, pages 1-14: incorporated herein by reference).

[0126] The length of the target site can vary, and includes, for example, target sites that are at least 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more nucleotides in length. It is further possible that the target site can be palindromic, that is, the sequence on one strand reads the same in the opposite direction on the complementary strand. The cleavage site can be within the target sequence or the cleavage site could be outside of the target sequence. In another variation, the cleavage could occur at nucleotide positions immediately opposite each other to produce a blunt end cut or, in other cases, the incisions could be staggered to produce single-stranded overhangs, also called "sticky ends", which can be either 5' overhangs, or 3' overhangs.

[0127] In some cases, active variant target sequences in the genome of the fungal cell can also be used, meaning that the target site is not 100% identical to the relevant sequence in the guide polynucleotide (within the crRNA sequence of the guide polynucleotide). Such active variants can comprise at least 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the given target site, wherein the active variant target sequences retain biological activity and hence are capable of being recognized and cleaved by a Cas endonuclease. Assays to measure the double-strand break of a target site by an endonuclease are known in the art and generally measure the

overall activity and specificity of the agent on DNA substrates containing recognition sites.

[0128] Target sites of interest include those located within a region of a gene of interest. Non-limiting examples of regions within a gene of interest include an open reading frame, a promoter, a transcriptional regulatory element, a translational regulatory element, a transcriptional terminator sequence, an mRNA splice site, a protein coding sequence, an intron site, and an intron enhancing motif. In certain cases, when the donor DNA comprises a region of homology to a genomic locus of the fungal cells, the Cas endonuclease and guide RNA introduced to the fungal cells are capable of forming a complex that enables the Cas endonuclease to act at a target site in or near the genomic locus of the fungal cells. In some embodiments, the Cas endonuclease cut site (or target site) on the genomic DNA is in the homologous region between the donor DNA and the genomic locus, where homologous recombination can occur. In other embodiments, the cut site is near the homologous region between the donor DNA and the genomic locus which can be anywhere from 1 bp to about 10 kb away from the homologous region, e.g., 1bp, 2 bp, 5 bp, 10 bp, 20 bp, 50 bp, 100 bp, 250bp, 500bp, 1 kb, 2 kb, 3 kb, 4 kb, 5 kb, 6 kb, 7 kb, 8 kb, 9 kb, or 10 kb away from the site of homologous region.

[0129] In certain embodiments, modification of the genome of the fungal cell results in a phenotypic effect that can be detected and, in many instances, is a desired outcome of the user. Non-limiting examples include acquisition of a selectable cell growth phenotype (e.g., resistance to or sensitivity to an antibiotic, gain or loss of an auxotrophic characteristic, increased or decreased rate of growth, etc.), expression of a detectable marker (e.g., fluorescent marker, cell-surface molecule, chromogenic enzyme, etc.), and the secretion of an enzyme the activity of which can be detected in culture supernatant.

[0130] When modification of the genome of the fungal cell results in a phenotypic effect, a donor DNA is often employed that includes a polynucleotide of interest that is (or encodes) a phenotypic marker. Any convenient phenotypic marker can be used, including any selectable or screenable marker that allows one to identify, select for, or screen for or against a fungal cell that contains it, often under particular culture conditions. Thus, in some aspects of the present invention, the identification of fungal cells having a desired genome modification incudes culturing the fungal population of cells that have received the Cas endonuclease and guide

polynucleotide (and optionally a donor DNA) under conditions to select for or screen for cells having the modification at the target site. Any type of selection system may be employed, including assessing for the gain or loss of an enzymatic activity in the fungal cell (also referred to as a selectable marker), e.g., the acquisition of antibiotic resistance or gain/loss of an auxotrophic marker.

[0131] In some instances, the genomic modification in the fungal cells is detected directly using any convenient method, including sequencing, PCR, Southern blot, restriction enzyme analysis, and the like, including combinations of such methods.

[0132] In some embodiments, specific genes are targeted for modification using the disclosed methods, including genes encoding enzymes, e.g., acetyl esterases, aminopeptidases, amylases, arabinases, arabinofuranosidases, carboxypeptidases, catalases, cellulases, chitinases, cutinase, deoxyribonucleases, epimerases, esterases, α-galactosidases, β-galactosidases, α-glucanases, glucan lysases, endo-β-glucanases, glucoamylases, glucose oxidases, α-glucosidases, β-glucosidases, glucuronidases, hemicellulases, hexose oxidases, hydrolases, invertases, isomerases, laccases, lipases, lyases, mannosidases, oxidases, oxidoreductases, pectate lyases, pectin acetyl esterases, pectin depolymerases, pectin methyl esterases, pectinolytic enzymes, peroxidases, phenoloxidases, phytases, polygalacturonases, proteases, rhamno-galacturonases, ribonucleases, transferases, transport proteins, transglutaminases, xylanases, hexose oxidases, and combinations thereof.

[0133] Aspects of the present disclosure are drawn to recombinant fungal cells produced by the methods described above as well as those for use as parental host cells in performing the methods.

[0134] Additional embodiments of the methods and compositions of the present disclosure are shown herein. Non-limiting examples or embodiments of the methods and compositions disclosed herein are as follows:

1. A method for homologous recombination of a donor DNA with a genomic locus in a fungal cell, the method comprising:

(a) generating a heterokaryon between a helper fungal strain and a target fungal strain, wherein the helper fungal strain comprises an expression construct that silences the non-homologous end joining (NHEJ) mechanism;

(b) introducing a donor DNA into the heterokaryon, wherein the donor DNA comprises a region of homology to a genomic locus in the target strain sufficient for homologous recombination at the genomic locus;

(c) generating and plating spores from the heterokaryon cells of (b); and

(d) identifying cells from the plated spores in which (i) the donor DNA has integrated into the genome by homologous recombination at the genomic locus, and (ii) the expression construct that silences the non-homologous end joining (NHEJ) mechanism is not present.

2. The method of embodiment 1, wherein the expression construct silences one or more of: ku80, ku70, rad50, mre11, xrs2, lig4, and xrs.

3. The method of embodiment 2, wherein the expression construct silences ku80, ku70, or both.

4. The method of any one of embodiments 1 to 3, further comprising introducing a functional Cas/guide RNA complex into the heterokaryon, wherein the Cas/guide RNA complex has a target site within the genomic locus.

5. The method of embodiment 4, wherein the Cas is a Cas nickase.

6. The method of any preceding embodiment, wherein the donor DNA comprises a polynucleotide sequence of interest, wherein homologous recombination at the genomic locus results in insertion of the polynucleotide sequence of interest in the genomic locus.

7. The method of any preceding embodiment, wherein the Cas endonuclease is a Cas9 endonuclease or variant thereof.

8. The method of embodiment 7, wherein the Cas9 endonuclease or variant thereof comprises a full length Cas9 or a functional fragment thereof from a species selected from the group consisting of: Streptococcus sp., S. pyogenes, S. mutans, S. thermophilus, Campylobacter sp., C. jejuni,

Neisseria sp., N. meningitides, Francisella sp., F. novicida, and Pasteurella sp., P. multocida.

9. The method of any one of embodiments 4 to 8, wherein introducing the functional Cas/guide RNA complex into the heterokaryon comprises introducing a DNA construct comprising an expression cassette for the Cas endonuclease into the fungal cells.

10. The method of any one of embodiments 4 to 9, wherein introducing the functional Cas/guide RNA complex into the heterokaryon comprises introducing a DNA construct comprising an expression cassette for the guide RNA into the fungal cells.

11. The method of any one of embodiments 4 to 8 or 10, wherein the introducing step comprises directly introducing the Cas endonuclease into the fungal cells.

12. The method of any one of embodiments 4 to 9 or 11, wherein the introducing step comprises directly introducing the guide RNA into the fungal cells.

13. The method of any preceding embodiment, wherein the fungal cell is a filamentous fungal cell.

14. The method of any preceding embodiment, wherein the fungal cell is a Eumycotina or Pezizomycotina fungal cell.

15. The method of any preceding embodiment, wherein filamentous fungal cell is selected from the group consisting of Trichoderma, Penicillium, Aspergillus, Humicola, Chrysosporium, Fusarium, Myceliophthora, Neurospora, Hypocrea, and Emericella.

16. The method of any preceding embodiment, wherein the fungal cell is a Trichoderma sp. cell.

17. The method of embodiment 16, wherein the Trichoderma sp. is Trichoderma reesei.

18. The method of any preceding embodiment, wherein the donor DNA has partially integrated into the genome at the genomic locus.

19. The method of any preceding embodiment, wherein integration of the donor DNA results in a modification of the genomic locus.

20. The method of embodiment 19, wherein the modification is selected from the group consisting of a deletion of one or more nucleotides, an insertion of one or more nucleotides, insertion of an expression cassette encoding a protein of interest, a substitution of one or more nucleotides, and any combination thereof.

21. The method of any preceding embodiment, wherein the identifying step comprises culturing cells grown from the spores from step (c) under conditions to select for or screen for the integration of the donor DNA at the genomic locus or the modification of the genomic locus.

22. A recombinant fungal cell produced by the method of any preceding embodiment.

EXAMPLES

[0135] In the following Examples, unless otherwise stated, parts and percentages are by weight and degrees are Celsius. It should be understood that these Examples, while indicating embodiments of the disclosure, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can make various changes and modifications of the disclosure to adapt it to various usages and conditions. Such modifications are also intended to fall within the scope of the appended claims.

[0136] This helper strain-based concept can provide benefits to any project where *T. reesei* or another filamentous fungus is used as a host, and where a high rate of homologous integration is required.

Example 1: Use of helper strain for complementing colonial growth and determining allele dominance

[0137] Experiments were performed to use a helper strain for complementing colonial growth and determining allele dominance. The work flow and the result of one of such experiments are shown in FIG. 7. Steps of this experiment include:

1) Fusing helper strain (HS) and target strain (CS, or Colonial Strain in FIG. 7) to form a heterokaryon;

2) Generating conidiospores from the heterokaryon;

3) Harvesting the conidiospores and diluting to appropriate concentration;

4) Plating conidiospores on selective media and counting colonies to score the phenotype and auxotrophy; and

5) Confirming the strains obtained.

In this particular experiment the helper strain (HS) is a RL-P37 Δcbh1 Δcbh2 Δegl1 Δegl2 Δhis3 strain with wild type colony growth rates and conidiation, and the target colonial strain (CS) is a RL-P37 Δcbh1 Δcbh2 Δegl1 Δegl2 Δpyr2 strain with deletion of a gene of interest which changes a feature of interest (in this experiment the feature of interest is normal colony growth rates and conidiation, so the gene deletion strain has reduced colony growth rates and conidiation). Protoplasts of the HS strain and the CS strain were co-inoculated on minimal medium plates supplemented with sorbitol. After the strains fused to form a heterokaryon and started growing, they were transferred to minimal medium plates without supplements and grown in conditions that promote production of conidiospores at 28ºC and a 12 h light-dark cycle. The heterokaryon had a wild type colony growth and conidiation equivalent to the helper strain, indicating that the reduced colony growth and conidiation of the CS strain are complemented and that the mutation in the gene of interest is recessive. Mature conidiospores were harvested from the heterokaryon, and serial dilutions of conidiospores were plated on selective minimal medium supplemented with either histidine or uridine. Colonies that grew upon transfer to minimal medium were identified as heterokaryons and eliminated. All the strains that grew on minimal medium supplemented with histidine had wild type

colony growth and conidiation, features associate with the helper strain. All the strains that grew on minimal medium supplemented with uridine had reduced colony growth and conidiation, features associated with the recessive mutation in the gene of interest. This indicated that the strain with sub-optimal reduced colony growth and conidiation containing the recessive mutation in the gene of interest could be rescued from the heterokaryon.

Example 2: Silencing of NHEJ mechanism in fungal cells with DNA constructs

Constructs

[0138] The pAVTrku80sil construct for silencing ku80 (FIG. 2A) contains a sense and antisense ku80 DNA sequence interrupted with an intron sequence, and is driven by a divergent promoter. The divergent promoter also drives the expression of *Ptilosarcus sp.* green fluorescent protein (PtGFP), which serves as an indicator of the antisense cassette expression. The construct also contains the *amdS* selective marker.

[0139] The intermediate construct for silencing both ku80 and ku70 (pAVTrku70ku80sil, FIG. 2B), and another construct for silencing ku70, lig4 and ku80 (pAVTrku70lig4ku80sil, FIG. 2C) have been generated and verified. They contain approximately 500 bp sense sequences of the respective genes, an intron and the equivalent antisense sequences, followed by the ku70 terminator sequence in the yeast pRS426 vector backbone.

[0140] The gene replacement construct fragments incorporated into the pRS426 vector have been assembled in vitro and cloned in yeast. The gene replacement cassette with the *pyr2* marker will be amplified from this construct.

Strains

[0141] A *T. reesei* strain (RL-P37 Δcbh1 Δcbh2 Δegl1 Δegl2) was transformed with the *T. reesei* ku80 gene silencing construct (FIG. 2A). Six stable candidates with a strong GFP signal were selected, verified and spore-purified. Four spore-purified isolates from each of the six candidates were grown in 24-well plates and the intensity of the GFP signal, indicative of the level of silencing, was assessed. One spore-purified isolate from each candidate with the highest GFP signal was selected for transformation with a *Δhis3::hph* deletion construct.

Protocol

[0142] Spore-purified strains from above containing the silencing constructs with the highest level of GFP expression were tested for the efficiency of homologous integration by transforming with a linear *his3* deletion cassette (FIG. 3) containing the *hph* selective marker. The silencing strain with the highest proportion of transformants containing the Δhis3 deletion (60%), indicating highest levels of NHEJ silencing, was selected and will be used as the helper strain. The helper strain will be fused with a host strain that has a non-functional *pyr2* locus (a nutritional marker), and optionally an altered colony morphology (a recessive feature). The forced heterokaryon grown on minimal medium will then be transformed by PEG transformation with a linear gene replacement construct (FIG. 4) containing a functional *pyr2* marker as the selective marker (this marker is also a nutritional marker), and plated on minimal medium. Conidiospores will be harvested and serial dilutions plated on minimal medium. Colonies that grow on minimal medium (and have altered colony morphology, if that feature was included) will be assessed for homologous recombination of the deletion construct, and efficiency of homologous recombination will be determined.

[0143] Specific, non-limiting examples of how the helper strain compositions and methods can be employed are described below.

Example 3: Use of cre *recombinase in a helper strain or heterokaryon for removal of DNA fragments in a target cell*

[0144] In one embodiment, a user can express *cre* recombinase in a helper strain and use it for removal of any DNA fragment in a target cell flanked by *loxP* sites.

[0145] In another embodiment, a user can introduce a telomeric vector expressing *cre* recombinase into a heterokaryon, and simultaneously remove one or more DNA fragments flanked by *loxP* sites in each of the helper and target strains. A schematic example is shown in FIG. 5. Two deletion strains, each with a deletion cassette containing the *hph* marker flanked by two *loxP* sites at the *ad3A* (Strain 2) or *his3* (Strain 1) loci of a wild type strain, were obtained by homologous recombination. To recycle the *hph* marker and concurrently remove it from one or

both strains, Strain 1 and Strain 2 were first fused into a forced heterokaryon using the procedure described in Example 1. The heterokaryon was then transformed with a *cre*-containing telomeric vector (FIG. 6) carrying an *amdS* marker by biolistic or PEG transformation, and then propagated on appropriate acetamide-containing plates to maintain the vector. While under selection, the telomeric vector provided Cre to both component strains without integrating into the genomes, allowing recombination at *loxP* sites and looping-out of the *hph* marker in one or both contributing genomes. Conidiospores from the transformed heterokaryon were harvested and plated on PDA medium supplemented with adenine and histidine. After a defined number of transfers on supplemented PDA medium, individual colonies were tested for auxotrophies, and resistance to hygromycin B. Strains with *ad3A* and *his3* deletions that have looped-out the *hph* cassette were not resistant to hygromycin B. To ensure that the rare event of telomeric vector incorporation into the genome did not occur, the strains were tested for the presence of a telomeric vector DNA fragment by PCR analysis, and candidates that had incorporated the *cre*-containing telomeric vector were eliminated. A wild type Δhis3 Δhph strain was confirmed by diagnostic PCR. The results indicate that the cre recombinase expressed from the telomeric vector introduced into the heterokaryon were able to successfully remove one or more DNA fragments flanked by *loxP* sites in each of the helper and target strains without integration of the vector into the genome.

Example 4: Use of Cas RNA-guided endonuclease in a helper strain for creating targeted double-strand DNA breaks in a target cell

[0146] In another embodiment a user can express a Cas RNA-guided endonuclease in a helper strain which preferably has silenced NHEJ- and use it to create targeted double-strand DNA breaks in a target cell. One benefit is that a double-strand DNA break stimulates integration of a donor DNA fragment by homologous recombination at the target site. Silencing NHEJ minimizes the frequency with which the donor DNA fragment integrates via the NHEJ pathway. In one experiment, a helper strain containing a NHEJ-silencing DNA construct as described in Example 2 and which has exhibited efficient homologous recombination is transformed with a Cas9 expression vector (FIG. 8) similar to those disclosed in PCT application no. PCT/CN2014/093914 (filed December 16, 2014, hereby incorporated by reference).

This vector uses a mutant version of the T. reesei als1 gene that confers resistance to chlorimuron ethyl as selectable marker and transformants that have stably integrated the vector into the chromosomal DNA are selected as a new helper strain. This helper strain is fused with a target strain (with non-functional pyr2 gene) to create a forced heterokaryon on selective medium lacking histidine and uridine. The heterokaryon is then co-transformed with a mix of sgRNA (single-guide RNA) and a donor DNA fragment for which integration at a defined target site within the genome of the target cell is desired. The sgRNA is designed to direct cas9 endonuclease to said defined target site and is generated using an in vitro transcription reaction as described in PCT application no. PCT/CN2014/093916 (filed December 16, 2014, hereby incorporated by reference)). Examples of sequences of sgRNAs designed to direct Cas9 to the ad3A, glucoamylase (TrGA), or pyr2 gene of T. reesei are provided as follows.

[0147] sgRNA: gAd3A TS1; SEQ ID NO:17
guccucgagcaaaaggugccGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUA GUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGGUGC.

[0148] sgRNA: gTrGA TS2; SEQ ID NO:18
guucagugcaauaggcgucuGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUA GUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGGUGC

[0149] sgRNA: gTrGA TS11; SEQ ID NO:19
gccaauggcgacggcagcacGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUA GUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGGUGC

[0150] sgRNA: gPyr2 TS6; SEQ ID NO:20
gcacagcgggaugcccuuguGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUA GUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGGUGC

[0151] Alternatively, in place of the sgRNA a DNA construct including a sgRNA expression cassette is included in the co-transformation. The DNA fragment for which integration at a defined target site is desired includes terminal regions of homology with the flanking DNA regions on either side of the cas9 target site. It also includes a selectable marker (such as pyr2) and an expression cassette for a gene of interest. Upon sporulation of the heterokaryon and plating of spores, colonies with the target cell phenotype but prototrophic for uridine (pyr2+) are isolated and

screened for integration of the DNA fragment at the target site. Advantageously, this method allows efficient generation of transformants having different genes of interest integrated at the same target site.

[0152] Although the foregoing compositions and methods have been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings herein that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

[0153] Accordingly, the preceding merely illustrates the principles of the present compositions and methods. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the present compositions and methods and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the present compositions and methods and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the present compositions and methods as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present compositions and methods, therefore, is not intended to be limited to the exemplary embodiments shown and described herein.

SEQUENCES

SEQ ID NO:1

Codon optimized *Streptococcus pyogenes* Cas9-encoding gene; with N- and C-terminal NLS sequences

atggcaccgaagaagaagcgcaaggtgatggacaagaagtacagcatcggcctcgacatcggcaccaactcgg
tgggctgggccgtcatcacggacgaatataaggtcccgtcgaagaagttcaaggtcctcggcaatacagaccgcc
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gctgggcggagacaagaagaagaagctcaagctctag

SEQ ID NO:2

Streptococcus pyogenes Cas9 encoded by SEQ ID NO:1; with N- and C-terminal NLS sequences

MAPKKKRKVMDKKYSIGLDIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLI
GALLFDSGETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEES
FLVEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMI
KFRGHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKS

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KALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVK
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SEQ ID NO:3

Full U6 gene promoter sequence (not including transcription start site)

AAAAAACACTAGTAAGTACTTACTTATGTATTATTAACTACTTTAGCTAACTTCTG
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GAAATGGTATTATGTAAGAGCTATAGTCTAAAGGCACTCTGCTGGATAAAAATA
GTGGCTATAAGTCTGCTGCAAAACTACCCCCAACCTCGTAGGTATATAAGTACT
GTTTGATGGTAGTCTATC

SEQ ID NO:4

Truncated/shorter U6 gene promoter sequence (not including transcription start site)

AATTCCTAAAGAAACAGCATGAAATGGTATTATGTAAGAGCTATAGTCTAAAGG
CACTCTGCTGGATAAAAATAGTGGCTATAAGTCTGCTGCAAAACTACCCCCAAC
CTCGTAGGTATATAAGTACTGTTTGATGGTAGTCTATC

SEQ ID NO:5

U6 gene intron

GTTCGTTTCGGCTTTTCCTCGGAACCCCCAGAGGTCATCAGTTCGAATCGCTA
ACAG

SEQ ID NO:6

U6 gene transcriptional terminator sequence

TTTTTTTTCTCTT

SEQ ID NO:7

Filamentous fungal cell Codon optimized Streptococcus pyogenes Cas9-encoding gene; no NLS

atggacaagaagtacagcatcggcctcgacatcggcaccaactcggtgggctgggccgtcatcacggacgaatat
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gtccagacgggcgggttcagcaaggagtccatcctcccgaagcgcaactccgacaagctcatcgcgaggaaga
aggattgggacccgaaaaaatatggcggcttcgacagcccgaccgtcgcatacagcgtcctcgtcgtggcgaagg
tggagaagggcaagtcaaagaagctcaagtccgtgaaggagctgctcgggatcacgattatggagcggtcctcctt
cgagaagaacccgatcgacttcctagaggccaagggatataaggaggtcaagaaggacctgattattaaactgcc
gaagtactcgctcttcgagctggaaaacggccgcaagaggatgctcgcctccgcaggcgagttgcagaagggca
acgagctcgccctcccgagcaaatacgtcaatttcctgtacctcgctagccactatgaaaagctcaagggcagccc

ggaggacaacgagcagaagcagctcttcgtggagcagcacaagcattacctggacgagatcatcgagcagatc
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gacaaaccaatacgcgagcaggccgaaaatatcatccacctcttcaccctcaccaacctcggcgctccggcagcc
ttcaagtacttcgacaccacgattgaccggaagcggtacacgagcacgaaggaggtgctcgatgcgacgctgatc
caccagagcatcacagggctctatgaaacacgcatcgacctgagccagctgggcggagac

SEQ ID NO:8

Streptococcus pyogenes Cas9 encoded by SEQ ID NO:7; no NLS

MDKKYSIGLDIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGE
TAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKH
ERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEG
DLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLP
GEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQY
ADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLP
EKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRK
QRTFDNGSIPHQIHLGELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGN
SRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPKHSLL
YEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFK
KIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDR
EMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKS
DGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVK
VVDELVKVMGRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKE
HPVENTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVDHIVPQSFLKDDSIDN
KVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAERGGLS
ELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSKLVSDFR
KDFQFYKVREINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYGDYKVYDVRKMIA
KSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFA
TVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSP
TVAYSVLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLII
KLPKYSLFELENGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDN
EQKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIH
LFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGD

SEQ ID NO:9

SV40 NLS

PKKKRKV

SEQ NO:10

T. reesei blr2 (blue light regulator 2) gene NLS

KKKKLKL

SEQ ID NO:11

Streptococcus thermophilus LMD-9 Cas9

MTKPYSIGLDIGTNSVGWAVTTDNYKVPSKKMKVLGNTSKKYIKKNLLGVLLFDSGI
TAEGRRLKRTARRRYTRRRNRILYLQEIFSTEMATLDDAFFQRLDDSFLVPDDKRD
SKYPIFGNLVEEKAYHDEFPTIYHLRKYLADSTKKADLRLVYLALAHMIKYRGHFLIE
GEFNSKNNDIQKNFQDFLDTYNAIFESDLSLENSKQLEEIVKDKISKLEKKDRILKLF
PGEKNSGIFSEFLKLIVGNQADFRKCFNLDEKASLHFSKESYDEDLETLLGYIGDDY
SDVFLKAKKLYDAILLSGFLTVTDNETEAPLSSAMIKRYNEHKEDLALLKEYIRNISLK
TYNEVFKDDTKNGYAGYIDGKTNQEDFYVYLKKLLAEFEGADYFLEKIDREDFLRK
QRTFDNGSIPYQIHLQEMRAILDKQAKFYPFLAKNKERIEKILTFRIPYYVGPLARGN
SDFAWSIRKRNEKITPWNFEDVIDKESSAEAFINRMTSFDLYLPEEKVLPKHSLLYE
TFNVYNELTKVRFIAESMRDYQFLDSKQKKDIVRLYFKDKRKVTDKDIIEYLHAIYGY
DGIELKGIEKQFNSSLSTYHDLLNIINDKEFLDDSSNEAIIEEIIHTLTIFEDREMIKQRL
SKFENIFDKSVLKKLSRRHYTGWGKLSAKLINGIRDEKSGNTILDYLIDDGISNRNFM
QLIHDDALSFKKKIQKAQIIGDEDKGNIKEVVKSLPGSPAIKKGILQSIKIVDELVKVM
GGRKPESIVVEMARENQYTNQGKSNSQQRLKRLEKSLKELGSKILKENIPAKLSKID
NNALQNDRLYLYYLQNGKDMYTGDDLDIDRLSNYDIDHIIPQAFLKDNSIDNKVLVS
SASNRGKSDDVPSLEVVKKRKTFWYQLLKSKLISQRKFDNLTKAERGGLSPEDKA
GFIQRQLVETRQITKHVARLLDEKFNNKKDENNRAVRTVKIITLKSTLVSQFRKDFEL
YKVREINDFHHAHDAYLNAVVASALLKKYPKLEPEFVYGDYPKYNSFRERKSATEK
VYFYSNIMNIFKKSISLADGRVIERPLIEVNEETGESVWNKESDLATVRRVLSYPQV
NVVKKVEEQNHGLDRGKPKGLFNANLSSKPKPNSNENLVGAKEYLDPKKYGGYA
GISNSFTVLVKGTIEKGAKKKITNVLEFQGISILDRINYRKDKLNFLLEKGYKDIELIIEL
PKYSLFELSDGSRRMLASILSTNNKRGEIHKGNQIFLSQKFVKLLYHAKRISNTINEN
HRKYVENHKKEFEELFYYILEFNENYVGAKKNGKLLNSAFQSWQNHSIDELCSSFI

GPTGSERKGLFELTSRGSAADFEFLGVKIPRYRDYTPSSLLKDATLIHQSVTGLYET
RIDLAKLGEG

SEQ ID NO:12

Streptococcus mutans UA159 Cas9

MKKPYSIGLDIGTNSVGWAVVTDDYKVPAKKMKVLGNTDKSHIEKNLLGALLFDSG
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GERHPIFGNLEEEVKYHENFPTIYHLRQYLADNPEKVDLRLVYLALAHIIKFRGHFLI
EGKFDTRNNDVQRLFQEFLAVYDNTFENSSLQEQNVQVEEILTDKISKSAKKDRVL
KLFPNEKSNGRFAEFLKLIVGNQADFKKHFELEEKAPLQFSKDTYEEELEVLLAQIG
DNYAELFLSAKKLYDSILLSGILTVTDVGTKAPLSASMIQRYNEHQMDLAQLKQFIR
QKLSDKYNEVFSDVSKDGYAGYIDGKTNQEAFYKYLKGLLNKIEGSGYFLDKIERE
DFLRKQRTFDNGSIPHQIHLQEMRAIIRRQAEFYPFLADNQDRIEKLLTFRIPYYVGP
LARGKSDFAWLSRKSADKITPWNFDEIVDKESSAEAFINRMTNYDLYLPNQKVLPK
HSLLYEKFTVYNELTKVKYKTEQGKTAFFDANMKQEIFDGVFKVYRKVTKDKLMDF
LEKEFDEFRIVDLTGLDKENKVFNASYGTYHDLCKILDKDFLDNSKNEKILEDIVLTL
TLFEDREMIRKRLENYSDLLTKEQVKKLERRHYTGWGRLSAELIHGIRNKESRKTIL
DYLIDDGNSNRNFMQLINDDALSFKEEIAKAQVIGETDNLNQVVSDIAGSPAIKKGIL
QSLKIVDELVKIMGHQPENIVVEMARENQFTNQGRRNSQQRLKGLTDSIKEFGSQI
LKEHPVENSQLQNDRLFLYYLQNGRDMYTGEELDIDYLSQYDIDHIIPQAFIKDNSID
NRVLTSSKENRGKSDDVPSKDVVRKMKSYWSKLLSAKLITQRKFDNLTKAERGGL
TDDDKAGFIKRQLVETRQITKHVARILDERFNTETDENNKKIRQVKIVTLKSNLVSNF
RKEFELYKVREINDYHHAHDAYLNAVIGKALLGVYPQLEPEFVYGDYPHFHGHKEN
KATAKKFFYSNIMNFFKKDDVRTDKNGEIIWKKDEHISNIKKVLSYPQVNIVKKVEEQ
TGGFSKESILPKGNSDKLIPRKTKKFYWDTKKYGGFDSPIVAYSILVIADIEKGKSKK
LKTVKALVGVTIMEKMTFERDPVAFLERKGYRNVQEENIIKLPKYSLFKLENGRKRL
LASARELQKGNEIVLPNHLGTLLYHAKNIHKVDEPKHLDYVDKHKDEFKELLDVVSN
FSKKYTLAEGNLEKIKELYAQNNGEDLKELASSFINLLTFTAIGAPATFKFFDKNIDR
KRYTSTTEILNATLIHQSITGLYETRIDLNKLGGD

SEQ ID NO:13

Campylobacter jejuni Cas9

MARILAFDIGISSIGWAFSENDELKDCGVRIFTKVENPKTGESLALPRRLARSARKR
LARRKARLNHLKHLIANEFKLNYEDYQSFDESLAKAYKGSLISPYELRFRALNELLS

KQDFARVILHIAKRRGYDDIKNSDDKEKGAILKAIKQNEEKLANYQSVGEYLYKEYF
QKFKENSKEFTNVRNKKESYERCIAQSFLKDELKLIFKKQREFGFSFSKKFEEEVLS
VAFYKRALKDFSHLVGNCSFFTDEKRAPKNSPLAFMFVALTRIINLLNNLKNTEGILY
TKDDLNALLNEVLKNGTLTYKQTKKLLGLSDDYEFKGEKGTYFIEFKKYKEFIKALG
EHNLSQDDLNEIAKDITLIKDEIKLKKALAKYDLNQNQIDSLSKLEFKDHLNISFKALK
LVTPLMLEGKKYDEACNELNLKVAINEDKKDFLPAFNETYYKDEVTNPVVLRAIKEY
RKVLNALLKKYGKVHKINIELAREVGKNHSQRAKIEKEQNENYKAKKDAELECEKL
GLKINSKNILKLRLFKEQKEFCAYSGEKIKISDLQDEKMLEIDHIYPYSRSFDDSYMN
KVLVFTKQNQEKLNQTPFEAFGNDSAKWQKIEVLAKNLPTKKQKRILDKNYKDKEQ
KNFKDRNLNDTRYIARLVLNYTKDYLDFLPLSDDENTKLNDTQKGSKVHVEAKSGM
LTSALRHTWGFSAKDRNNHLHHAIDAVIIAYANNSIVKAFSDFKKEQESNSAELYAK
KISELDYKNKRKFFEPFSGFRQKVLDKIDEIFVSKPERKKPSGALHEETFRKEEEFY
QSYGGKEGVLKALELGKIRKVNGKIVKNGDMFRVDIFKHKKTNKFYAVPIYTMDFAL
KVLPNKAVARSKKGEIKDWILMDENYEFCFSLYKDSLILIQTKDMQEPEFVYYNAFT
SSTVSLIVSKHDNKFETLSKNQKILFKNANEKEVIAKSIGIQNLKVFEKYIVSALGEVT
KAEFRQREDFKK

SEQ ID NO:14

Neisseria meningitides Cas9

MAAFKPNSINYILGLDIGIASVGWAMVEIDEEENPIRLIDLGVRVFERAEVPKTGDSL
AMARRLARSVRRLTRRRAHRLLRTRRLLKREGVLQAANFDENGLIKSLPNTPWQL
RAAALDRKLTPLEWSAVLLHLIKHRGYLSQRKNEGETADKELGALLKGVAGNAHAL
QTGDFRTPAELALNKFEKESGHIRNQRSDYSHTFSRKDLQAELILLFEKQKEFGNP
HVSGGLKEGIETLLMTQRPALSGDAVQKMLGHCTFEPAEPKAAKNTYTAERFIWLT
KLNNLRILEQGSERPLTDTERATLMDEPYRKSKLTYAQARKLLGLEDTAFFKGLRY
GKDNAEASTLMEMKAYHAISRALEKEGLKDKKSPLNLSPELQDEIGTAFSLFKTDE
DITGRLKDRIQPEILEALLKHISFDKFVQISLKALRRIVPLMEQGKRYDEACAEIYGDH
YGKKNTEEKIYLPPIPADEIRNPVVLRALSQARKVINGVVRRYGSPARIHIETAREVG
KSFKDRKEIEKRQEENRKDREKAAAKFREYFPNFVGEPKSKDILKLRLYEQQHGKC
LYSGKEINLGRLNEKGYVEIDHALPFSRTWDDSFNNKVLVLGSENQNKGNQTPYE
YFNGKDNSREWQEFKARVETSRFPRSKKQRILLQKFDEDGFKERNLNDTRYVNRF
LCQFVADRMRLTGKGKKRVFASNGQITNLLRGFWGLRKVRAENDRHHALDAVVV
ACSTVAMQQKITRFVRYKEMNAFDGKTIDKETGEVLHQKTHFPQPWEFFAQEVMI
RVFGKPDGKPEFEEADTLEKLRTLLAEKLSSRPEAVHEYVTPLFVSRAPNRKMSG

QGHMETVKSAKRLDEGVSVLRVPLTQLKLKDLEKMVNREREPKLYEALKARLEAH
KDDPAKAFAEPFYKYDKAGNRTQQVKAVRVEQVQKTGVWVRNHNGIADNATMVR
VDVFEKGDKYYLVPIYSWQVAKGILPDRAVVQGKDEEDWQLIDDSFNFKFSLHPN
DLVEVITKKARMFGYFASCHRGTGNINIRIHDLDHKIGKNGILEGIGVKTALSFQKYQ
IDELGKEIRPCRLKKRPPVR

SEQ ID NO:15

Francisella tularensis subsp. novicida Cas9

MNFKILPIAIDLGVKNTGVFSAFYQKGTSLERLDNKNGKVYELSKDSYTLLMNNRTA
RRHQRRGIDRKQLVKRLFKLIWTEQLNLEWDKDTQQAISFLFNRRGFSFITDGYSP
EYLNIVPEQVKAILMDIFDDYNGEDDLDSYLKLATEQESKISEIYNKLMQKILEFKLM
KLCTDIKDDKVSTKTLKEITSYEFELLADYLANYSESLKTQKFSYTDKQGNLKELSY
YHHDKYNIQEFLKRHATINDRILDTLLTDDLDIWNFNFEKFDFDKNEEKLQNQEDKD
HIQAHLHHFVFAVNKIKSEMASGGRHRSQYFQEITNVLDENNHQEGYLKNFCENL
HNKKYSNLSVKNLVNLIGNLSNLELKPLRKYFNDKIHAKADHWDEQKFTETYCHWI
LGEWRVGVKDQDKKDGAKYSYKDLCNELKQKVTKAGLVDFLLELDPCRTIPPYLD
NNNRKPPKCQSLILNPKFLDNQYPNWQQYLQELKKLQSIQNYLDSFETDLKVLKSS
KDQPYFVEYKSSNQQIASGQRDYKDLDARILQFIFDRVKASDELLLNEIYFQAKKLK
QKASSELEKLESSKKLDEVIANSQLSQILKSQHTNGIFEQGTFLHLVCKYYKQRQRA
RDSRLYIMPEYRYDKKLHKYNNTGRFDDDNQLLTYCNHKPRQKRYQLLNDLAGVL
QVSPNFLKDKIGSDDDLFISKWLVEHIRGFKKACEDSLKIQKDNRGLLNHKINIARNT
KGKCEKEIFNLICKIEGSEDKKGNYKHGLAYELGVLLFGEPNEASKPEFDRKIKKFN
SIYSFAQIQQIAFAERKGNANTCAVCSADNAHRMQQIKITEPVEDNKDKIILSAKAQR
LPAIPTRIVDGAVKKMATILAKNIVDDNWQNIKQVLSAKHQLHIPIITESNAFEFEPAL
ADVKGKSLKDRRKKALERISPENIFKDKNNRIKEFAKGISAYSGANLTDGDFDGAKE
ELDHIIPRSHKKYGTLNDEANLICVTRGDNKNKGNRIFCLRDLADNYKLKQFETTDD
LEIEKKIADTIWDANKKDFKFGNYRSFINLTPQEQKAFRHALFLADENPIKQAVIRAIN
NRNRTFVNGTQRYFAEVLANNIYLRAKKENLNTDKISFDYFGIPTIGNGRGIAEIRQL
YEKVDSDIQAYAKGDKPQASYSHLIDAMLAFCIAADEHRNDGSIGLEIDKNYSLYPL
DKNTGEVFTKDIFSQIKITDNEFSDKKLVRKKAIEGFNTHRQMTRDGIYAENYLPILIH
KELNEVRKGYTWKNSEEIKIFKGKKYDIQQLNNLVYCLKFVDKPISIDIQISTLEELRN
ILTTNNIAATAEYYYINLKTQKLHEYYIENYNTALGYKKYSKEMEFLRSLAYRSERVKI
KSIDDVKQVLDKDSNFIIGKITLPFKKEWQRLYREWQNTTIKDDYEFLKSFFNVKSIT
KLHKKVRKDFSLPISTNEGKFLVKRKTWDNNFIYQILNDSDSRADGTKPFIPAFDISK

NEIVEAIIDSFTSKNIFWLPKNIELQKVDNKNIFAIDTSKWFEVETPSDLRDIGIATIQY
KIDNNSRPKVRVKLDYVIDDDSKINYFMNHSLLKSRYPDKVLEILKQSTIIEFESSGF
NKTIKEMLGMKLAGIYNETSNN

SEQ ID NO:16

Pasteurella multocida Cas9

MQTTNLSYILGLDLGIASVGWAVVEINENEDPIGLIDVGVRIFERAEVPKTGESLALS
RRLARSTRRLIRRRAHRLLLAKRFLKREGILSTIDLEKGLPNQAWELRVAGLERRLS
AIEWGAVLLHLIKHRGYLSKRKNESQTNNKELGALLSGVAQNHQLLQSDDYRTPAE
LALKKFAKEEGHIRNQRGAYTHTFNRLDLLAELNLLFAQQHQFGNPHCKEHIQQYM
TELLMWQKPALSGEAILKMLGKCTHEKNEFKAAKHTYSAERFVWLTKLNNLRILED
GAERALNEEERQLLINHPYEKSKLTYAQVRKLLGLSEQAIFKHLRYSKENAESATF
MELKAWHAIRKALENQGLKDTWQDLAKKPDLLDEIGTAFSLYKTDEDIQQYLTNKV
PNSVINALLVSLNFDKFIELSLKSLRKILPLMEQGKRYDQACREIYGHHYGEANQKT
SQLLPAIPAQEIRNPVVLRTLSQARKVINAIIRQYGSPARVHIETGRELGKSFKERREI
QKQQEDNRTKRESAVQKFKELFSDFSSEPKSKDILKFRLYEQQHGKCLYSGKEINI
HRLNEKGYVEIDHALPFSRTWDDSFNNKVLVLASENQNKGNQTPYEWLQGKINSE
RWKNFVALVLGSQCSAAKKQRLLTQVIDDNKFIDRNLNDTRYIARFLSNYIQENLLL
VGKNKKNVFTPNGQITALLRSRWGLIKARENNNRHHALDAIVVACATPSMQQKITR
FIRFKEVHPYKIENRYEMVDQESGEIISPHFPEPWAYFRQEVNIRVFDNHPDTVLKE
MLPDRPQANHQFVQPLFVSRAPTRKMSGQGHMETIKSAKRLAEGISVLRIPLTQLK
PNLLENMVNKEREPALYAGLKARLAEFNQDPAKAFATPFYKQGGQQVKAIRVEQV
QKSGVLVRENNGVADNASIVRTDVFIKNNKFFLVPIYTWQVAKGILPNKAIVAHKNE
DEWEEMDEGAKFKFSLFPNDLVELKTKKEYFFGYYIGLDRATGNISLKEHDGEISK
GKDGVYRVGVKLALSFEKYQVDELGKNRQICRPQQRQPVR

SEQ ID NO:17

sgRNA: gAd3A TS1

guccucgagcaaaaggugccGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUA
GUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGGUGC.

SEQ ID NO:18

sgRNA: gTrGA TS2

guucagugcaauaggcgucuGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUA
GUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGGUGC

SEQ ID NO:19

sgRNA: gTrGA TS11

gccaauggcgacggcagcacGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUA
GUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGGUGC

SEQ ID NO:20

sgRNA: gPyr2 TS6

gcacagcgggaugcccuuguGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUA
GUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGGUGC

THAT WHICH IS CLAIMED:

1. A method for homologous recombination of a donor DNA with a genomic locus in a fungal cell, the method comprising:
(a) generating a heterokaryon between a helper fungal strain and a target fungal strain, wherein the helper fungal strain comprises an expression construct that silences the non-homologous end joining (NHEJ) mechanism;
(b) introducing a donor DNA into the heterokaryon, wherein the donor DNA comprises a region of homology to a genomic locus in the target strain sufficient for homologous recombination at the genomic locus;
(c) generating and plating spores from the heterokaryon cells of (b); and
(d) identifying cells from the plated spores in which (i) the donor DNA has integrated into the genome by homologous recombination at the genomic locus, and (ii) the expression construct that silences the non-homologous end joining (NHEJ) mechanism is not present.

2. The method of claim 1, wherein the expression construct silences one or more of: ku80, ku70, rad50, mre11, xrs2, lig4, and xrs.

3. The method of claim 2, wherein the expression construct silences ku80, ku70, or both.

4. The method of any one of claims 1 to 3, further comprising introducing a functional Cas/guide RNA complex into the heterokaryon, wherein the Cas/guide RNA complex has a target site within the genomic locus.

5. The method of claim 4, wherein the Cas is a Cas nickase.

6. The method of any preceding claim, wherein the donor DNA comprises a polynucleotide sequence of interest, wherein homologous recombination at the genomic locus results in insertion of the polynucleotide sequence of interest in the genomic locus.

7. The method of any preceding claim, wherein the Cas endonuclease is a Cas9 endonuclease or variant thereof.

8. The method of claim 7, wherein the Cas9 endonuclease or variant thereof comprises a full length Cas9 or a functional fragment thereof from a species selected from the group consisting of: *Streptococcus sp., S. pyogenes, S. mutans, S. thermophilus, Campylobacter sp., C. jejuni, Neisseria sp., N. meningitides, Francisella sp., F. novicida*, and *Pasteurella sp., P. multocida*.

9. The method of any one of claims 4 to 8, wherein introducing the functional Cas/guide RNA complex into the heterokaryon comprises introducing a DNA construct comprising an expression cassette for the Cas endonuclease into the fungal cells.

10. The method of any one of claims 4 to 9, wherein introducing the functional Cas/guide RNA complex into the heterokaryon comprises introducing a DNA construct comprising an expression cassette for the guide RNA into the fungal cells.

11. The method of any one of claims 4 to 8 or 10, wherein the introducing step comprises directly introducing the Cas endonuclease into the fungal cells.

12. The method of any one of claims 4 to 9 or 11, wherein the introducing step comprises directly introducing the guide RNA into the fungal cells.

13. The method of any preceding claim, wherein the fungal cell is a filamentous fungal cell.

14. The method of any preceding claim, wherein the fungal cell is a Eumycotina or Pezizomycotina fungal cell.

15. The method of any preceding claim, wherein filamentous fungal cell is selected from the group consisting of Trichoderma, Penicillium, Aspergillus, Humicola, Chrysosporium, Fusarium, Myceliophthora, Neurospora, Hypocrea, and Emericella.

16. The method of any preceding claim, wherein the fungal cell is a *Trichoderma sp.* cell.

17. The method of claim 16, wherein the *Trichoderma sp.* is *Trichoderma reesei*.

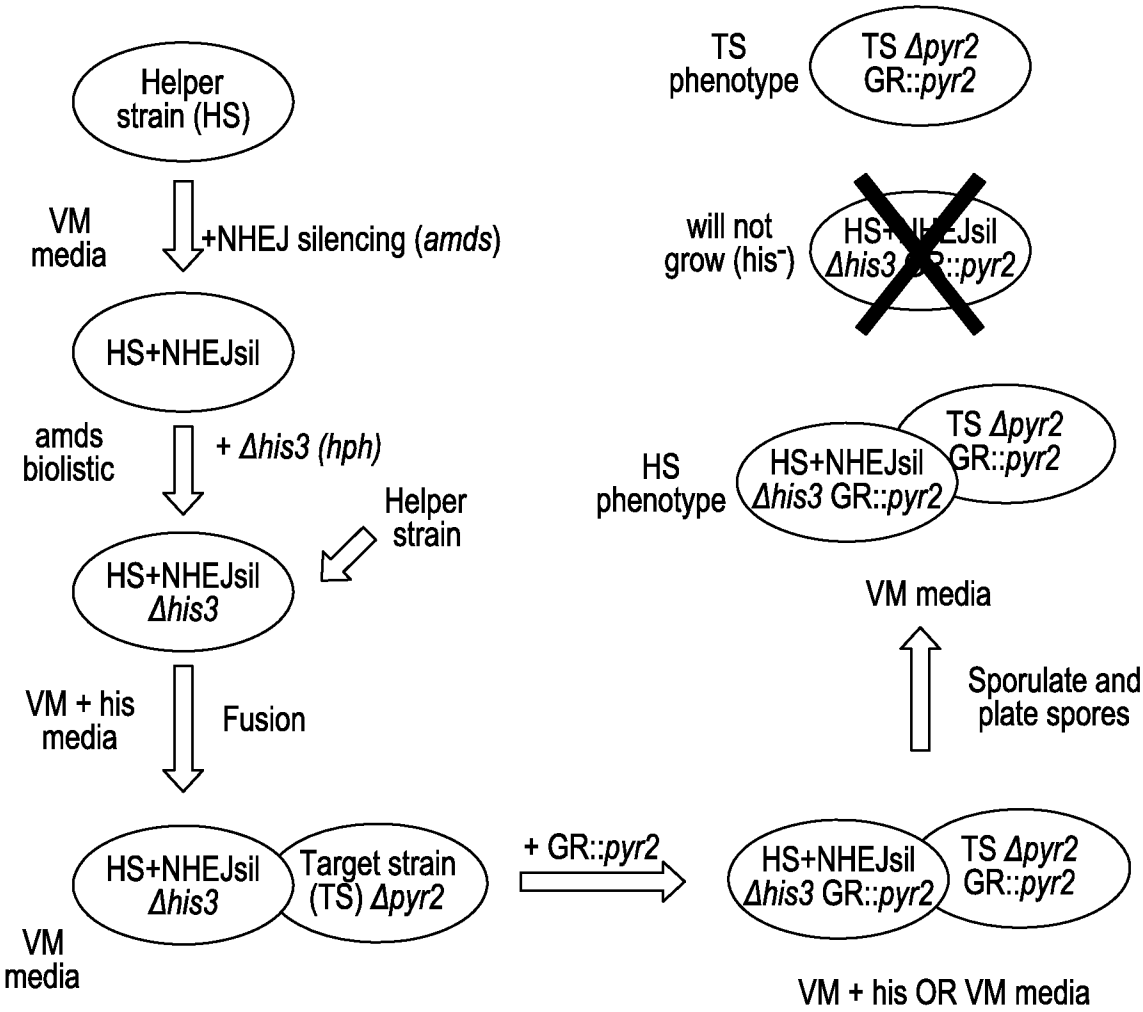
18. The method of any preceding claim, wherein the donor DNA has partially integrated into the genome at the genomic locus.

19. The method of any preceding claim, wherein integration of the donor DNA results in a modification of the genomic locus.

20. The method of claim 19, wherein the modification is selected from the group consisting of a deletion of one or more nucleotides, an insertion of one or more nucleotides, insertion of an expression cassette encoding a protein of interest, a substitution of one or more nucleotides, and any combination thereof.

21. The method of any preceding claim, wherein the identifying step comprises culturing cells grown from the spores from step (c) under conditions to select for or screen for the integration of the donor DNA at the genomic locus or the modification of the genomic locus.

22. A recombinant fungal cell produced by the method of any preceding claim.


Helper strain (HS)
VM media
+NHEJ silencing (amds)
HS+NHEJsil
amds biolistic
+ Δhis3 (hph)
Helper strain
HS+NHEJsil Δhis3
VM + his media
Fusion
HS+NHEJsil Δhis3
Target strain (TS) Δpyr2
VM media
+ GR::pyr2
TS phenotype
TS Δpyr2 GR::pyr2
will not grow (his-)
HS+NHEJsil Δhis3 GR::pyr2
HS phenotype
HS+NHEJsil Δhis3 GR::pyr2
TS Δpyr2 GR::pyr2
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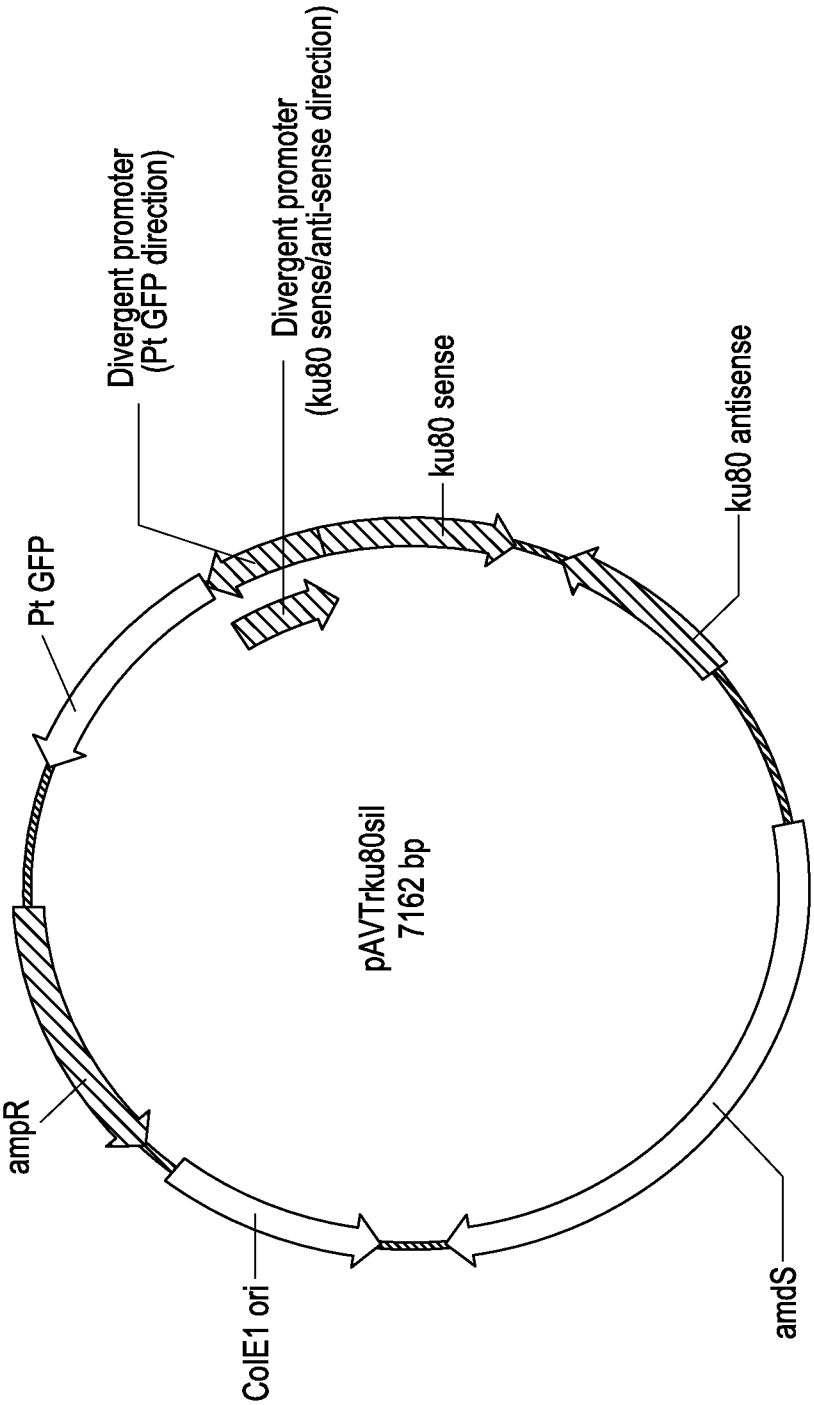
Sporulate and plate spores
HS+NHEJsil Δhis3 GR::pyr2
TS Δpyr2 GR::pyr2
VM + his OR VM media

FIG. 1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(Pt GFP direction)
Divergent promoter
(ku80 sense/anti-sense direction)
ku80 sense
ku80 antisense
Pt GFP
pAVTrku80sil
7162 bp
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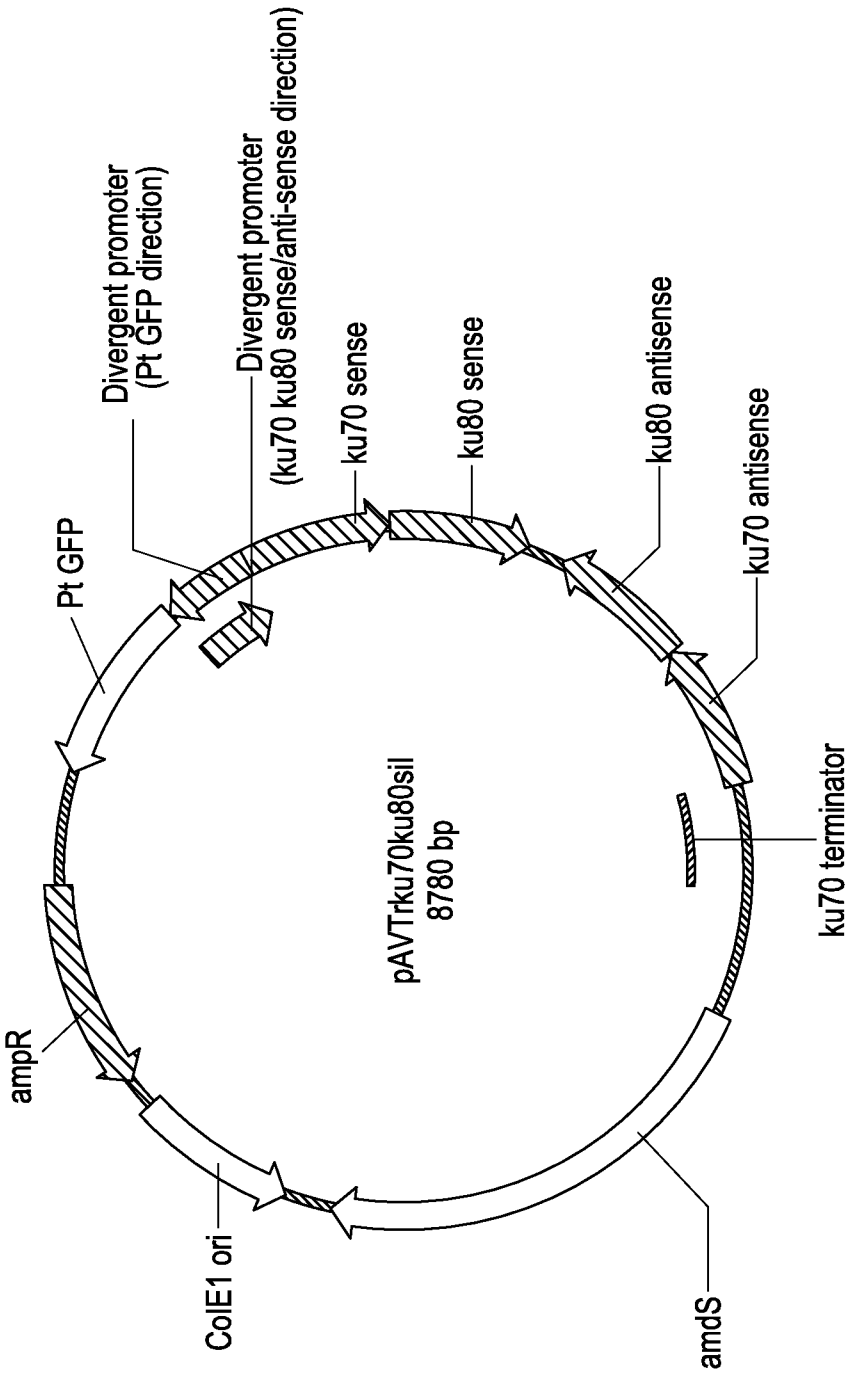
amdS

FIG. 2A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(Pt GFP direction)
Divergent promoter
(ku70 ku80 sense/anti-sense direction)
ku70 sense
ku80 sense
ku80 antisense
ku70 antisense
Pt GFP
pAVTrku70ku80sil
8780 bp
ku70 terminator
ampR
ColE1 ori
amdS

FIG. 2B

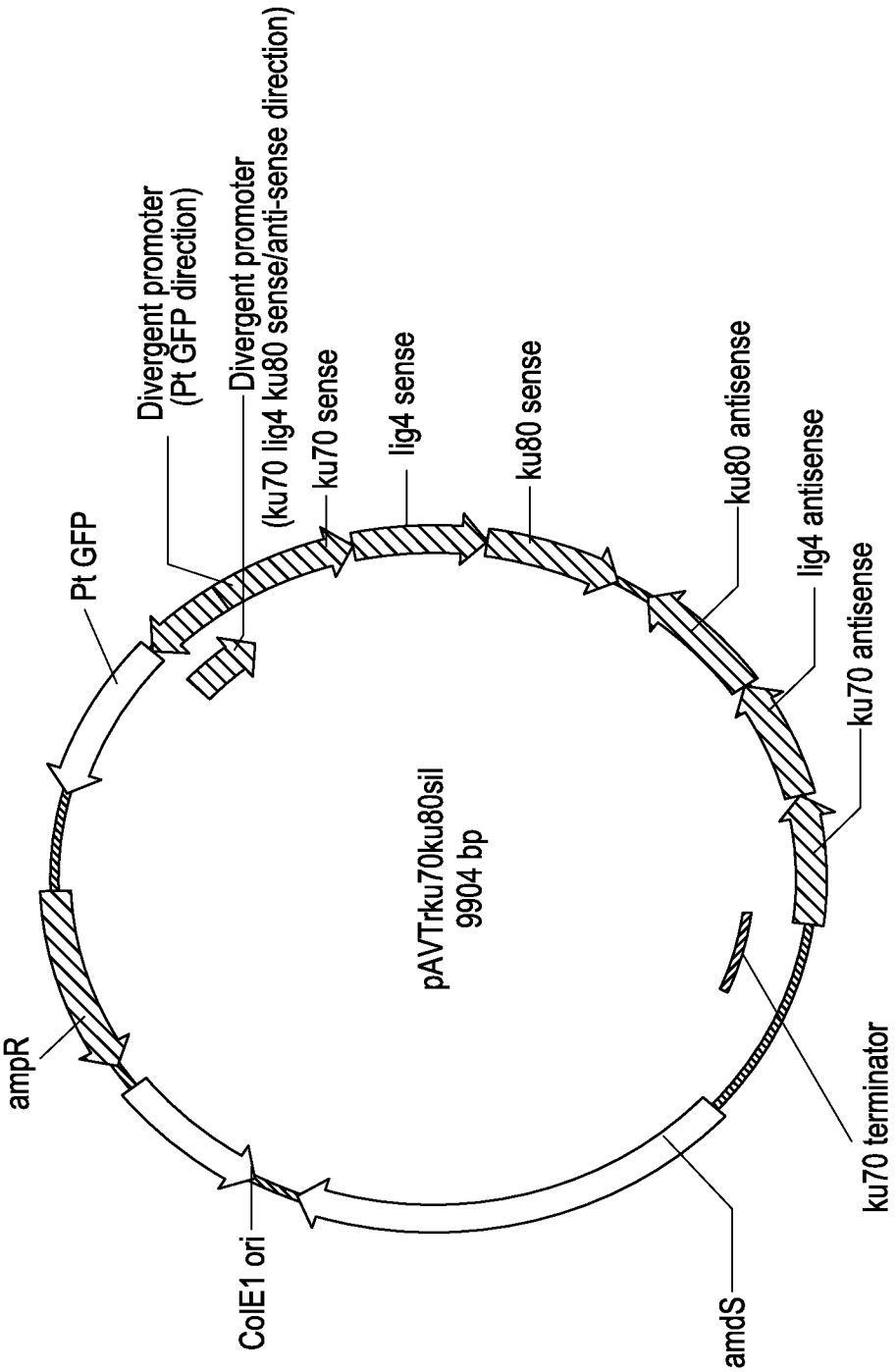
Divergent promoter
(Pt GFP direction)
Divergent promoter
(ku70 lig4 ku80 sense/anti-sense direction)
ku70 sense
lig4 sense
ku80 sense
ku80 antisense
lig4 antisense
ku70 antisense
Pt GFP
pAVTrku70ku80sil
9904 bp
ampR
ColE1 ori
amdS
ku70 terminator

FIG. 2C

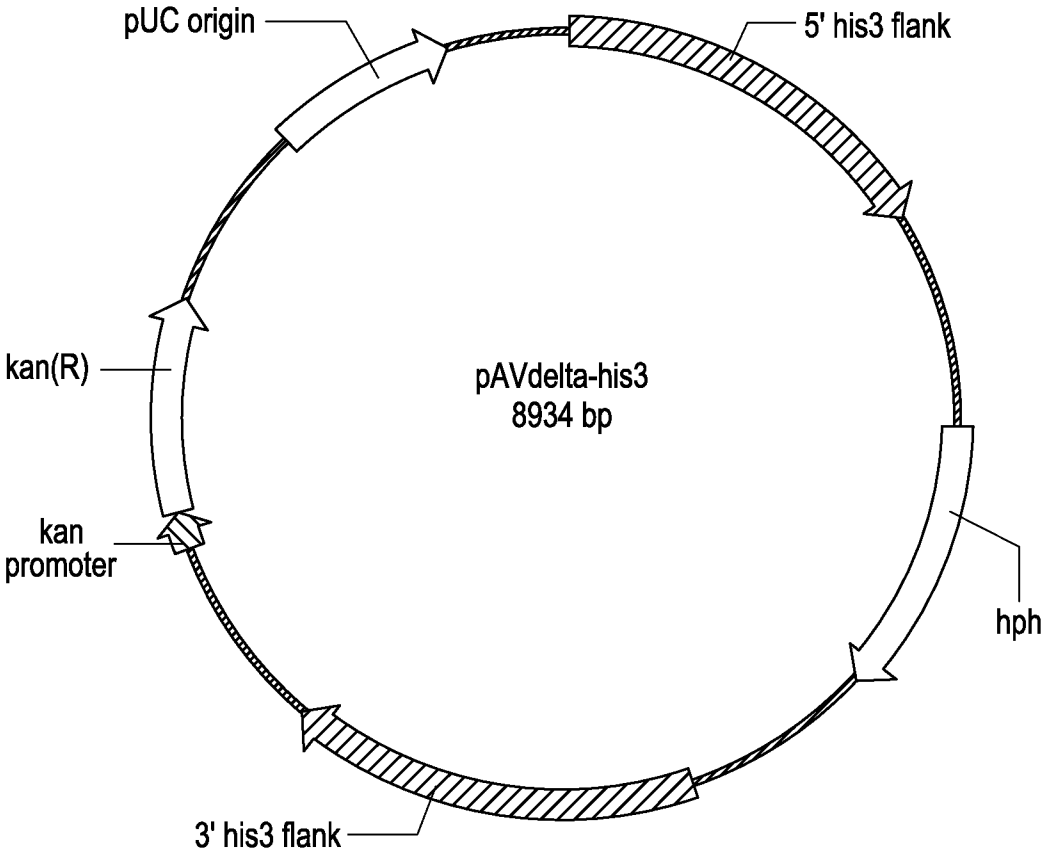
pUC origin
5' his3 flank
kan(R)
pAVdelta-his3
8934 bp
kan
promoter
hph
3' his3 flank

FIG. 3

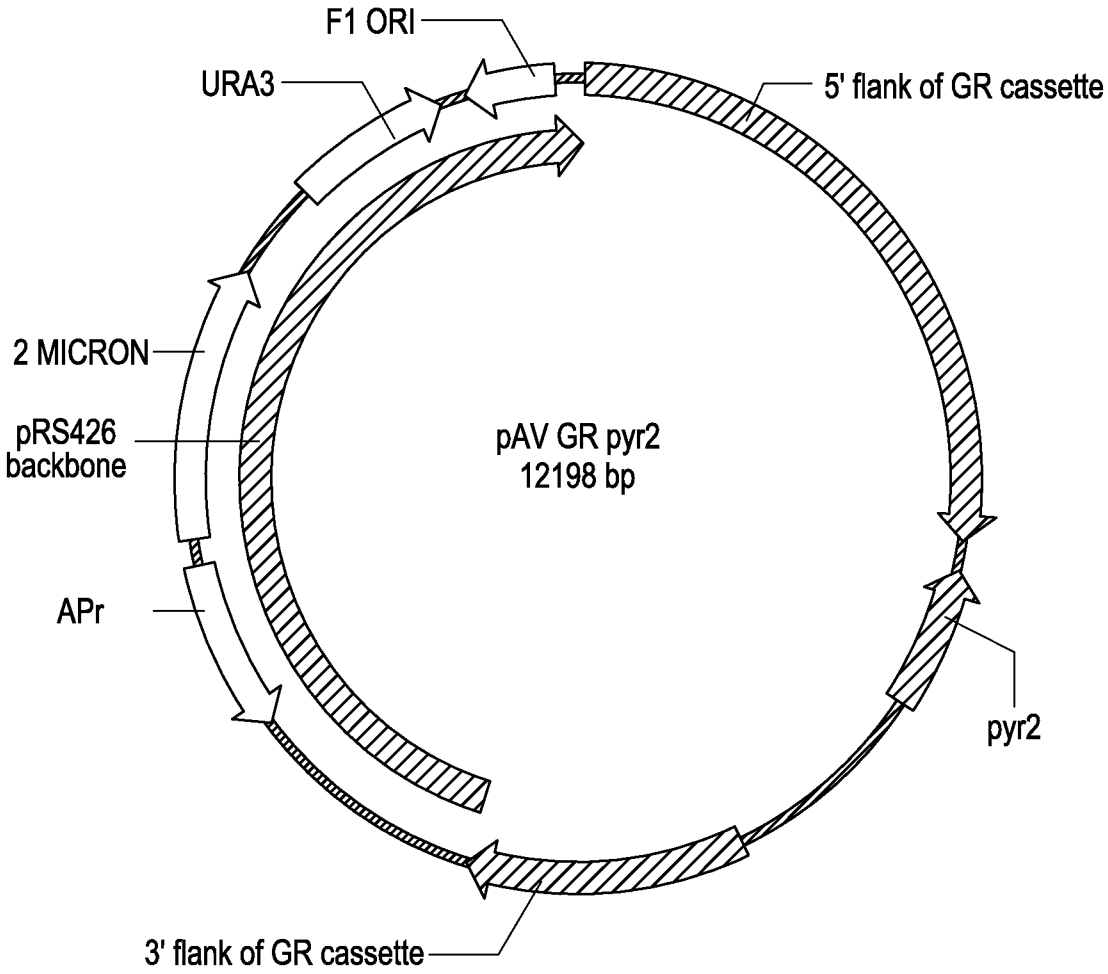
F1 ORI
URA3
5' flank of GR cassette
2 MICRON
pRS426
backbone
pAV GR pyr2
12198 bp
APr
pyr2
3' flank of GR cassette

FIG. 4

X

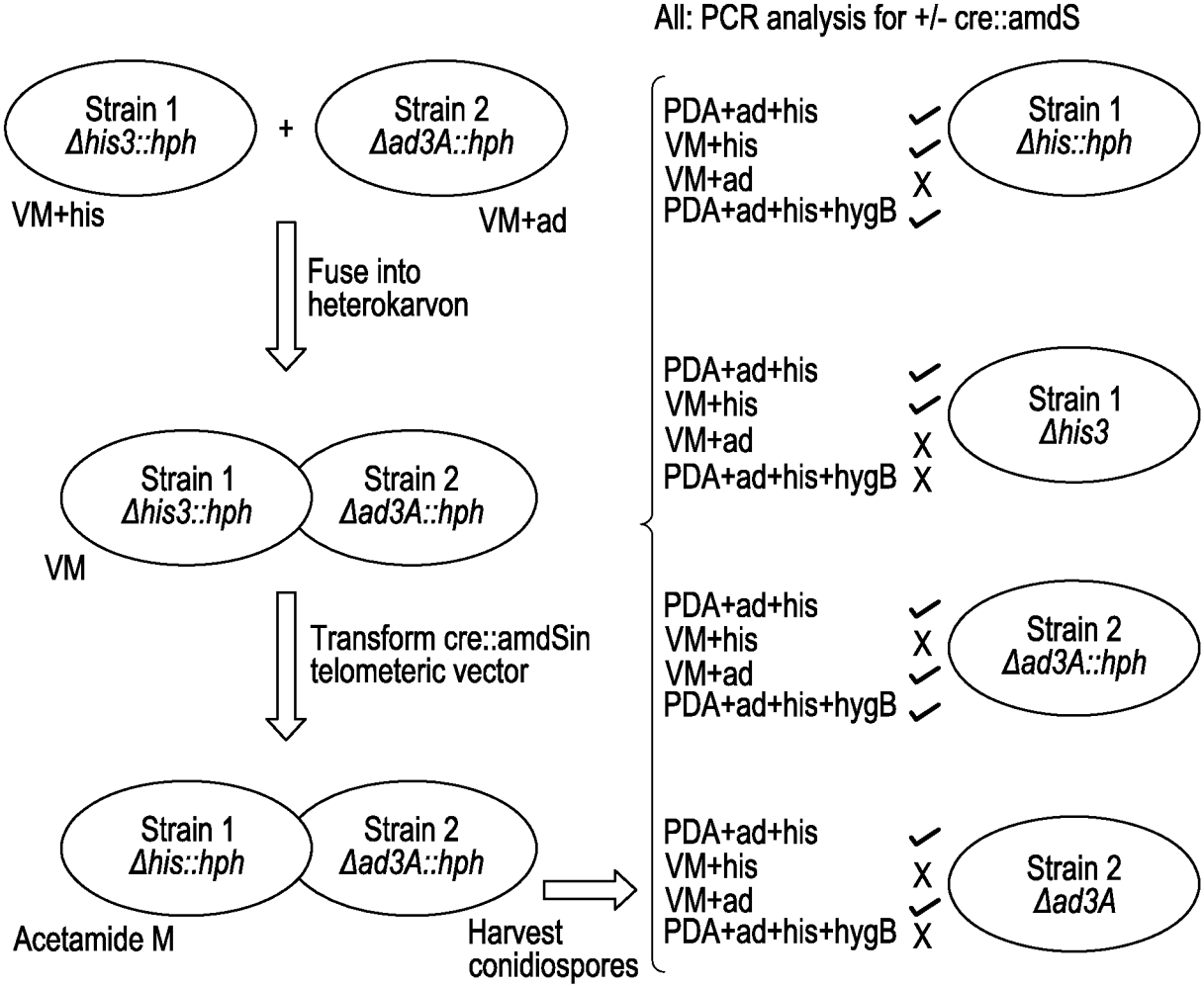
All: PCR analysis for +/- cre::amdS
Strain 1
Δhis3::hph
+
Strain 2
Δad3A::hph
VM+his
VM+ad
Fuse into
heterokarvon
Strain 1
Δhis3::hph
Strain 2
Δad3A::hph
VM
Transform cre::amdSin
telometeric vector
Strain 1
Δhis::hph
Strain 2
Δad3A::hph
Acetamide M
Harvest
conidiospores
PDA+ad+his ✓
VM+his ✓
VM+ad X
PDA+ad+his+hygB ✓
Strain 1
Δhis::hph
PDA+ad+his ✓
VM+his ✓
VM+ad X
PDA+ad+his+hygB X
Strain 1
Δhis3
PDA+ad+his ✓
VM+his X
VM+ad ✓
PDA+ad+his+hygB ✓
Strain 2
Δad3A::hph
PDA+ad+his ✓
VM+his X
VM+ad ✓
PDA+ad+his+hygB X
Strain 2
Δad3A

FIG. 5

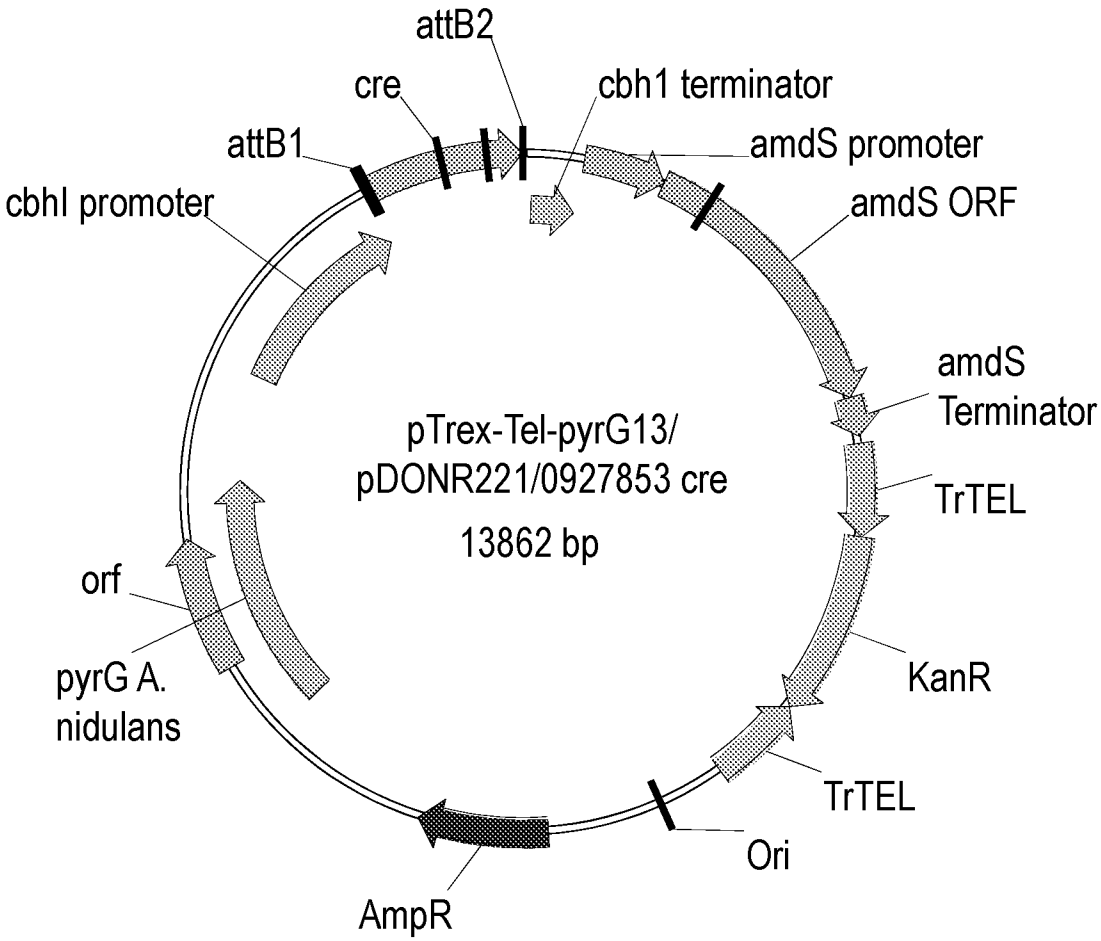
attB2
cre
cbh1 terminator
attB1
amdS promoter
cbhI promoter
amdS ORF
amdS
Terminator
pTrex-Tel-pyrG13/
pDONR221/0927853 cre
13862 bp
TrTEL
orf
pyrG A.
nidulans
KanR
TrTEL
Ori
AmpR

FIG. 6

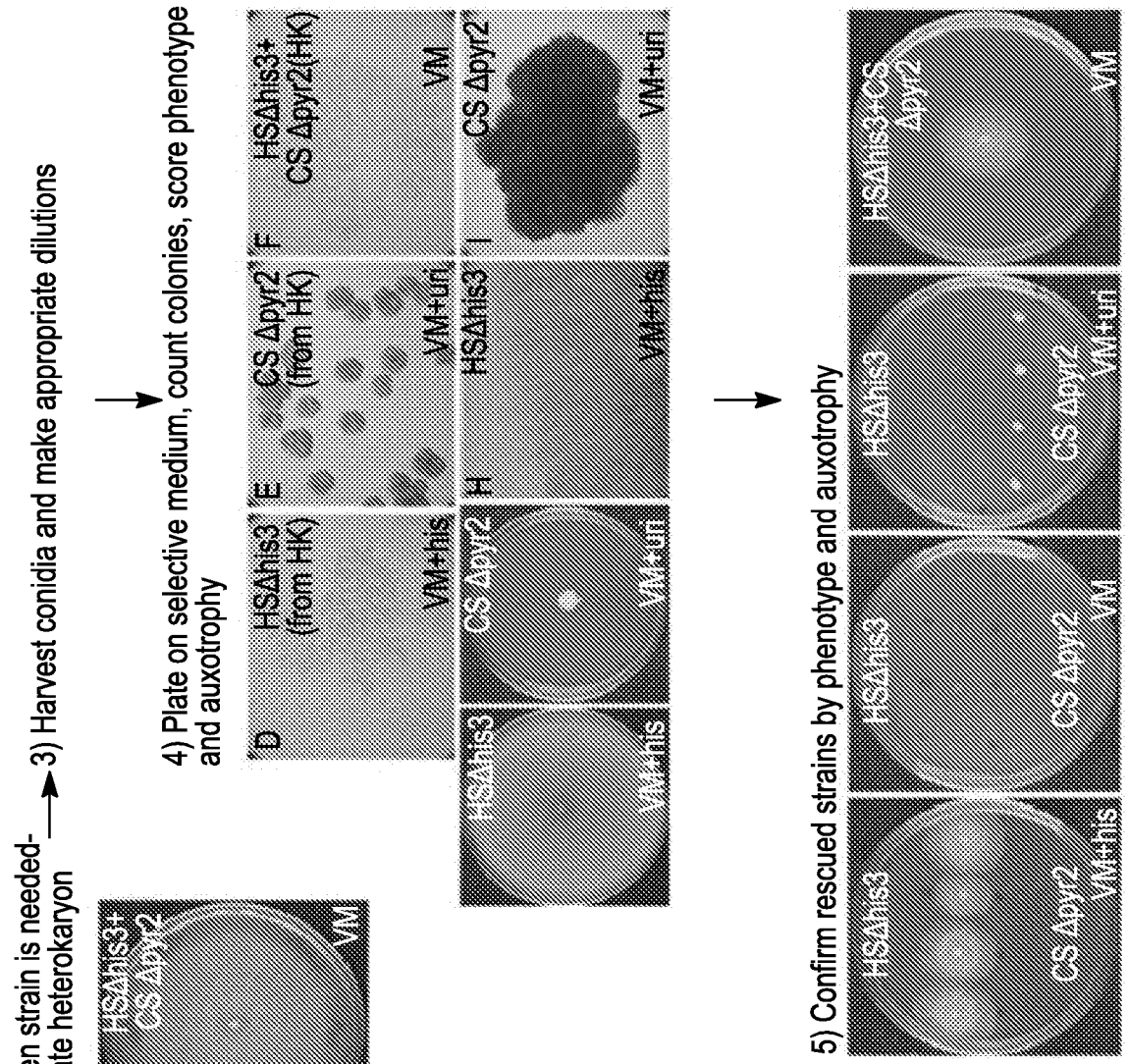
1) Fuse component strains into heterokaryon
2) When strain is needed- conidiate heterokaryon
3) Harvest conidia and make appropriate dilutions
4) Plate on selective medium, count colonies, score phenotype and auxotrophy
5) Confirm rescued strains by phenotype and auxotrophy
HS=Helper Strain
CS=Colonial Strain
VM=Minimal Medium
HK=Heterokaryon
his=L-histidine
uri=Uridine
HSΔnis3+ CS Δpyr2
VMS
VM
HSΔnis3 (from HK)
CS Δpyr2 (from HK)
HSΔnis3+ CS Δpyr2(HK)
VM+his
VM+uri
VM+his+uri
HSΔnis3
CS Δpyr2
HSΔnis3+CS Δpyr2

FIG. 7

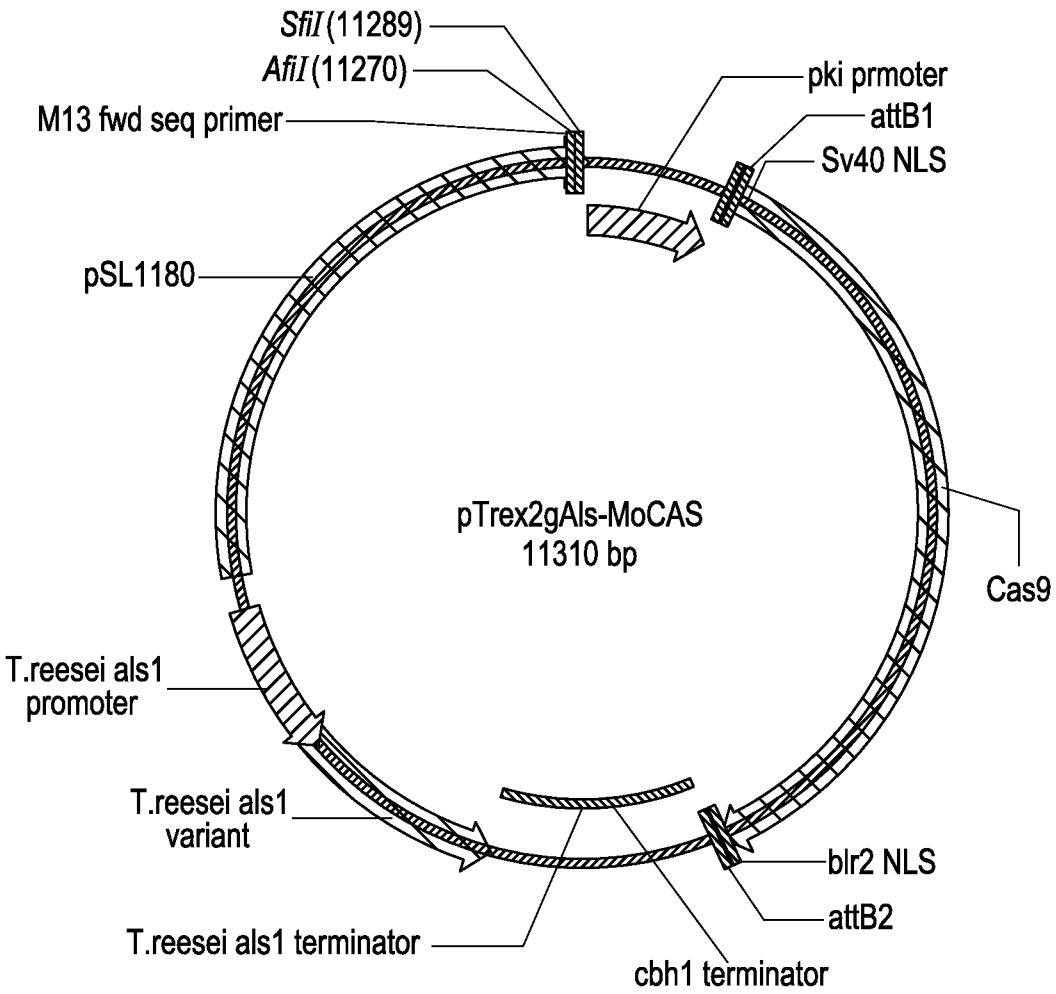
SfiI (11289)
AfiI (11270)
M13 fwd seq primer
pki prmoter
attB1
Sv40 NLS
pSL1180
pTrex2gAls-MoCAS
11310 bp
Cas9
T.reesei als1
promoter
T.reesei als1
variant
blr2 NLS
attB2
T.reesei als1 terminator
cbh1 terminator

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2015/066178

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/90
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, BIOSIS, COMPENDEX, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GUANGTAO Z ET AL: "Gene targeting in a nonhomologous end joining deficient Hypocrea jecorina", JOURNAL OF BIOTECHNOLOGY, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 139, no. 2, 15 January 2009 (2009-01-15), pages 146-151, XP025842466, ISSN: 0168-1656, DOI: 10.1016/J.JBIOTEC.2008.10.007 [retrieved on 2008-11-05] cited in the application the whole document, in particular abstract, Fig. 1 and paragraph 3.1. ----- -/--	22

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 April 2016	21/04/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bassias, Ioannis

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE BOER P ET AL: "Highly efficient gene targeting in Penicillium chrysogenum using the bi-partite approach in DELTAlig4 or DELTAku70 mutants", FUNGAL GENETICS AND BIOLOGY, SAN DIEGO, CA, US, vol. 47, no. 10, 1 October 2010 (2010-10-01), pages 839-846, XP027264914, ISSN: 1087-1845 [retrieved on 2010-07-24] the whole document, in particular abstract and Fig. 1 and 3	22
X	TADASHI TAKAHASHI ET AL: "Enhanced gene targeting frequency in ku70 and ku80 disruption mutants of Aspergillus sojae and Aspergillus oryzae", MOLECULAR GENETICS AND GENOMICS, SPRINGER, BERLIN, DE, vol. 275, no. 5, 10 February 2006 (2006-02-10), pages 460-470, XP019428009, ISSN: 1617-4623, DOI: 10.1007/S00438-006-0104-1 the whole document, in particular abstract and Fig. 1 and 2	22
X	J. E. DICARLO ET AL: "Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems", NUCLEIC ACIDS RESEARCH, vol. 41, no. 7, 4 March 2013 (2013-03-04), pages 4336-4343, XP055086617, ISSN: 0305-1048, DOI: 10.1093/nar/gkt135 the whole document	22
X	M. LEVY ET AL: "Efficient gene replacement and direct hyphal transformation in Sclerotinia sclerotiorum", MOLECULAR PLANT PATHOLOGY, vol. 9, no. 5, 1 September 2008 (2008-09-01), pages 719-725, XP055263206, GB ISSN: 1464-6722, DOI: 10.1111/j.1364-3703.2008.00483.x the whole document	22

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MAIER F J ET AL: "Development of a highly efficient gene targeting system for Fusarium graminearum using the disruption of a polyketide synthase gene as a visible marker", FEMS YEAST RESEARCH, WILEY-BLACKWELL PUBLISHING LTD, GB, NL, vol. 5, no. 6-7, 1 April 2005 (2005-04-01) , pages 653-662, XP027680477, ISSN: 1567-1356 [retrieved on 2005-04-01] the whole document, in particular abstract and Fig. 1	22
X	MIZUTANI O ET AL: "A defect of LigD (human Lig4 homolog) for nonhomologous end joining significantly improves efficiency of gene-targeting in Aspergillus oryzae", FUNGAL GENETICS AND BIOLOGY, SAN DIEGO, CA, US, vol. 45, no. 6, 1 June 2008 (2008-06-01), pages 878-889, XP022665003, ISSN: 1087-1845, DOI: 10.1016/J.FGB.2007.12.010 [retrieved on 2008-01-11] the whole document, in particular abstract and Fig. 2	22
X	WO 2008/138835 A1 (NOVOZYMES AS [DK]; VIND JESPER [DK]) 20 November 2008 (2008-11-20) the whole document, in particular the claims	22
A	DEWEI JIANG ET AL: "Molecular tools for functional genomics in filamentous fungi: Recent advances and new strategies", BIOTECHNOLOGY ADVANCES., vol. 31, no. 8, 1 December 2013 (2013-12-01), pages 1562-1574, XP055250956, GB ISSN: 0734-9750, DOI: 10.1016/j.biotechadv.2013.08.005 the whole document, in particular p. 1569, paragraph 3.3.	1-22
A	US 5 683 899 A (STUART W DORSEY [US]) 4 November 1997 (1997-11-04)	1-22

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PRASHANT MALI ET AL: "Cas9 as a versatile tool for engineering biology", NATURE METHODS, NATURE PUBLISHING GROUP, GB, vol. 10, no. 10, 1 October 2013 (2013-10-01), pages 957-963, XP002718606, ISSN: 1548-7105, DOI: 10.1038/NMETH.2649 [retrieved on 2013-09-27] -----	1-22

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2015/066178

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		DK 2147107 T3	24-10-2011
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		WO 9801534 A1	15-01-1998