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(54) Title: ASPERGILLUS NIGER BETA-GLUCOSIDASE GENE, PROTEIN AND USES THEREOF

(57) Abrégé/Abstract:
A polypeptide having β-glucosidase enzymatic activity, a polynucleotide encoding the polypeptide, a nucleic acid constructs carrying the polynucleotide, transformed or infected cells, such as yeast cells, and transgenic organisms expressing the polynucleotide and various uses of the polypeptide, the polynucleotide, cells and/or organisms, including, producing a recombinant polypeptide having the β-glucosidase enzymatic activity, increasing the level of aroma compounds in alcoholic beverages, as well as other fermentation products of plant material hydrolyzing cellobiose and thus increasing the level of fermentable glucose, increasing the production of alcohol, such as ethanol from plant material increasing the aroma released from a plant or a plant product, and hydrolysis or transglycosylation of glycosides

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(54) Title: ASPEGILLUS NIGER BETA-GLUCOSIDASE GENE, PROTEIN AND USES THEREOF

(57) Abstract: A polypeptide having β-glucosidase enzymatic activity, a polynucleotide encoding the polypeptide, a nucleic acid constructs carrying the polynucleotide, transformed or infected cells, such as yeast cells, and transgenic organisms expressing the polynucleotide and various uses of the polypeptide, the polynucleotide, cells and/or organisms, including, producing a recombinant polypeptide having the β-glucosidase enzymatic activity, increasing the level of aroma compounds in alcoholic beverages, as well as other fermentation products of plant material, hydrolyzing cellobiose and thus increasing the level of fermentable glucose, increasing the production of alcohol, such as ethanol from plant material, increasing the aroma released from a plant or a plant product, and hydrolysis or transglycosylation of glycosides.

ASPEGILLUS NIGER β-GLUCOSIDASE GENE, PROTEIN AND USES THEREOF

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to a polypeptide having β-glucosidase enzymatic activity, to a polynucleotide encoding the polypeptide, to nucleic acid constructs carrying the polynucleotide, to transformed or infected cells, such as yeast cells, and organisms expressing the polynucleotide and to various uses of the polypeptide, the polynucleotide, cells and/or organisms, including, but not limited to, producing a recombinant polypeptide having β-glucosidase enzymatic activity, increasing the level of aroma compounds in alcoholic beverages, as well as other fermentation products of plant material, hydrolyzing cellobiose and thus increasing the level of fermentable glucose, to increase production of alcohol, such as ethanol from plant material, increasing the aroma released from a plant or a plant product, and hydrolysis or transglycosylation of glycosides.

Abbreviations used herein include: BGL1 - *Aspergillus niger* B1 β-glucosidase; bgl1 - a cDNA encoding same; 2FGlcF - 2-deoxy-2-fluoro β-glucosyl fluoride; DNP - 2,4-dinitrophenol; DNPGlc - 2,4-dinitrophenyl β-D-glucopyranoside; pNP - p-nitrophenol; pNPGlc - p-nitrophenyl β-D-glucopyranoside; MUGlc - 4-methylumbeliferyl-β-D-glucopyranoside; YNB - yeast nitrogen base without amino acids; and X-glu - 5-bromo-4-chloro-3-indolyl β-D-glucopyranoside.

β-Glucosidases (EC 3.2.1.21; β-D-glucoside glucohydrolase) play a number of different important roles in biology, including the degradation of cellulosic biomass by fungi and bacteria, degradation of glycolipids in mammalian lysosomes and the cleavage of glucosylated flavonoids in plants. These enzymes are therefore of considerable industrial interest, not only as constituents of cellulose-degrading systems, but also in the food industry (2, 3).

Aspergillus species are known as a useful source of β-glucosidases (4-6), and *Aspergillus niger* is by far the most efficient producer of β-glucosidase among the microorganisms investigated (4). Shoseyov *et al.* (7) have previously described a β-glucosidase from *Aspergillus niger* B1 (CMI CC 324626) which is active at low pHs, as well as in the presence of high ethanol concentrations. This enzyme effectively hydrolyzes flavor-compound glycosides in certain low-pH products, such as wine and passion fruit juice, thereby enhancing their flavor (8-12), and is particularly attractive for use in the food industry, as *A. niger* is considered non-toxic (3). In addition, β-glucosidase was found useful in enzymatic synthesis of glycosides (13-15). Other *A. niger* β-glucosidases have also been purified (16-18), however, differences in their properties have been reported, including ranges of molecular weights (116-137 kDa), isoelectric points (pI values of 3.8-4) and pH optima (3.4-4.5). Indeed, at least two β-glucosidases, with distinct substrate specificities, have been identified in commercial *A. niger* β-glucosidase preparations (19). Attempts to clear this confusion by cloning and expression of a functional *A. niger* β-glucosidase gene in *S. cerevisiae* has been previously reported (20), however the protein was not characterized, and the sequence was not published.

Glycosidases have been assigned to families on the basis of sequence similarities, there now being some 77 different such families defined containing over 2,000 different enzymes (21, see also http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html). With the exception of the glucosylceramidases (Family 30), all simple β-glucosidases belong to either Family 1 or 3. Family 1 contains enzymes from bacteria, plants and mammals, including also 6-phospho-glucosidases and thioglucosidases. Furthermore, most Family 1 enzymes also have significant galactosidase activity. Family 3 contains β-glucosidases and hexosaminidases of fungal, bacterial and plant origin. Enzymes of both families hydrolyze their substrates with net retention of

anomeric configuration, presumably via a two-step, double-displacement mechanism, involving two key active site carboxylic acid residues (for reviews of mechanism, see 22-24). In the first step, one of the carboxylic acids (the nucleophile) attacks at the substrate anomeric center, while the other (the acid/base catalyst) protonates the glycosidic oxygen, thereby assisting the departure of the aglycone. This results in the formation of a covalent α-glycosyl-enzyme intermediate. In a second step this intermediate is hydrolyzed by general base-catalyzed attack of water at the anomeric center of the glycosyl-enzyme, to release the β-glucose product and regenerate free enzyme. Both the formation and the hydrolysis of this intermediate proceed via transition states with substantial oxocarbenium ion character.

Given that Family 3 contains fungal enzymes of similar mass, including those from other *Aspergillus* sp., it is likely that the *Aspergillus niger* β-glucosidase would be a member of this family. Mechanistic information on this family is relatively sparse: the best characterized being the glycosylated 170 kDa β-glucosidase from *Aspergillus wentii*. By labeling the active site with conduritol B-epoxide, this enzyme was shown to carry out hydrolysis, with net retention of anomeric configuration. This study has demonstrated that the labeled aspartic acid residue was the same as that derivatized by the slow substrate D-glucal (1, 25). Furthermore, it was shown that the 2-deoxyglucosyl-enzyme, trapped by use of D-glucal, was kinetically identical to that formed during the hydrolysis of PNP-2-deoxy-β-D- glucopyranoside (26). Further detailed kinetic analysis of the enzyme was performed by Legler *et al.* (27), including measurement of Hammett relationships, kinetic isotope effects and studies of the binding of potent reversible inhibitors, such as gluconolactone and nojirimycin.

While reducing the present invention to practice, the β-glucosidase protein was isolated from *Aspergillus niger*, purified, cloned, sequenced, expressed in yeast host cells and its enzymatic function characterized. In

addition, the protein as well as signal peptide fused thereto and optionally an endoplasmic reticulum retaining peptide fused thereto were expressed in transgenic plants and the release of aroma substances therefrom following homogenization monitored. The enzyme encoded by the isolated gene, as described above, is of known usefulness in plant and/or plant products, as well as in biotechnological processes, including the food industry. Several unexpected advantages were uncovered, including, but not limited to, pH and temperature stability of the β-glucosidase from *Aspergillus niger*, requirement for a signal peptide for obtaining catalytic activity when expressed in plants. Advantage for an endoplasmic retaining peptide or for a lack thereof when expressed in plants, depending on the application.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided an isolated nucleic acid comprising a genomic, complementary or composite polynucleotide preferably being derived from *Aspergillus niger*, encoding a polypeptide having a β-glucosidase catalytic activity and preferably further encoding, in frame, a signal peptide and an endoplasmic reticulum retaining peptide.

According to another aspect of the present invention there is provided a recombinant protein comprising a polypeptide having a β-glucosidase catalytic activity, the polypeptide is preferably derived from *Aspergillus niger* and it preferably fused to a signal peptide and optionally also to an endoplasmic reticulum retaining peptide.

According to yet another aspect of the present invention there is provided a nucleic acid construct comprising the isolated nucleic acid described herein.

According to still another aspect of the present invention there is provided host cell or an organism, such as a plant, comprising the nucleic acid or nucleic acid construct described herein.

According to further features in preferred embodiments of the invention described below, the polynucleotide is as set forth in SEQ ID NOs:1, 3 or a portion thereof.

According to still further features in the described preferred embodiments, the nucleic acid construct further comprising at least one cis acting control element for regulating expression of the polynucleotide.

According to still further features in the described preferred embodiments, the host cell is selected from the group consisting of a prokaryotic cell and a eukaryotic cell.

According to still further features in the described preferred embodiments the prokaryotic cell is E. coli.

According to still further features in the described preferred embodiments the eukaryotic cell is selected from the group consisting of a yeast cell, a fungous cell, a plant cell and an animal cell.

According to still further features in the described preferred embodiments the polypeptide is as set forth in SEQ ID NO: 2 or a portion thereof having the β-glucosidase catalytic activity.

According to an additional aspect of the present invention there is provided a method of producing recombinant β-glucosidase, the method comprising the step of introducing, in an expressible form, a nucleic acid construct into a host cell, the nucleic acid construct including a genomic, complementary or composite polynucleotide preferably derived from *Aspergillus niger*, encoding a polypeptide having a β-glucosidase catalytic activity and preferably further encoding, in frame, a signal peptide and an endoplasmic reticulum retaining peptide.

According to further features in preferred embodiments of the invention described below, the method further comprising the step of extracting the polypeptide having the β-glucosidase catalytic activity.

According to yet an additional aspect of the present invention there is provided a method of producing a recombinant β-glucosidase overexpressing cell, the method comprising the step of introducing, in an overexpressible form, a nucleic acid construct into a host cell, the nucleic acid construct including a genomic, complementary or composite polynucleotide preferably derived from *Aspergillus niger*, encoding a polypeptide having a β-glucosidase catalytic activity and preferably further encoding, in frame, a signal peptide and an endoplasmic reticulum retaining peptide.

According to still an additional aspect of the present invention there is provided a method of increasing a level of at least one fermentation substance in a fermentation product, the method comprising the step of fermenting a glucose containing fermentation starting material by a yeast cell overexpressing a nucleic acid construct including a genomic, complementary or composite polynucleotide being preferably derived from *Aspergillus niger*, encoding a polypeptide having a β-glucosidase catalytic activity and preferably further encoding, in frame, a signal peptide and an endoplasmic reticulum retaining peptide, thereby increasing the level of the at least one fermentation substance in the fermentation product.

According to a further aspect of the present invention there is provided a method of increasing a level of at least one fermentation substance in a fermentation product, the method comprising the step of fermenting a plant derived glucose containing fermentation starting material by a yeast cell, the plant overexpressing a nucleic acid construct including a genomic, complementary or composite polynucleotide preferably derived from *Aspergillus niger*, encoding a polypeptide having a β-glucosidase catalytic activity and preferably further encoding, in frame, a signal peptide and an endoplasmic reticulum retaining peptide, thereby increasing the level of the at least one fermentation substance in the fermentation product.

According to a further aspect of the present invention there is provided a method of increasing a level of at least one aroma substance in a plant

derived product, the method comprising the step of incubating a glucose containing plant starting material with a yeast cell overexpressing a nucleic acid construct including a genomic, complementary or composite polynucleotide preferably derived from *Aspergillus niger*, encoding a polypeptide having a β-glucosidase catalytic activity and preferably further encoding, in frame, a signal peptide and an endoplasmic reticulum retaining peptide, thereby increasing the level of the at least one aroma substance in the plant derived product.

According to yet a further aspect of the present invention there is provided a method of increasing a level of at least one aroma substance in a plant derived product, the method comprising the step of incubating a glucose containing plant starting material with a yeast cell, said plant overexpressing a nucleic acid construct including a genomic, complementary or composite polynucleotide preferably derived from *Aspergillus niger*, encoding a polypeptide having a β-glucosidase catalytic activity and preferably further encoding, in frame, a signal peptide and an endoplasmic reticulum retaining peptide, thereby increasing the level of the at least one aroma substance in the plant derived product.

According to still further features in the described preferred embodiments the plant derived product is a fermentation product, such as, but not limited to, an alcoholic beverage.

According to still a further aspect of the present invention there is provided a method of increasing a level of free glucose in a glucose containing fermentation starting material, the method comprising the step of fermenting the glucose containing fermentation starting material by a cell overexpressing a nucleic acid construct including a genomic, complementary or composite polynucleotide preferably derived from *Aspergillus niger*, encoding a polypeptide having a β-glucosidase catalytic activity and preferably further encoding, in frame, a signal peptide and an endoplasmic reticulum retaining

peptide, thereby increasing the level of the free glucose in the glucose containing fermentation starting material.

According to another aspect of the present invention there is provided a method of increasing a level of free glucose in a plant derived glucose containing fermentation starting material, the method comprising the step of fermenting the plant derived glucose containing fermentation starting material by a cell, the plant overexpressing a nucleic acid construct including a genomic, complementary or composite polynucleotide preferably derived from *Aspergillus niger*, encoding a polypeptide having a β-glucosidase catalytic activity and preferably further encoding, in frame, a signal peptide and an endoplasmic reticulum retaining peptide, thereby increasing the level of the free glucose in the plant.

According to yet another aspect of the present invention there is provided a method of increasing a level of free glucose in a plant, the method comprising the step of overexpressing in the plant a nucleic acid construct including a genomic, complementary or composite polynucleotide preferably derived from *Aspergillus niger*, encoding a polypeptide having a β-glucosidase catalytic activity and preferably further encoding, in frame, a signal peptide and an endoplasmic reticulum retaining peptide, thereby increasing the level of the free glucose in the plant.

According to still another aspect of the present invention there is provided a method of producing an alcohol, the method comprising the step of fermenting a glucose containing fermentation starting material by a cell overexpressing a nucleic acid construct including a genomic, complementary or composite polynucleotide preferably derived from *Aspergillus niger*, encoding a polypeptide having a β-glucosidase catalytic activity and preferably further encoding, in frame, a signal peptide and an endoplasmic reticulum retaining peptide, and extracting the alcohol therefrom.

According to an additional aspect of the present invention there is provided a method of producing an alcohol, the method comprising the step of

fermenting a plant derived glucose containing fermentation starting material by a cell, the plant overexpressing a nucleic acid construct including a genomic, complementary or composite polynucleotide preferably derived from *Aspergillus niger*, encoding a polypeptide having a β-glucosidase catalytic activity and preferably further encoding, in frame, a signal peptide and an endoplasmic reticulum retaining peptide, and extracting the alcohol therefrom.

According to an additional aspect of the present invention there is provided a method of producing an aroma spreading plant, the method comprising the step of overexpressing in the plant a nucleic acid construct including a genomic, complementary or composite polynucleotide preferably derived from *Aspergillus niger*, encoding a polypeptide having a β-glucosidase catalytic activity and preferably further encoding, in frame, a signal peptide and an endoplasmic reticulum retaining peptide, thereby increasing aroma spread from the plant.

According to further features in preferred embodiments of the invention described below, overexpressing the nucleic acid construct is performed in a tissue specific manner.

According to still further features in the described preferred embodiments overexpressing the nucleic acid construct is limited to at least one tissue selected from the group consisting of flower, fruit, seed, root, stem, pollen and leaves.

The present invention successfully addresses the shortcomings of the presently known configurations by providing a polypeptide having β-glucosidase enzymatic activity, a polynucleotide encoding the polypeptide, a nucleic acid constructs carrying the polynucleotide, transformed or infected cells, such as yeast cells, and organisms expressing the polynucleotide and various uses of the polypeptide, the polynucleotide, cells and/or organisms, including, but not limited to, producing a recombinant polypeptide having β-glucosidase enzymatic activity, increasing the level of aroma compounds in alcoholic beverages, as well as other fermentation products of plant material,

hydrolyzing cellobiose and thus increasing the level of fermentable and/or free glucose, to increase production of a fermentation product, such as ethanol from plant material, increasing the aroma released from a plant or a plant product, and hydrolysis or transglycosylation of glycosides.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIGs. 1a-c demonstrate plasmid maps employed as expression vectors for bgl1 cDNA. Figure 1a - *E. coli* expression vector containing bgl1 cDNA, inserted into the *NcoI*/*Bam*HI sites of pET3d. Figure 1b - *S. cerevisiae* expression vector containing bgl1 cDNA, inserted into the *Hin*dIII/*Bam*HI sites of pYES2-bgl1 plasmid. Figure 1c - *P. pastoris* expression vector containing bgl1 cDNA, inserted into the *Eco*RI/*Bam*HI sites of pHIL-S1.

FIGs. 2a-b demonstrates SDS-PAGE analysis of active protein samples eluted from a Mono-Q*column, stained with coomassie blue (Figure 2a), or β-glucosidase zymogram (Figure 2b) using MUGlc as a substrate. Lanes (for both Figures 2a and 2b): 1 - Electroeluted band of BGL1 from preparative PAGE-SDS gel stabs; 2, 3, 4, 5 - acetone precipitates from Mono-Q separation of BGL1.

*Trademark

FIG. 3 demonstrates SDS-PAGE analysis of purified β-glucosidase by Mono-Q and Resource-S.* Lanes: 1 - crude (27.5 μg protein); 2 - active fraction after Mono-Q (7 μg protein); and 3 - active fraction after Resource-S (10 μg protein).

FIG. 4 demonstrates SDS-PAGE analysis of β-glucosidase deglycosylated by N-glycosidase-F. Lanes: 1 - molecular weight marker; 2 - native β-glucosidase; and 3 - deglycosylated protein.

FIG. 5a demonstrates the DNA and amino acid sequences of bgl1. Amino acid sequences determined by Edman degradation are underlined. DNA sequences of introns are underlined. Signal peptide is indicated by italic letters.

FIG 5b. demonstrates bgl1 gene organization. Exons (E1-7) are indicated by filled boxes, introns by solid lines, restriction sites and the stop codon by arrows.

FIG. 6a demonstrates a Western blot analysis of recombinant BGL1 expressed in *S. cerevisiae*. Lanes: 1 - native BGL1 (positive control); 2 - total protein extract of *S. cerevisiae* expressing recombinant BGL1; 3 - total protein extract of *S. cerevisiae* without the bgl1 expression vector (negative control).

FIG. 6b demonstrates a Western blot analysis of recombinant BGL1 secreted from *P. pastoris*. Lanes: 1 - molecular weight marker; 2 - medium supernatant of *P. pastoris* expressing recombinant BGL1; 3 - medium supernatant of *P. pastoris* host without the vector (negative control).

FIG. 7 demonstrates proton-NMR spectra, illustrating the stereochemical course of pNPGlc hydrolysis by *A. niger* β-glucosidase. Spectra are for the anomeric proton region of the substrate at different time intervals relative to addition of the enzyme.

FIG. 8 demonstrates inactivation of recombinant BGL1 by 2FGlcF. Pure enzyme was incubated in the presence of various concentrations of the inactivator, and residual enzyme activity was determined at different time

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intervals. Residual activity is presented, semilogarithmically, versus time, in the presence of the indicated concentrations of inactivator.

FIG. 9 demonstrates reactivation of 2-deoxy-2-fluoroglucosyl-recombinant BGL1 by linamarin. Activity is plotted versus incubation time in the presence of the indicated concentrations of linamarin.

FIG. 10 demonstrates the stability of recombinant *A. niger* β-glucosidase at various temperatures. Activity is calculated as percent of a recombinant enzyme solution kept at 4 °C.

FIGs. 11a-c show schematic depictions of expression cassettes used for expression of *A. niger* β-glucosidase in tobacco plants. Figure 11a - a cassette encoding BGL1 without a signal peptide (see, SEQ ID NO:13 for the nucleotide sequence and SEQ ID NO:14 for the amino acid sequence); Figure 11b - a cassette encoding a BGL1 fused to a Cel1 signal peptide for secretion into the apoplast (see, SEQ ID NO:15 for the nucleotide sequence and SEQ ID NO:16 for the amino acid sequence); and Figure 11c - a cassette encoding a BGL1 fused to Cel1 signal peptide as in Figure 11b and in addition to HDEL (SEQ ID NO:17) ER-retaining peptide at the C-terminus for accumulation in the ER (see, SEQ ID NO:18 for the nucleotide sequence and SEQ ID NO:19 for the amino acid sequence).

FIG. 12 demonstrate PCR amplification results of bgl1 cDNA indicating the presence of bgl1 cDNA in transgenic plants. CB10 and CB11 - transgenic plants transformed with bgl1 and Cel1 signal peptide without HDEL ER retaining peptide. CBT3, CBT8 and CBT15 - different transgenic lines transformed with bgl1, Cel1 signal peptide and HDEL. B1 - a transgenic plants transformed with bgl1. 1kb - 1 kb DNA marker. WT - wild type non transgenic plant. pETB1 - bgl1 plasmid DNA.

FIGs. 13a-b show Western blot analyses of transgenic plants containing BGL1 without signal peptide (13a), and BGL1 with Cel1 signal peptide (13b), with and without HDEL ER retaining peptide. An gluco - purified *A. niger* beta-glucosidase. WT - nontransgenic control plant. B1, B15, B16, B20, B27,

B33 and B34 - different transgenic lines transformed with bgl1. CBT1, CBT 3, CBT 7 and CBT 8 - different transgenic lines transformed with bgl1, Cel1 signal peptide and HDEL. CB10 and CB12 - transgenic plants transformed with bgl1 and Cel1 signal peptide without HDEL ER retaining peptide.

FIG. 14 show activity gel analysis of transgenic tobacco plant extracts in SDS-PAGE incubated with MUGlu. WT - non-transgenic control plant. CB10 and CB11 - two independent lines of transgenic plants expressing BGL1 fused to Cel1 signal peptide (without HDEL). CBT3, CBT8 and CBT15 - independent lines of transgenic plants expressing BGL1 fused to Cel1 signal peptide at the N terminus and HDEL ER retaining peptide at the C terminus. B1 and B34 - transgenic plant expressing BGL1 without signal peptide or HDEL ER retaining peptide and which were positive for BGL1 protein in Western blot analysis. An Glu - control *A. niger* native beta-glucosidase.

FIG. 15 demonstrates level of BGL1 activity in different transgenic plants. WT - non-transgenic control plant. B1 and B21 - transgenic plants expressing BGL1 without signal peptide or HDEL ER retaining peptide and which were positive for BGL1 in Western blot analysis. CBT8, CBT21, CBT0 and CBT15 - independent lines of transgenic plants expressing BGL1 fused to Cel1 signal peptide at the N terminus and HDEL ER retaining peptide at the C terminus. CB12, CB13, CB14 and CB15 - four independent lines of transgenic plants expressing BGL1 fused to Cel1 signal peptide (without HDEL).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of a polypeptide having β-glucosidase enzymatic activity, a polynucleotide encoding the polypeptide, a nucleic acid constructs carrying the polynucleotide, transformed or infected cells, such as yeast cells, and organisms expressing the polynucleotide and various uses of the polypeptide, the polynucleotide, cells and/or organisms, including, but not limited to, producing a recombinant polypeptide having the β-glucosidase

enzymatic activity, increasing the level of aroma compounds in alcoholic beverages, as well as other fermentation products of plant material, hydrolyzing cellobiose and thus increasing the level of fermentable glucose, increasing the production of alcohol, such as ethanol from plant material, increasing the aroma released from a plant or a plant product, and hydrolysis or transglycosylation of glycosides.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of the components set forth in the following description or exemplified in the examples that follow. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

According to one aspect of the present invention there is provided an isolated nucleic acid comprising a genomic, complementary or composite polynucleotide encoding a polypeptide having a β-glucosidase catalytic activity. Preferably the polynucleotide is derived from *Aspergillus niger*, however other sources are applicable. These include all isolated polynucleotides encoding polypeptide having β-glucosidase catalytic activity. Such polynucleotides and polypeptides identified by their GenBank Accession Nos. are listed in Table 1 below, all of which can be used while implementing the present invention.

TABLE 1

Accession numbers of cDNA and their encoded beta-glucosidases (EC.3.2.1.21)

Organism	SWISS-PROT	EMBL
Acetobacter xylinus	O24749	AB003689; AB010645
Agrobacterium sp.	P12614	M19033; AAA22085.1
Agrobacterium tumefaciens	P27034	M59852; AAA22082.1

Arabidopsis thaliana	O82772, O24433, O23656	AF082157; AF082158; AC009327; U72153; U72155 AC020665; AC066691
Aspergillus aculeatus	P48825	D64088, BAA10968.1
Aspergillus kawachi	P87076	AB003470
Aspergillus niger B1		AJ132386; CAB75696.1
Aspergillus niger AMS1	Q9P456	AF268911
Avena sativa	Q38786, Q9ZP27	X78433; AF082991
Azospirillum irakense		AF090429; AAF21798.1
Bacillus circulans	Q03506	M96979; AAA22266.1
Bacillus sp. GL1	Q9ZNN7	AB009411; BAA36161.1; AB009410
Bacillus polymyxa	P22073, P22505	M60210; M60211
Bacillus subtilis	P40740	Z34526; CAA84287.1
Bacillus subtilis	P42403	D30762; BAA06429.1
Bacteroides fragilis	O31356	AF006658; AAB62870.1
Bifidobacterium breve	P94248, O08487	D84489; D88311
Botryotinia fuckeliana		AJ130890; CAB61489.1
Brassica napus	Q42618	X82577
Brassica nigra	O24434	U72154
Butyrivibrio fibrisolvens	P16084	M31120; AAA23008.1
Caldocellum saccharolyticum	P10482	X12575; CAA31087.1
Caldicellulosiruptor sp. 14B	Q9ZEN0	AJ131346
Candida wickerhamii	Q12601	U13672
Cavia porcellus	P97265	U50545
Cellulomonas biazotea	O51843	AF005277; AAC38196.1
Cellulomonas fimi	Q46043	M94865
Cellvibrio gilvus	P96316	D14068; BAA03152.1
Chryseobacterium meningosepticum	O30713	AF015915
Clostridium stercorarium	O08331	Z94045
Clostridium thermocellum	P26208	X60268; CAA42814.1
Clostridium thermocellum	P14002	X15644; CAA33665.1
Coccidioides immitis	O14424	U87805; AF022893
Costus speciosus	Q42707	D83177
Dalbergia cochinchinensis	Q9SPK3	AF163097
Dictyostelium discoideum	Q23892	L21014
Digitalis lanata	Q9ZPB6	AJ133406
Erwinia chrysanthemi	Q46684	U08606; AAA80156.1
Erwinia herbicola	Q59437	X79911; CAA56282.1
Escherichia coli	P33363	U15049; AAB38487.1
Escherichia coli K12/ MG1655	E65074, Q46829	U28375; AE000373
Glycine max		AF000378; AAD09291.1
Hansenula anomala	P06835	X02903; CAA26662.1
Homo sapiens		AJ278964; CAC08178.1
Hordeum vulgare	Q40025	L41869
Humicola grisea var.thermoidea	O93784	AB003109
Kluyveromyces marxianus	P07337	X05918; CAA29353.1
Lactobacillus plantarum	O86291	Y15954; AJ250202; CAB71149.1
Manihot esculenta	Q40283	X94986
Microbispora bispora	P38645	M97265; AAA25311.1
Nicotiana tabacum	O82151	AB017502; BAA33065.1
Orpinomyces sp. PC-2		AF016864; AAD45834.1
Oryza sativa	Q42975	U28047

Paenibacillus polymyxa	P22073	M60210; AAA22263.1
Paenibacillus polymyxa	P22505	M60211; AAA22264.1
Phaeosphaeria avenaria		AJ276675; CAB82861.1
Phanerochaete chrysosporium	O74203	AF036872; AF036873
Pichia anomala (Candida pelliculosa)	P06835	X02903
Pinus contorta		AF072736; AAC696.1
Polygonum tinctorium		AB003089; BAA78708.1
Prunus avium	Q43014	U39228
Prunus serotina	Q43073, Q40984	U50201; U26025
Prevotella albensis M384		AJ276021; CAC07184.1
Prevotella ruminicola	Q59716	U35425
Pyrococcus furiosus	Q51723	AF013169; U37557
Ruminococcus albus	P15885 O66050	X15415; CAA33461.1 U92808
Saccharomycopsis fibuligera	P22506	M22475; AAA34314.1
Saccharomycopsis fibuligera	P22507	M22476; AAA34315.1
Saccharopolyspora erythraea	O70021	Y14327
Salmonella typhimurium	Q56078	D86507; BAA13102.1
Schizophyllum commune	P29091	M27313; AAA33925.1
Schizosaccharomyces pombe		AL355920; CAB91163.1
Secale cereale		AF293849; AAG00614.1
Septoria lycopersici	Q99324	U24701; U35462
Sorghum bicolor	Q41290	U33817
Spodoptera frugiperda	O61594	AF052729
Streptomyces coelicolor A3(2)		AL121596; CAB56653.1
Streptomyces reticuli	Q9X9R4	AJ009797
Streptomyces rochei A2	Q55000	X74291
Streptomyces sp. QM-B814	Q59976	Z29625
Thermoanaerobacter brockii	P96090, Q60026	Z56279; Z56279
Thermobifida fusca ER1		AF086819; AAF37727.1
Thermococcus sp.	O08324	Z70242
Thermotoga maritima	Q08638	X74163; CAA52276.1
Thermotoga neapolitana	O33843, Q60038	Z97212; Z77856; CAB10165.1
Thermus sp. Z-1	Q9RA58	AB034947
Thermus thermophilus	Q9X9D4	Y16753
Trichoderma reesei (Hypocrea Jecorina)	Q12715, O93785	U09580; AAA18473.1, AB003110
Trifolium repens	P26204	X56734; CAA40058.1
Trifolium repens	P26205	X56733; CAA40057.1
Tropaeolum majus	O82074	AJ006501; CAA07070.1
Zea mays	P49235, Q41761	X74217, U25157; CAA52293.1 U33816, U44087, U44773
Unidentified bacterium	Q60055	U12011

As used herein in the specification and in the claims section that follows, the term "isolated" refers to a biological component (such as a nucleic acid or protein or organelle) that has been substantially separated or purified away from other biological components in the cell of the organism in which the component naturally occurs, i.e., other chromosomal and extra-

chromosomal DNA and RNA, proteins and organelles. Nucleic acids and proteins that have been "isolated" include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids.

As used herein and in the claims section that follows the terms and phrases "polynucleotide" and “polynucleotide sequence” are used interchangeably and refer to a nucleotide sequence which can be DNA or RNA of, for example, genomic or synthetic origin, which may be single- or double-stranded, and which may represent the sense or antisense strand. Similarly, the terms "polypeptide" and "polypeptide sequence" are interchangeably used herein and refer to an amino acid sequence of any length.

As used herein in the specification and in the claims section that follows, the phrase "complementary polynucleotide sequence" includes sequences, which originally result from reverse transcription of messenger RNA using a reverse transcriptase or any other RNA dependent DNA polymerase. Such sequences can be subsequently amplified *in vivo* or *in vitro* using a DNA dependent DNA polymerase.

As used herein in the specification and in the claims section that follows, the phrase "genomic polynucleotide sequence" includes sequences which originally derive from a chromosome and reflect a contiguous portion of a chromosome.

As used herein in the specification and in the claims section that follows, the phrase "composite polynucleotide sequence" includes sequences which are at least partially complementary and at least partially genomic. A composite sequence can include some exonal sequences required to encode the polypeptide having the β-glucosidase catalytic activity, as well as some intronic sequences interposing therebetween. The intronic sequences can be of any source, including of other genes, and typically will include conserved

splicing signal sequences. Such intronic sequences may further include *cis* acting expression regulatory elements, as hereinbelow described.

As used herein in the specification and in the claims section that follows, the phrase "having a β-glucosidase catalytic activity" refers to a polypeptide sequence, protein or fragments thereof capable of serving as catalysts to a chemical reaction involving hydrolysis of the O-glycosidic bond of glucosides, the result of which is the release of a β-D-glucose residue(s), or an aglycon, in addition to the β-D-glucose residue. Specifically, hydrolysis by retaining enzymes is performed while maintaining the β-configuration of the anomeric center of the carbohydrate. A wide specificity for β-glucosides exists, thus, some examples also hydrolyze one or more of the following: β-D-galactosides, α-L- arabinosides, β-D-xylosides, and β-D-fucosides.

As used herein the term "catalyst" refers to a substance that accelerates a chemical reaction, but is not consumed or changed permanently thereby.

As used herein the term "glucoside" refers to a compound of at least two monomers, at least one of which is a glucose, including a glycoside bond. Examples of glucosides include, but are not limited to, glucose containing backbones, such as the diglucose cellobiose, and the glucose polymer, cellulose.

According to preferred embodiments, the polynucleotide according to this aspect of the present invention encodes a polypeptide as set forth in SEQ ID NO:2 or a portion thereof which retains β-glucosidase catalytic activity.

Alternatively or additionally, the polynucleotide according to this aspect of the present invention is as set forth in SEQ ID NO: 1, 3 or a portion thereof, the portion encodes a polypeptide retaining β-glucosidase catalytic activity.

In a broader aspect the polynucleotides according to the present invention encode a polypeptide which is at least 75 %, at least 80 %, at least 85 %, at least 90 %, at least 95 % or more, say 95 % - 100 % homologous to SEQ ID NO:2 as determined using the BestFit software of the Wisconsin sequence

analysis package, utilizing the Smith and Waterman algorithm, where gap creation penalty equals 8 and gap extension penalty equals 2.

According to preferred embodiments, the polynucleotides according to the broader aspect of the present invention encodes a polypeptide as set forth in SEQ ID NOs:1 or 3 or a portion thereof which retains activity.

Alternatively or additionally, the polynucleotides according to this broader aspect of the present invention are hybridizable with SEQ ID NOs: 1 or 3.

Hybridization for long nucleic acids (e.g., above 200 bp in length) is effected according to preferred embodiments of the present invention by stringent or moderate hybridization, wherein stringent hybridization is effected by a hybridization solution containing 10 % dextrane sulfate, 1 M NaCl, 1 % SDS and 5 x 10^6 cpm ^{32}p labeled probe, at 65 °C, with a final wash solution of 0.2 x SSC and 0.1 % SDS and final wash at 65°C; whereas moderate hybridization is effected by a hybridization solution containing 10 % dextrane sulfate, 1 M NaCl, 1 % SDS and 5 x 10^6 cpm ^{32}p labeled probe, at 65 °C, with a final wash solution of 1 x SSC and 0.1 % SDS and final wash at 50 °C.

Yet alternatively or additionally, the polynucleotides according to this broad aspect of the present invention is preferably at least 70 %, at least 75 %, at least 80 %, at least 85 %, at least 90 %, at least 95 % or more, say 95 % - 100 %, identical with SEQ ID NOs: 1 or 3 as determined using the BestFit software of the Wisconsin sequence analysis package, utilizing the Smith and Waterman algorithm, where gap weight equals 50, length weight equals 3, average match equals 10 and average mismatch equals -9.

Thus, this broad aspect of the present invention encompasses (i) polynucleotides as set forth in SEQ ID NOs:1 or 3; (ii) fragments thereof; (iii) sequences hybridizable therewith; (iv) sequences homologous thereto; (v) sequences encoding similar polypeptides with different codon usage; (vi) altered sequences characterized by mutations, such as deletion, insertion or

substitution of one or more nucleotides, either naturally occurring or man induced, either randomly or in a targeted fashion.

According to another aspect of the present invention there is provided a nucleic acid construct comprising the isolated nucleic acid described herein.

According to a preferred embodiment, the nucleic acid construct according to this aspect of the present invention further comprising at least one *cis* acting control (regulatory) element for regulating the expression of the isolated nucleic acid. Such *cis* acting regulatory elements include, for example, promoters, which are known to be sequence elements required for transcription, as they serve to bind DNA dependent RNA polymerase, which transcribes sequences present downstream thereof. Further details relating to various regulatory elements are described hereinbelow.

While the isolated nucleic acid described herein is an essential element of the invention, it is modular and can be used in different contexts. The promoter of choice that is used in conjunction with this invention is of secondary importance, and will comprise any suitable promoter. It will be appreciated by one skilled in the art, however, that it is necessary to make sure that the transcription start site(s) will be located upstream of an open reading frame. In a preferred embodiment of the present invention, the promoter that is selected comprises an element that is active in the particular host cells of interest. These elements may be selected from transcriptional regulators that activate the transcription of genes essential for the survival of these cells in conditions of stress or starvation, including the heat shock proteins.

A construct according to the present invention preferably further includes an appropriate selectable marker. In a more preferred embodiment according to the present invention the construct further includes an origin of replication. In another most preferred embodiment according to the present invention the construct is a shuttle vector, which can propagate both in *E. coli* (wherein the construct comprises an appropriate selectable marker and origin of replication) and be compatible for propagation in cells, or integration in the

genome, of an organism of choice, such as a plant. The construct according to this aspect of the present invention can be, for example, a plasmid, a bacmid, a phagemid, a cosmid, a phage, a virus or an artificial chromosome.

According to an additional aspect of the present invention there is provided a recombinant protein comprising a polypeptide having a β-glucosidase catalytic activity. The polypeptide is preferably derived from an *Aspergillus niger* and preferably includes a signal peptide and optionally an endoplasmic reticulum retaining peptide.

According to preferred embodiments, the polypeptide according to this aspect of the present invention is as set forth in SEQ ID NO:2 or a portion thereof which retains β-glucosidase catalytic activity.

SEQ ID NO:2 of *A. niger* β-glucosidase is similar to the amino acid sequence of the β-glucosidase of *A. kawachii*. However, while the former is highly stable at wide range of temperatures and pH treatments, the latter is relatively unstable, and thus has certain disadvantages, rendering its use for the purpose of the present invention as is further detailed and described hereinunder, unfeasible and/or much less attractive.

Recently, Iwashita and coworkers have published the sequence of a β-glucosidase (GenBank/EMBL AB003470) obtained from *Aspergillus kawachii* strain: IFO4308. Sequence comparison between *Aspergillus kawachii* β-glucosidase and *A. niger* β-glucosidase revealed that the two share 98 % homology.

Enzymes of the two *Aspergillus* sp. contain seven cysteine residues and identical number of glycosylation sites, while differing in their degree of glycosylation (35).

The physical and kinetic properties of three β-glucosidases from *Aspergillus kawachii* were described (35), and the three were shown to be products of the same gene, differing solely by the degree of glycosylation. The three purified *A. kawachii* β-glucosidases were readily inactivated, even at

moderate pH and temperature conditions. In sharp distinction, while examining the stability of the recombinant *A. niger* β-glucosidase according to the present invention under conditions identical to those described by Iwashita et al. and as described hereinbelow in the Examples section, revealed that the enzyme is highly stable, retaining majority of the enzymatic activity even after 1 hour incubation at 60 °C (68 % activity, as defined by percent activity of an enzyme kept at 4 °C).

Thus, despite the similarity between the *A. kawachii* and *A. niger* β-glucosidases, the *A. niger* enzyme unexpectedly exhibits significantly higher thermal and pH stability.

According to yet another aspect of the present invention there is provided a host cell comprising a nucleic acid construct as described herein. The term "host cell" refers to a recipient of a heterologous nucleic acid, which host cell can be either a prokaryotic cell, such as *E. coli*, or a eukaryotic cell, such as a yeast cell, a filamentous fungus cell, a plant cell or an animal cell. Examples for a yeast cell include, but not limited to, *Pichia* sp. such as *P. pastoris*, and *Saccharomyces* sp. such as *S. cervisiae*.

As used herein and in the claims section which follows, the term "heterologous" when used in context of a nucleic acid sequence or a protein found within a plant, plant derived tissue or plant cells, or alternatively, within a eukaryotic cell, such as yeast, or a prokaryotic cell such as bacteria, refers to nucleic acid or amino acid sequences typically not native to the plant, plant derived tissue or plant cells, or alternatively, to the eukaryotic cell, such as yeast, or the prokaryotic cell, such as bacteria. Interchangeably, nucleic acid or amino acid sequences typically not native to the plant, plant derived tissue or plant cells, or alternatively, to the eukaryotic cell, such as yeast, or the prokaryotic cell, such as bacteria, are referred to by “recombinant nucleic acid” and “recombinant protein”, respectively. Thus, a recombinant nucleic acid is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of

sequence. This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques.

As used herein in the specification and in the claims section that follows, the term "eukaryotic cell" refers to a cell containing a diploid genome through at least a portion of its life cycle, having membrane-bound nucleus with chromosomes made of DNA, with cell division involving a form of mitosis in which spindles are involved. Possession of a eukaryote type of cell characterizes the four kingdoms, *Protoctista*, *Fungi*, *Plantae* and *Animalia*.

As used herein in the specification and in the claims section that follows, the term "prokaryotic cell" refers to various bacteria and blue-green algae, characterized by the absence of the nuclear organization, mitotic capacities and complex organelles that typify the eukaryote superkingdom. Examples of prokaryotic cell according to the present invention are bacteria, such as, but not limited to, *E. coli*.

According to still another aspect of the present invention there is provided an organism comprising a nucleic acid construct as described herein, such as, but not limited to, a plant. Such an organism is said to be transformed or virally infected.

As used herein the term "transformed" and its conjugations such as transformation, transforming and transform, all relate to the process of introducing heterologous nucleic acid sequences into a cell or an organism, which nucleic acid sequences are propagatable to the offspring. The term thus reads on, for example, "genetically modified", "transgenic" and "transfected", which may be used herein to further describe and/or claim the present invention. The term relates both to introduction of a heterologous nucleic acid sequence into the genome of an organism and/or into the genome of a nucleic acid containing organelle thereof, such as into a genome of chloroplast or a mitochondrion.

As used herein the phrase "viral infected" includes infection by a virus carrying a heterologous nucleic acid sequence. Such infection typically results in transient expression of the nucleic acid sequence, which nucleic acid sequence is typically not integrated into a genome and therefore not propagatable to offspring, unless further infection of such offspring is experienced.

There are various methods of introducing foreign genes into both monocotyledonous and dicotyledenous plants (Potrykus, I., Annu. Rev. Plant. Physiol., Plant. Mol. Biol. (1991) 42:205-225; Shimamoto *et al.,* Nature (1989) 338:274-276). The principle methods of causing stable integration of exogenous DNA into plant genomic DNA include two main approaches:

(i) *Agrobacterium*-mediated gene transfer: Klee *et al.* (1987) Annu. Rev. Plant Physiol. 38:467-486; Klee and Rogers in Cell Culture and Somatic Cell Genetics of Plants, Vol. 6, Molecular Biology of Plant Nuclear Genes, eds. Schell, J., and Vasil, L. K., Academic Publishers, San Diego, Calif. (1989) p. 2-25; Gatenby, in Plant Biotechnology, eds. Kung, S. and Arntzen, C. J., Butterworth Publishers, Boston, Mass. (1989) p. 93-112.

(ii) direct DNA uptake: Paszkowski *et al.,* in Cell Culture and Somatic Cell Genetics of Plants, Vol. 6, Molecular Biology of Plant Nuclear Genes eds. Schell, J., and Vasil, L. K., Academic Publishers, San Diego, Calif. (1989) p. 52-68; including methods for direct uptake of DNA into protoplasts, Toriyama, K. *et al.* (1988) Bio/Technology 6:1072-1074. DNA uptake induced by brief electric shock of plant cells: Zhang *et al.* Plant Cell Rep. (1988) 7:379-384. Fromm *et al.* Nature (1986) 319:791-793. DNA injection into plant cells or tissues by particle bombardment, Klein *et al.* Bio/Technology (1988) 6:559-563; McCabe *et al.* Bio/Technology (1988) 6:923-926; Sanford, Physiol. Plant. (1990) 79:206-209; by the use of micropipette systems: Neuhaus *et al.,* Theor. Appl. Genet. (1987) 75:30-36; Neuhaus and Spangenberg, Physiol. Plant. (1990) 79:213-217; or by the direct incubation of DNA with germinating pollen, DeWet *et al.* in Experimental

Manipulation of Ovule Tissue, eds. Chapman, G. P. and Mantell, S. H. and Daniels, W. Longman, London, (1985) p. 197-209; and Ohta, Proc. Natl. Acad. Sci. USA (1986) 83:715-719.

The *Agrobacterium* system includes the use of plasmid vectors that contain defined DNA segments that integrate into the plant genomic DNA. Methods of inoculation of the plant tissue vary depending upon the plant species and the *Agrobacterium* delivery system. A widely used approach is the leaf disc procedure, which can be performed with any tissue explant that provides a good source for initiation of whole plant differentiation. Horsch *et al.* in Plant Molecular Biology Manual A5, Kluwer Academic Publishers, Dordrecht (1988) p. 1-9. A supplementary approach employs the *Agrobacterium* delivery system in combination with vacuum infiltration. The *Agrobacterium* system is especially viable in the creation of transgenic dicotyledenous plants.

There are various methods of direct DNA transfer into plant cells. In electroporation, the protoplasts are briefly exposed to a strong electric field. In microinjection, the DNA is mechanically injected directly into the cells using very small micropipettes. In microparticle bombardment, the DNA is adsorbed on microprojectiles such as magnesium sulfate crystals or tungsten particles, and the microprojectiles are physically accelerated into cells or plant tissues.

Following transformation plant propagation is exercised. The most common method of plant propagation is by seed. Regeneration by seed propagation, however, has the deficiency that due to heterozygosity there is a lack of uniformity in the crop, since seeds are produced by plants according to the genetic variances governed by Mendelian rules. Basically, each seed is genetically different and each will grow with its own specific traits. Therefore, it is preferred that the transformed plant be produced such that the regenerated plant has the identical traits and characteristics of the parent transgenic plant. Therefore, it is preferred that the transformed plant be

regenerated by micropropagation, which provides a rapid, consistent reproduction of the transformed plants.

Micropropagation is a process of growing new generation plants from a single piece of tissue that has been excised from a selected parent plant or cultivar. This process permits the mass reproduction of plants having the preferred tissue expressing the protein. The new generation plants, which are produced, are genetically identical to, and have all of the characteristics of, the original plant. Micropropagation allows mass production of quality plant material in a short period of time and offers a rapid multiplication of selected cultivars in the preservation of the characteristics of the original transgenic or transformed plant. The advantages of cloning plants are the speed of plant multiplication and the quality and uniformity of plants produced.

Micropropagation is a multi-stage procedure that requires alteration of culture medium or growth conditions between stages. Thus, the micropropagation process involves four basic stages: Stage one, initial tissue culturing; stage two, tissue culture multiplication; stage three, differentiation and plant formation; and stage four, greenhouse culturing and hardening. During stage one, initial tissue culturing, the tissue culture is established and certified contaminant-free. During stage two, the initial tissue culture is multiplied until a sufficient number of tissue samples are produced to meet production goals. During stage three, the tissue samples grown in stage two are divided and grown into individual plantlets. At stage four, the transformed plantlets are transferred to a greenhouse for hardening where the plants' tolerance to light is gradually increased so that it can be grown in the natural environment.

Sequences suitable for permitting integration of the heterologous sequence into the plant genome are recommended. These might include transposon sequences and the like for homologous recombination as well as Ti sequences which permit random insertion of a heterologous expression cassette into a plant genome.

Suitable prokaryote selectable markers include resistance toward antibiotics such as ampicillin or tetracycline. Other DNA sequences encoding additional functions may also be present in the vector, as is known in the art.

The constructs of the subject invention will include an expression cassette for expression of the protein of interest. Usually, there will be only one expression cassette, although two or more are feasible. The recombinant expression cassette will contain in addition to the heterologous sequence one or more of the following sequence elements, a promoter region, plant 5' untranslated sequences which can include regulatory elements, initiation codon depending upon whether or not the structural gene comes equipped with one, and a transcription and translation termination sequence. Unique restriction enzyme sites at the 5' and 3' ends of the cassette allow for easy insertion into a pre-existing vector.

As used herein, the phrase "regulatory element" refers to a nucleotide sequence which are typically included within an expression cassette and function in regulating (i.e., enhancing or depressing) the expression of a coding sequence therefrom. This regulation can be effected either at the transcription or the translation stages. Examples of regulatory elements include, but are not limited to, enhancers, suppressers and transcription terminators.

As used herein the term "promoter" refers to a nucleotide sequence, which can direct gene expression in cells. Such a promoter can be derived from a plant, a plant virus, or from any other living organism including bacteria and animals.

A plant promoter can be a constitutive promoter, such as, but not limited to, CaMV35S and CaMV19S promoters, FMV34S promoter, sugarcane bacilliform badnavirus promoter, CsVMV promoter, *Arabidopsis* ACT2/ACT8 actin promoter, *Arabidopsis* ubiquitin UBQ1 promoter, barley leaf thionin BTH6 promoter, and rice actin promoter.

The promoter can alternatively be a tissue specific promoter. Examples of plant tissue specific promoters include, without being limited to, bean phaseolin storage protein promoter, DLEC promoter, PHSβ promoter, zein stprotein promoter, conglutin gamma promoter from soybean, AT2S1 gene promoter, ACT11 actin promoter from *Arabidopsis*, napA promoter from *Brassica napus*, potato patatin gene promoter and the Tob promoter.

The promoter may also be a promoter which is active in a specific developmental stage of a plant's life cycle, for example, a promoter active in late embryogenesis, such as: the LEA promoter; Endosperm-specific expression promoter (the seed storage prolamin from rice is expressed in tobacco seed at the developmental stage about 20 days after flowering) or the promoter controlling the FbL2A gene during fiber wall synthesis stages.

In case of a tissue-specific promoter, it ensures that the heterologous protein is expressed only in the desired tissue, for example, only in the flower, the fruit, the root, the seed, etc.

Both the tissue-specific and the non-specific promoters may be constitutive, i.e., may cause continuous expression of the heterologous protein.

The promoter may also be an inducible promoter, i.e., a promoter which is activated by the presence of an inducing agent, and only upon said activation, causes expression of the heterologous protein. An inducing agent can be for example, light, chemicals, drought, high salinity, osmotic shock, oxidant conditions or in case of pathogenicity and include, without being limited to, the light-inducible promoter derived from the pea rbcS gene, the promoter from the alfalfa rbcS gene, the promoters DRE, MYC and MYB active in drought; the promoters INT, INPS, prxEa, Ha hsp17.7G4 and RD21 active in high salinity and osmotic stress, the promoters hsr303J and str246C active in pathogenic stress, the copper-controllable gene expression system and the steroid-inducible gene system

Alternatively, an inducing agent may be an endogenous agent which is normally present in only certain tissues of the plant, or is produced only at

certain time periods of the plant's life cycle, such as ethylene or steroids. By using such an endogenous tissue-specific inducing agent, it is possible to control the expression from such inducible promoters only in those specific tissues. By using an inducing agent produced only during a specific period of the life cycle, it is possible to control the expression from an inducible promoter to the specific phase in the life-cycle in which the inducing agent is produced.

Bacterial and yeast derived promoters are well known in the art.

Viruses are a unique class of infectious agents whose distinctive features are their simple organization and their mechanism of replication. In fact, a complete viral particle, or virion, may be regarded mainly as a block of genetic material (either DNA or RNA) capable of autonomous replication, surrounded by a protein coat and sometimes by an additional membranous envelope such as in the case of alpha viruses. The coat protects the virus from the environment and serves as a vehicle for transmission from one host cell to another.

Viruses that have been shown to be useful for the transformation of plant hosts include CaV, TMV and BV. Transformation of plants using plant viruses is described in U.S. Pat. No. 4,855,237 (BGV), EP-A 67,553 (TMV), Japanese Published Application No. 63-14693 (TMV), EPA 194,809 (BV), EPA 278,667 (BV); and Gluzman, Y. *et al.*, Communications in Molecular Biology: Viral Vectors, Cold Spring Harbor Laboratory, New York, pp. 172-189 (1988). Pseudovirus particles for use in expressing foreign DNA in many hosts, including plants, is described in WO 87/06261.

Construction of plant RNA viruses for the introduction and expression of non-viral foreign genes in plants is demonstrated by the above references as well as by Dawson, W. O. *et al.*, Virology (1989) 172:285-292; Takamatsu *et al.* EMBO J. (1987) 6:307-311; French *et al.* Science (1986) 231:1294-1297; and Takamatsu *et al.* FEBS Letters (1990) 269:73-76.

When the virus is a DNA virus, the constructions can be made to the virus itself. Alternatively, the virus can first be cloned into a bacterial plasmid for ease of constructing the desired viral vector with the foreign DNA. The virus n then be excised from the plasmid. If the virus is a DNA virus, a bacterial origin of replication can be attached to the viral DNA, which is then replicated by the bacteria. Transcription and translation of this DNA will produce the coat protein which will encapsidate the viral DNA. If the virus is an RNA virus, the virus is generally cloned as a cDNA and inserted into a plasmid. The plasmid is then used to make all of the constructions. The RNA virus is then produced by transcribing the viral sequence of the plasmid and translation of the viral genes to produce the coat protein(s) which encapsidate the viral RNA.

Construction of plant RNA viruses for the introduction and expression of non-viral foreign genes in plants is demonstrated by the above references as well as in U.S. Pat. No. 5,316,931.

In one embodiment, a plant viral nucleic acid is provided in which the native coat protein coding sequence has been deleted from a viral nucleic acid, a non-native plant viral coat protein coding sequence and a non promoter, preferably the subgenomic promoter of the non-native coat protein coding sequence, capable of expression in the plant host, packaging of the recombinant plant viral nucleic acid, and ensuring a systemic infection of the host by the recombinant plant viral nucleic acid, has been inserted. Alternatively, the coat protein gene may be inactivated by insertion of the non-native nucleic acid sequence within it, such that a protein is produced. The recombinant plant viral nucleic acid may contain one or more additional non-native subgenomic promoters. Each non-native subgenomic promoter is capable of transcribing or expressing adjacent genes or nucleic acid sequences in the plant host and incapable of recombination with each other and with native subgenomic promoters. Non-native (foreign) nucleic acid sequences may be inserted adjacent the native plant viral subgenomic promoter or the

native and a non-native plant viral subgenomic promoters if more than one nucleic acid sequence is included. The non-native nucleic acid sequences are transcribed or expressed in the host plant under control of the subgenomic promoter to produce the desired products.

In a second embodiment, a recombinant plant viral nucleic acid is provided as in the first embodiment except that the native coat protein coding sequence is placed adjacent one of the non-native coat protein subgenomic promoters instead of a non-native coat protein coding sequence.

In a third embodiment, a recombinant plant viral nucleic acid is provided in which the native coat protein gene is adjacent its subgenomic promoter and one or more non-native subgenomic promoters have been inserted into the viral nucleic acid. The inserted non-native subgenomic promoters are capable of transcribing or expressing adjacent genes in a plant host and are incapable of recombination with each other and with native subgenomic promoters. Non-native nucleic acid sequences may be inserted adjacent the non-native subgenomic plant viral promoters such that said sequences are transcribed or expressed in the host plant under control of the subgenomic promoters to produce the desired product.

In a fourth embodiment, a recombinant plant viral nucleic acid is provided as in the third embodiment except that the native coat protein coding sequence is replaced by a non-native coat protein coding sequence.

The viral vectors are encapsidated by the coat proteins encoded by the recombinant plant viral nucleic acid to produce a recombinant plant virus. The recombinant plant viral nucleic acid or recombinant plant virus is used to infect appropriate host plants. The recombinant plant viral nucleic acid is capable of replication in the host, systemic spread in the host, and transcription or expression of foreign gene(s) in the host to produce the desired protein.

In many instances it is desired to target the expression of a recombinant protein. Such targeting can be into a cellular organelle or outside of the cell. This can be effected, as is well known in the art, by appropriate signal

peptides, which are fused to the polypeptide to be targeted, typically at the N terminus.

Thus, as used herein and in the claims section which follows, the phrase "signal peptide" refers to a stretch of amino acids which is effective in targeting a protein expressed in a cell into a target location. Different signal peptides, which are known in the art, are effective in secreting a protein from bacteria, yeast, plant and animal cells.

It should be noted in this respect that signal peptides serve the function of translocation of produced protein across the endoplasmic reticulum membrane. Similarly, transmembrane segments halt translocation and provide anchoring of the protein to the plasma membrane, see, Johnson *et al.* The Plant Cell (1990) 2:525-532; Sauer *et al.* EMBO J. (1990) 9:3045-3050; Mueckler *et al.* Science (1985) 229:941-945. Mitochondrial, nuclear, chloroplast, or vacuolar signals target expressed protein correctly into the corresponding organelle through the secretory pathway, see, Von Heijne, Eur. J. Biochem. (1983) 133:17-21; Yon Heijne, J. Mol. Biol. (1986) 189:239-242; Iturriaga *et al.* The Plant Cell (1989) 1:381-390; McKnight *et al.*, Nucl. Acid Res. (1990) 18:4939-4943; Matsuoka and Nakamura, Proc. Natl. Acad. Sci. USA (1991) 88:834-838. A recent book by Cunningham and Porter (Recombinant proteins from plants, Eds. C. Cunningham and A.J.R. Porter, 1998 Humana Press Totowa, N.J.) describe methods for the production of recombinant proteins in plants and methods for targeting the proteins to different compartments in the plant cell. In particular, two chapters therein (14 and 15) describe different methods to introduce targeting sequences that results in accumulation of **recombinant proteins in compartments such as ER, vacuole, plastid, nucleus** and cytoplasm. Presently, the preferred site of accumulation of the fusion protein according to the present invention is the ER using signal peptide such as Cel 1 or the rice amylase signal peptide at the N-terminus and an ER retaining peptide (HDEL, SEQ ID NO:17; or KDEL, SEQ ID NO:24) at the C-terminus.

According to an additional aspect of the present invention there is provided a method of producing recombinant β-glucosidase. The method according to this aspect of the present invention is effected by introducing, in an expressible or overexpressible form, a nucleic acid construct into a host cell. The nucleic acid construct includes a genomic, complementary or composite polynucleotide preferably derived from *Aspergillus niger* and encoding a polypeptide having a β-glucosidase catalytic activity. The polynucleotide preferably further encodes a signal peptide in frame with the polypeptide. Still preferably, the polynucleotide further encodes an endoplasmic reticulum retaining peptide in frame with the polypeptide.

As used herein the term "introducing" refers both to transforming and to virally infecting, as these terms are further defined hereinabove. As used herein the terms "expressible form" and "overexpressible form" refers to a recombinant form which includes the required regulatory elements to effect expression or over expression of a coding region, all as is further detailed hereinabove.

According to a preferred embodiment of this aspect of the present invention, after sufficient expression has been detected, the polypeptide having the β-glucosidase catalytic activity is extracted from the expressing host cell.

Thus host cells, expressing the polypeptide according to the present invention, provide an immediate, easy and indefinite source of the polypeptide.

Any number of well-known liquid or solid culture media may be used for appropriately culturing host cells of the present invention, although growth on liquid media is preferred as the secretion of the polypeptide into the media results in simplification of polypeptide recovery. As is further detailed hereinabove, such secretion can be effected by the incorporation of a suitable signal peptide. The β-glucosidase may be isolated or separated or purified from host cell preparations using techniques well known in the art, such as, but not limited to, centrifugation filtration, chromatography, electrophoresis and

dialysis. Further concentration and/or purification of the β-glucosidase may be effected by use of conventional techniques, including, but not limited to, ultrafiltration, further dialysis, ion-exchange chromatography, HPLC, size-exclusion chromatography, cellobiose-sepharose affinity chromatography, and electrophoresis, such as polyacrylamide-gel-electrophoresis (PAGE). Using these techniques, β-glucosidase may be recovered in pure or substantially pure form.

According to an additional aspect of the present invention there is provided a method of increasing a level of at least one fermentation substance in a fermentation product. The method according to this aspect of the present invention is effected by fermenting a glucose containing fermentation starting material by a yeast cell overexpressing a nucleic acid construct which includes a genomic, complementary or composite polynucleotide preferably derived from *Aspergillus niger* and which encodes a polypeptide having a β-glucosidase catalytic activity, thereby increasing the level of the at least one fermentation substance in the fermentation product. The polynucleotide preferably further encodes a signal peptide in frame with the polypeptide. Still preferably, the polynucleotide further encodes an endoplasmic reticulum retaining peptide in frame with the polypeptide.

According an alternative aspect of the present invention there is provided a method of increasing a level of at least one fermentation substance in a fermentation product. The method according to this aspect of the present invention is effected by fermenting a plant derived glucose containing fermentation starting material by a yeast cell, the plant overexpressing a nucleic acid construct which includes a genomic, complementary or composite polynucleotide preferably derived from *Aspergillus niger* and which encodes a polypeptide having a β-glucosidase catalytic activity, thereby increasing the level of the at least one fermentation substance in the fermentation product. The polynucleotide preferably further encodes a signal peptide in frame with

the polypeptide. Still preferably, the polynucleotide further encodes an endoplasmic reticulum retaining peptide in frame with the polypeptide.

As used herein in the specification and in the claims section that follows, the term "fermentation" refers to a chemical change induced in a complex organic compound by the action of an enzyme, whereby the substance is split into simpler compounds. Specifically, the term "fermentation" includes the anaerobic dissimilation of substrates with the production of energy and reduced compounds, the final products thereof are organic acids, alcohols, such as ethanol, isopropanol, butanol, etc., and CO_2. Such products, are typically secreted and each of which is referred to herein as a "fermentation substance", i.e., any known fermentation resultant of either microbial or yeast fermentation.

As used herein in the specification and in the claims section that follows, the phrase "fermentation product" refers to the resultant material of a fermentation process. Examples include, but are not limited to, alcohol containing fermentation medium and alcoholic beverages, such a, but not limited to, fruit-based alcohol-containing beverages, wines and beers.

When used in conjunction with, for example, a β-glucanase, the β-glucosidase is effective for hydrolyzing a variety of cellulose containing materials to glucose. The glucose produced by enzymatic hydrolysis of the cellulose and other glucose containing saccharides, may be recovered and stored, or it may be subsequently fermented to ethanol using conventional techniques. Many processes for the fermentation of glucose generated from cellulose are well known, and are suitable for use herein. Briefly, the hydrolyzate containing the glucose from the enzymatic reaction is contacted with an appropriate microorganism under conditions effective for the fermentation of the glucose to ethanol. This fermentation may be separate from and follow the enzymatic hydrolysis of the cellulose (sequentially processed), or the hydrolysis and fermentation may be concurrent and conducted in the same vessel (simultaneously processed). Details of the various fermentation

techniques, conditions, and suitable microorganisms have been described, for example, by Wyman (1994, Bioresource Technol., 50:3-16) or Olsson and Hahn-Hagerdal (1996, Enzyme Microbial Technol., 18:312-331), the content of each of which is incorporated herein by reference. Following the completion of a fermentation, the alcohol may be recovered by extraction, and optionally purified e.g., by distillation.

Thus, according to still another aspect of the present invention there is provided a method of producing an alcohol. The method according to this aspect of the present invention is effected by fermenting a glucose containing fermentation starting material by a cell overexpressing a nucleic acid construct including a genomic, complementary or composite polynucleotide preferably derived from *Aspergillus niger*, encoding a polypeptide having a β-glucosidase catalytic activity, and extracting the alcohol therefrom. The polynucleotide preferably further encodes a signal peptide in frame with the polypeptide. Still preferably, the polynucleotide further encodes an endoplasmic reticulum retaining peptide in frame with the polypeptide.

According to an additional aspect of the present invention there is provided a method of producing an alcohol. The method according to this aspect of the present invention is effected by fermenting a plant derived glucose containing fermentation starting material by a cell, the plant overexpressing a nucleic acid construct including a genomic, complementary or composite polynucleotide preferably derived from *Aspergillus niger*, encoding a polypeptide having a β-glucosidase catalytic activity, and extracting the alcohol therefrom. The polynucleotide preferably further encodes a signal peptide in frame with the polypeptide. Still preferably, the polynucleotide further encodes an endoplasmic reticulum retaining peptide in frame with the polypeptide.

Plants contain aroma and flavor compounds of glycosidic nature, their inherent aroma property can be released by degrading enzymes, turning a non-volatile aroma compound into its volatile form. Thus, for example, α-L-

arabinofuranosidases, assist in the liberation of aroma compounds from substrates such as juices or wines, as described by Gunata et al. ("purification and some properties of an alpha-L-arabinofuranosidase from *A. niger* action on grape monoterpenyl arabinofuranosylglucosides. J. Agric. Food Chem. 38: 772-776, 1990). This outcome is achieved, for example, in a two step process wherein the first step comprises the use of an α-L-arabinofuranosidase, to catalyze the release of arabinose residues from monoterpenyl α-L-arabinofuranosyl glucosides contained in, for example, the fruit or vegetable juice via the cleavage of the (1→6) linkage between a terminal arabinofuranosyl unit and the intermediate glucose of a monoterpenyl α-L-arabinofuranosylglucoside. The α-L-arabinofuranosidase is preferably in a purified form so as to avoid the undesirable degradation of other components of the juice which may be detrimental to its ultimate quality. In the second step, ß-glucosidase is required to yield the free terpenol from the resulting desarabinosylated monoterpenyl glucoside. If desired, both reaction steps may be performed in the same reaction vessel without the need to isolate the intermediate product (Gunata et al. (1989), supra). Thus, ß-glucosidase is an essential contributor when the liberation of these aroma compounds for improving the flavor of the juice or wine is desired. Moreover, in the case of wine, the control of the liberation of aroma compounds provides wines with a more consistent flavor, thus reducing or eliminating the undesirable effect of "poor vintage years". Additional information is contained in: "Cloning and expression of DNA molecules encoding arabinan degrading enzyme of fungal origin", U.S. Pat. No. 5,863,783; Y. Gueguen, et al. "A Very Efficient ß-Glucosidase Catalyst for the Hydrolysis of Flavor Precursors of Wines and Fruit Juices", J. Agric. Food Chem. 44:2336-2340, 1996.

Thus, according to a further aspect of the present invention there is provided a method of increasing a level of at least one aroma substance in a

plant derived product, such as, but not limited to, an alcoholic beverage. The method according to this aspect of the present invention is effected by incubating a glucose containing plant starting material with a yeast cell overexpressing a nucleic acid construct including a genomic, complementary or composite polynucleotide preferably derived from *Aspergillus niger* which encodes a polypeptide having a β-glucosidase catalytic activity, thereby increasing the level of the at least one aroma substance in the plant derived product. The polynucleotide preferably further encodes a signal peptide in frame with the polypeptide. Still preferably, the polynucleotide further encodes an endoplasmic reticulum retaining peptide in frame with the polypeptide.

While reducing the present invention to practice it was discovered that in order to obtain activity of a β-glucosidase in a transgenic plant, the expression construct should include a signal peptide. In addition, it was found that retaining the enzyme in the endoplasmic reticulum results in higher release of aroma compounds following homogenization and incubation. It is assumed that compartmentalization of the enzyme in for example the ER prevents it from interacting with its substrates which are mainly outside the cells, limiting such interaction following homogenization. Indeed, directing the enzyme to the apoplast resulted in increased release of aroma *in vivo*. Thus, depending on the specific application, one can chose weather to include in the construct an endoplasmic reticulum retaining peptide or not.

According to yet a further aspect of the present invention there is provided a method of increasing a level of at least one aroma substance in a plant derived product, such as, but not limited to, an alcoholic beverage. The method according to this aspect of the present invention is effected by incubating a glucose containing plant starting material with a yeast cell, said plant overexpressing a nucleic acid construct including a genomic, complementary or composite polynucleotide preferably derived from *Aspergillus niger* which encodes a polypeptide having a β-glucosidase

catalytic activity, thereby increasing the level of the at least one aroma substance in the plant derived product. The polynucleotide preferably further encodes a signal peptide in frame with the polypeptide. Still preferably, the polynucleotide further encodes an endoplasmic reticulum retaining peptide in frame with the polypeptide.

As used herein in the specification and in the claims section that follows, the phrase "glucose containing starting material" refers to any source of energy, in the form of glucose containing compounds, other than free glucose, including, but not limited to, crushed, minced, diced or extracted plant material, plant, or portions thereof, such as fruits, examples thereof are tropical fruits and grapes.

According to an additional aspect of the present invention there is provided a method of producing an aroma spreading plant. As used herein in the specification and in the claims section that follows, the phrase "aroma spreading plant" refers to substantially any part of a plant, in which volatile compounds are generated by the catalytic activity of the β-glucosidase polypeptide of the present invention, release of volatile compounds therefrom is perceived by the olfactory system of an organism, such as a human.

The method according to this aspect of the present invention is effected by overexpressing in the plant a nucleic acid construct including a genomic, complementary or composite polynucleotide derived from *Aspergillus niger*, which encodes a polypeptide having a β-glucosidase catalytic activity, thereby increasing aroma spread from the plant. Such overexpression is preferably performed in a tissue specific manner by, for example, employing a tissue specific promoter, as hereinabove described, to thereby overexpress a heterologous protein in a selected portion of the plant. The tissue in which such overexpression is effected is selected according to the availability of glucose containing non-volatile aroma substrates therein. Thus, such an overexpression will cause the release of a volatile and aroma constituent of the substrate. Thus, according to preferred embodiments overexpressing the

nucleic acid construct is limited to at least one tissue, such as a flower, a fruit, a seed, a root, a stem, pollen and leaves.

According to still a further aspect of the present invention there is provided a method of increasing a level of free glucose in a glucose containing fermentation starting material. The method according to this aspect of the present invention is effected by fermenting the glucose containing fermentation starting material by a cell overexpressing a nucleic acid construct including a genomic, complementary or composite polynucleotide preferably derived from *Aspergillus niger*, which encodes a polypeptide having a β-glucosidase catalytic activity, thereby increasing the level of the free glucose in the glucose containing fermentation starting material. The polynucleotide preferably further encodes a signal peptide in frame with the polypeptide. Still preferably, the polynucleotide further encodes an endoplasmic reticulum retaining peptide in frame with the polypeptide.

According to another aspect of the present invention there is provided a method of increasing a level of free glucose in a plant derived glucose containing fermentation starting material. The method according to this aspect of the present invention is effected by fermenting the plant derived glucose containing fermentation starting material by a cell, the plant overexpressing a nucleic acid construct including a genomic, complementary or composite polynucleotide preferably derived from *Aspergillus niger*, which encodes a polypeptide having a β-glucosidase catalytic activity, thereby increasing the level of the free glucose in the plant. The polynucleotide preferably further encodes a signal peptide in frame with the polypeptide. Still preferably, the polynucleotide further encodes an endoplasmic reticulum retaining peptide in frame with the polypeptide.

As used herein in the specification and in the claims section that follows, the term "free glucose" refers to glucose residues in the form of a monosaccharide, the levels of which are increased by the catalytic activity of β -glucosidase.

As used herein in the specification and in the claims section that follows, the phrase "glucose containing fermentation starting material" refers to any source of energy, in the form of glucose containing compounds, other than free glucose, including, but not limited to, crushed, minced, diced or extracted plant material, plant, or portions thereof, used in industrial fermentation processes.

According to yet another aspect of the present invention there is provided a method of increasing a level of extra- or intracellular free glucose in a plant. The method according to this aspect of the present invention is effected by overexpressing in the plant a nucleic acid construct including a genomic, complementary or composite polynucleotide preferably derived from *Aspergillus niger*, which encodes a polypeptide having a β-glucosidase catalytic activity, thereby increasing the level of the free glucose in the plant. Thus, sweeter fruits can be produced. The polynucleotide preferably further encodes a signal peptide in frame with the polypeptide. Still preferably, the polynucleotide further encodes an endoplasmic reticulum retaining peptide in frame with the polypeptide.

Glycosidases, including β-glucosidase, catalyze reactions involving the hydrolysis of O-glycosidic bond of glycosides, and synthesize oligosaccharides when the reaction is run in reverse from the normal direction, a result achieved by, for example, site directed mutagenesis, and Km reversal. As described in the Background section hereinabove, the hydrolysis reaction mechanism of glycosidases involves two catalytic steps, the second of which involves a base catalyzed H_2O attack, resulting in the regeneration of the enzyme, and the release of the saccharide residue. Thus, in addition, oligosaccharide synthesis can be achieved by adding a second saccharide to the reaction mixture, which competes with the H_2O molecule, and reacts in its place with the first saccharide in, what is known as, a transglycosylation reaction. Hence, as glycosidases are generally available and easy to handle, these enzymes have the potential to catalyze the production of many different products using

inexpensive substrates. For further detail see U.S. Pat. No. 5,716,812.

Thus, according to yet an additional aspect of the present invention there is provided a method of synthesizing oligosaccharides. The method according to this aspect of the present invention is effected by mixing a polypeptide having a β-glucosidase catalytic activity with first and second saccharide molecules to thereby join the first and second saccharide molecules into an oligosaccharide.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook *et al.*, (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel *et al.*, "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson *et al.*, "Recombinant DNA", Scientific American Books, New York; Birren *et al.* (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; available

immunoassays are extensively described in the patent and scientific liter, see, for example, U. S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281.521. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader.

MATERIALS AND EXPERIMENTAL METHODS

Purification of A. niger β-glucosidase:

A crude preparation of *A. niger* B1 (CMI CC 324626) β-glucosidase was obtained from Shaligal Ltd. (Tel-Aviv, Israel). A sample (10 ml) of the crude enzyme (140 Units/ml) was first diafiltered through a 50 kDa cut-off Amicon membrane (Amicon Corp., Danvers, MA), with 20 mM citrate buffer pH=5. The proteins were then separated on an FPLC equipped with a Mono-Q RH 5/5 *column (Amersham Pharmacia Biotech AB, Uppsala, Sweden),

*Trademark

equilibrated with the same buffer. The enzyme was eluted with a linear gradient of 0 to 350 mM NaCl. Active fractions (see below, enzyme assays) were monitored and pooled (between 80-110 mM NaCl). The partially purified enzyme was dialyzed against 20 mM citrate buffer pH=3.5, applied to a Resource-S column equilibrated with the same buffer, and eluted with a gradient of 0-1 M NaCl. The purified enzyme (eluted at 155 mM NaCl) was concentrated by ultrafiltration (50 kDa cut-off membrane, Amicon).

Enzyme assays:

β-glucosidase enzyme activity was monitored using a plate assay as follows. 4-methylumbelife β-D-glucopyranoside (MUGlc, Sigma Chemical Inc. St. Louis, Missouri) to a final concentration of 0.5 mM, was dissolved in PC buffer (50 mM phosphate, 12 mM citric acid, pH=3.4) at 45 °C. The solution was mixed with 3 % agar in water, previously boiled and then cooled to 45 °C. The resulting solution (20 ml) was poured into a petri dish and after solidification, 10 µl enzyme samples were spotted. The plate was incubated at 50 °C for one hour, and then illuminated with long UV. An intense fluorescence was indicative of β-glucosidase activity.

Detection of β-glucosidase in polyacrylamide gels was carried out by washing the SDS-polyacrylamide gel with 1:1 isopropanol:PC buffer to remove SDS and renature the enzyme. The gel was washed once in PC buffer and incubated in a thin layer of a solution of 0.5 mM MUGlc. After incubation at 50 °C for one hour, the active protein band was visualized by UV light.

Quantitative assays were performed using pNPGlc as a substrate according to Shoseyov (7).

Determination of thermal stability of A. niger β-glucosidase:

Recombinant enzyme (40 µg/ml) was dissolved in 20 mM citrate phosphate buffer, pH=5. Each tested sample (8 µl) was covered by 15 µl mineral oil. The activity was determined by the standard pNPGlc assay (7).

Deglycosylation of A. niger β-glucosidase by N-glycosidase-F:

A N-glycosidase-F (Boehringer Mannheim, Mannheim, Germany) reaction mixture, containing 0.125 µg pure β-glucosidase (previously denatured by boiling for 3 minutes in 1 % SDS and 5 % β-mercaptoethanol), 0.2 units of the N-glycosidase-F, sodium phosphate buffer (50 mM, pH=7.5), EDTA (25 mM), 1 % Triton X-100* and 0.02 % sodium azide, in a total volume of 12.5 µl, was incubated for 4 hours at 37 °C. Reaction was stopped by addition of PAGE sample application buffer followed by 3 minutes of boiling.

Proteolysis and N-terminal sequences of A. niger B1 β-glucosidase:

Partial enzymatic proteolysis with *Staphylococcus aureus* V8 protease was carried out as described by Cleveland (28). Briefly, FPLC-purified β-glucosidase (5 µg), was concentrated by acetone precipitation. The protein was separated on a preparative 10 % SDS-PAGE. The gel was stained with coomassie blue, destained and rinsed with cold water, and the β-glucosidase protein band was excised. The resulting gel slice was applied to a second SDS-PAGE gel (15 % acrylamide) and overlaid with *Staphylococcus aurous* V8 protease. Digestion was carried out within the stacking gel by turning off the current for 30 min. As the bromophenol blue dye neared the bottom of the stacking gel, the current was restored. The electrophoresed cleavage products were electroblotted to PVDF membranes. The native protein was transferred to PVDF in parallel. The N-terminal sequence of the native protein and two of the numerous cleavage products were analyzed by Edman degradation using a gas-phase protein sequencer (Applied Biosystems model 475A microsequencer).*

Cloning of bgl1 cDNA and genomic gene:

Total RNA isolation: Total RNA was isolated from *Aspergillus niger* B1 as follows: *A. niger* B1 was grown in liquid culture consisting of mineral media $(NH_4)_2SO_4 \cdot 3H_2O$ (0.5 g/l), KH_2PO_4 (0.2 g/l), $MgSO_4$ (0.2 g/l), $CaCl_2 \cdot H_2O$ (0.1 g/l), $FeSO_4 \cdot 6H_2O$ (0.001 g/l), $ZnSO_4 \cdot 7H_2O$ (0.001 g/l), and

*Trademarks

2 mM citric acid, at pH=3.5 with 1 % w/v bran as a carbon source. The medium was autoclaved, cooled and inoculated with *A. niger* B1 (10^6 spores/ml). Baffled flasks were used with constant shaking (200 RPM) at 37 ° C. The appearance of β-glucosidase activity was monitored by placing 5 µl of growth medium on 1 % agar plates containing 0.5 mM MUGlc, as described above. Activity was detected following 15 hours incubation. The mycelium was harvested following 24 hours growth period, and the medium removed by filtering through GFA glass microfibre (Whatman Inter. Ltd., Maidstone, England). The mycelium was then frozen with liquid nitrogen and ground to fine powder with mortar and pestle. Total RNA was produced from this powder by the Guanidine thiocyanate (TriReagent™) method (Molecular Research Center, Inc.).

RNA reverse-transcription reaction: cDNA was obtained by reverse transcribing total RNA (10 µg) using Stratagene RT-PCR** kit (Stratagene, La Jolla, CA). The reaction mixture (50 µl) additionally consisted of: Oligo dT18 (1 µg), RNase Block Ribonuclease Inhibitor (20 units), 1x buffer (50 mM Tris-HCl, pH=8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$), dNTPs (500 µM each) and reverse transcriptase (300 units). Total RNA was initially denatured at 70 °C, allowed to cool to room temperature (for primers annealing), and added to the reaction mixture. The reaction mixture was incubated for 1 hour at 37 °C, followed by heating (95 °C, 5 minutes) and stored at -70 °C until further use.

DNA amplification: Degenerate primers for DNA amplification reaction by PCR methods were synthesized, based on part of the amino acid N-terminal sequence and an internal sequence, as determined by the Edman degradation, following V8 proteolysis (hereinbelow, experimental results). The partial sequence from β-glucosidase N-terminal derived amino acid sequence was Ser-Pro-Pro-Tyr-Tyr-Pro (SEQ ID NO:4), yielding the following primer: 5'-(C/G)(A/C/G/T)CC(A/C/G/T) CC(A/C/G/T)TA(C/T)TA(C/T)CC-3'

*Trademark

(SEQ ID NO:5). The partial sequence from E2 internal cleavage product amino acid sequence was Gln-Pro-Ile-Leu-Pro-Ala-Gly-Gly (SEQ ID NO:6), yielding the following primer: 5'-TCCIGC(T/G/C/A)GG(TG/C/A)A(G/A)(T/G/A)AT(T/G/C/A)GG(T/C)TG-3' (SEQ ID NO: 7).

DNA amplification reaction mixture (25 µl) contained: reverse transcriptase reaction product (1 µl), 10 x PCR buffer (2.5 µl, Promega Corp., Madison, WI), dNTPs (250 µM each), $MgCl_2$ (2.0 mM), degenerate primers (250 pmol each), DNA polymerase (3 units, Stratagene, La Jolla, CA) and overlaid with mineral oil (25 µl). The reaction was performed in an automated heating block (Programmable thermal controller - MJ Research, Inc.). PCR cycling conditions were 30 seconds denaturing at 94 °C, 60 seconds annealing at 50 °C, and 150 seconds elongation at 72 °C, repeated 36 times. The resulting amplified product was electrophoresed on a 1.2 % (w/v) agarose/TBE gel, resulting in a 2.2 kb cDNA gene fragment, which was further isolated using Gel Extraction* Kit (QIAGEN, Hilden, Germany) and cloned directly into the single 3'-T PCR insertion site of pGEM-T cloning vector* (Promega Corp., Madison, WI).

Probe preparation: The 2.2 kb partial cDNA was digested with *Pst*I to produce a 1.2 kb fragment DNA probe. A sample (25 ng) of the fragment was labeled with [^{32}P]dCTP, using the random sequence nanonucleotide rediprime DNA labeling system (Amersham Pharmacia Biotech AB, Buckinghamshire, England).

Preparation of genomic DNA plasmid library: An *A. niger* B1 genomic library was constructed in the pYEAUra3 yeast/*E. coli* shuttle vector (Clontech Lab. Inc. Palo Alto, CA). *A. niger* B1 was grown in liquid culture as described above, the mycelium harvested following 48 hours of growth, frozen in liquid nitrogen and grounded. The mycelium ground was used to produce genomic DNA by the CTAB method of Murray and Thompson (29). The library was constructed from partially digested *Sau*3A genomic DNA, cloned into the *Bam*HI site of the pYEUra3* yeast shuttle vector (Clontech Lab.

*Trademarks

Inc. Palo Alto, CA). pYEAUra3 yeast/*E. coli* shuttle vector was digested with *Bam*HI and dephosphorylated with CIP to prevent self ligation. The partially digested genomic DNA was cloned into the shuttle vector with T4 ligase and used to transform TOP10 *E. coli* electro-competent cells, which were then plated on LB-agar containing ampicillin (50 μg/ml). A total of 4 x 10^4 colonies were grown on LB-agar plates, blotted to Hybond-N* membranes (Amersham Pharmacia Biotech AB, Buckinghamshire, England) and screened using the above described 1.2 kb probe. Positive clones were subcloned in pUC18 and sequenced (Biological Services, The Weizmann Institute of Science, Rehovot, Israel).

Expression of bgl1 cDNA in E. coli:

Two specific primers were designed according to the 5' and the 3' sequences, corresponding to the N-terminal and C-terminal region of the mature protein: sense primer: 5'-' (SEQ ID NO:8). Antisense primer: 5'-AAAGGATCCTTAGTGAACAGTAGGCAGAGACGC-3' (SEQ ID NO:9). The isolated cDNA was digested with *Nco*I and *Bam*HI and cloned into a pET3d* expression vector (Figure 1A, Novagen Inc., Madison, WI). Positive *E. coli* BL21(DE3) pLysS colonies, containing the bgl1 cDNA, were confirmed by enzyme restriction and sequence analysis. Recombinant BGL1 was expressed according to the manufacturer's protocol.

Expression of bgl1 cDNA in Saccharomyces cerevisiae and Pichia pastoris:

The pYES2* vector (Invitrogen Inc., San Diego, CA) was used to successfully clone the bgl1 cDNA gene into the *Hin*dIII/*Bam*HI of pYES2-bgl1 plasmid (Figure 1b), and transform *Saccharomyces cerevisiae* using the lithium acetate method (30). The BGL1 was expressed by inducing the Gal1 promoter according to the manufacturer's protocol. *Saccharomyces cerevisiae* strain INVSc2 (MATa, his3-D200, ura3-167) was used as the host. *Pichia pastoris* strain GS115 (his4 mutant) was used as the host for shuttle and expression vector plasmid pHIL-S1 (Invitrogen Inc., San Diego, CA). The

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bgl1 cDNA was cloned into the *Eco*RI/*Bam*HI sites of pHIL-S1, yielding the pHIL-S1-bgl1 expression and secretion vector (Figure 1c). Expression in *P. pastoris* was carried out according to the manufacturer's protocol. Screening of β-glucosidase-expressing clones was facilitated by top-agar, containing 50 mg X-Glc, 30 ml methanol and 1 % agar per liter. Blue color indicated a colony producing active β-glucosidase.

Western blot analysis:

Antibodies were produced from rabbit serum 36 days following a second injection of 100 µg purified protein and adjuvant (AniLab Biological Services, Tal-Sachar, Israel). High molecular weight ladder was from Sigma Chemical Inc. St. Louis, Missouri. Western blot conditions were as described in reference 36.

Determination of the Stereochemical Course of Hydrolysis:

The method was essentially as described by Wong *et al.* (31). PNPGlc (10 µmols) was dissolved in 0.5 ml of 25 mM acetate buffer pH=3.5 in D_2O in an NMR tube. β-Glucosidase was lyophilized and redissolved in 100 µl D_2O (35 units/ml). The 1H-NMR spectrum of the substrate was recorded, enzyme added (10 µl), and spectra recorded at specified time intervals on a Bruker AMX400 at 25 °C.

Inactivation and reactivation studies:

Pure *A. niger* β-Glucosidase enzyme (0.47 mg/ml) was incubated in the presence of various concentrations of 2-deoxy-2-fluoro-β-glucosyl fluoride (2FGlcF, 0.5-6 mM) in 30 mM citrate buffer pH=4.8 at 50 °C. Residual enzyme activity was determined at different time intervals by addition of an aliquot (10 µl) of the inactivation mixture, to a solution containing citrate buffer (30 mM, pH=4.8), BSA (8 µg) and 2,4-dinitrophenyl β-D-glucopyranoside (DNPGlc, 0.625 mM, 830 µl). Release of DNP was determined spectrophotometrically by measuring the absorbance at 400 nm one minute after the addition of the substrate.

Reactivation rates were determined as follows: pure *A. niger* β-glucosidase (0.34 mg/ml) was preincubated with 2FGlcF (5 mM) for 15 min, after which the excess of the inactivator was diafiltered by 20-kDa nominal molecular mass cutoff centrifugal concentrators (Sartorius Inc., Goettingen, Germany). Samples of the purified, inactivated enzyme were incubated in the presence linamarin (0-16 mM) in citrate buffer (30 mM, pH=4.8) at 50 °C for 0, 10, 20 and 30 minutes, and the activity of each sample was determined using p-nitrophenyl β-D-glucopyranoside (pNPGlc) as a substrate.

Expression of bgl1 cDNA in tobacco plants:

Genetic constructs:

Bgl1 cDNA was cloned in pETB1 (37). pJD330 and pBINPlus (38) were used as an intermediate and binary vector, respectively. Cel1 signal sequence as well as 35S plus Ω fragment were retrieved from pB21, modified pBluescript SK (39). *Nicotiana tabacum* cv. Samson was used as a model plant for gene transformation. Three gene constructs were employed (Figures 11a-c): (i) bgl1 without any signal peptide which served for cytoplasmic expression (Figure 11a, plasmid pJDB1); (ii) bgl1 including a cel1 signal peptide at the N terminus for secretion into the apoplast (Figure 11b, plasmid pJDCB1); and (iii) bgl1 including the cel1 signal peptide and the KDEL (SEQ ID NO:24) ER-retaining peptide at the C-terminus for accumulation in the ER (Figure 11c, plasmid pJDCB1T).

To this end, bgl1 cDNA (2.5 kb) was released from pETB1 (37) with *Nco*I and *Bam*HI and inserted into pJD330 between the 35S promoter Ω fragment and the *nos* terminator, eliminating the *gus* gene, resulting in plasmid pJDB1. Endoplasmic reticulum retaining signal tetrapeptide HDEL (SEQ ID NO:17) was synthesized and fused with *bgl*1 at the C-terminal in pJDB1 by a fidelity PCR reaction with the following pair of primers: Forward primer (23 mer), starting from nucleotide 1248 of bgl1 cDNA 5' - (1248) - CAGTGACCGTGGATGCGACAATG - (1270') - 3' (SEQ ID NO:20); Reverse primer (40 mer), starting at nucleotide 2506 of bgl1 cDNA encoding

also for the HDEL (SEQ ID NO:17) peptide 5' - (2506) - AGAGACGGATGACAAGTACTACTTGAAATTGGGCCCAAAA-3' (SEQ ID NO:21). For pJDCB1T (35S Ω + Cel1 + bgl1 + HDEL), the 35S Ω fragment of pJDB1 was replaced by a 35S Ω + Cel1 fragment digested from pB21 with *Bam*HI and *Xba*I. For pJDCB1 (35S Ω + Cel1 + bgl1), the fragment containing 35S Ω and Cel1 as well as part of bgl1 was cut from pJDCB1T with *Hind*III and *Nru*I and ligated with the vector of pJDB1 digested with the same pair of restriction enzymes. The nucleotide sequence of all of the genetic constructs was confirmed by DNA sequencing.

Gene cassettes in the intermediate vectors of pJDB1, pJDCB1 and pJDCB1T were further isolated with *Hind*III and *Eco*RI and inserted into multiple cloning sites of the binary vector pBINPlus. Disarmed *Agrobateruim* LB4404 was transformed with pBINPlus containing bgl1 gene cassettes.

Tobacco plant transformation:

The young leaves of *in vitro* grown plantlets were excised and cut into 0.5 cm^2 pieces and then immersed for 5 minutes in an overnight grown culture of *Agrobacterium*. After blotted with sterile Whatman filter paper, the infected leaves were co-cultured for 2 days with *Agrobacterium* on MS medium plus 2.0 mg/L of Zeatin and 0.1 mg/L of IAA as well as 0.35 % (w/v) phytagel and then transferred to the same medium but with 300 mg/L kanamycin and 300 mg/L carbenicillin. Regenerates were then transferred to the rooting media, containing only MS salts, vitamins and the same antibiotics. Rooted plants were transferred to greenhouse after PCR screening.

Screening for transgenic plants:

DNA and protein of plants were extracted according to Nagy *et al*. (40). PCR verification of gene insertion into plant genome was done with the following pairs of primers, which cover the DNA fragment from position 1248 to the end of bgl1: 5'-CAGTGACCGTGGATGCGACAATG-3' (SEQ ID NO:22) and 5'-AAAGGATCCTTAGTGAACAGTAGGCAGAGACGC-3' (SEQ ID NO:23).

Identifying transgenic plants expressing BGL1 protein and activity:

Western blot (40) and SDS-PAGE activity gel staining (37) were employed to screen successful transgenic lines, using the purified *A. niger* BGL1 protein as positive controls and non-transgenic plant as negative control.

SPMI-GC/MS analysis:

The effect of bgl1 on flavor compound evolution and composition was studied. Fresh leaves of transgenic plants and of wild type control plants were excised and ground in liquid nitrogen. Ice-cold extraction buffer, containing 10 mM EDTA, 4 mM DTT in 50 mM phosphate buffer, pH 4.3, was added in a ratio of 1:3 w/w. The mixture was then shaken for 0.5 hours. 0.75 ml of supernatant from each of the centrifuged mixtures was taken into a glass vial. All manipulations were at 4 °C. After 9 hours of incubation at 37 °C, the volatiles in the vial were analyzed according to Clark *et al.* (41) using a Saturn Varian 3800 SPMI-GC-MS* apparatus, equipped with a DB-5 capillary column.* The temperature of splitless injections was 250 °C and the transfer line was maintained at 280 °C. Helium was used as a carrier gas. The oven was programmed as follows: 1 minute at 40 °C with gradually heating to 250 °C at a rate of 5 °C/minute.

EXPERIMENTAL RESULTS

Purification of wild type A. niger β-glucosidase:

A. niger β-glucosidase enzyme preparation was purified by Mono-Q FPLC. Active protein samples eluted from the Mono-Q column were separated on a 10 % SDS-PAGE gel, stained with coomassie blue, and incubated in the presence of MUGlc to demonstrate activity of the enzyme. At this stage of purification, a discrete band, having an apparent molecular mass of approximately 160 kDa and β-glucosidase activity could be detected (Figure 2b, lanes 1-5: 1 - electroeluted band of BGL1 from preparative PAGE-SDS gel stabs; 2-5 - acetone precipitates from Mono-Q separation of BGL1). However,

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the apparent mass of the denatured enzyme (boiled for 10 min in the presence of β-mercaptoethanol), was shown to be 120 kDa on 10 % SDS-PAGE (Figure 2a). The enzyme was designated BGL1 was further purified to homogeneity on a Resource-S column* (Figure 3). Deglycosylation of *A. niger* β-glucosidase was performed by N-glycosidase-F. As demonstrated in Figure 4, SDS-PAGE analysis indicated that approximately 20 kDa of the *A. niger* β-glucosidase mass can be attributed to N-linked carbohydrates.

Proteolysis and N-terminal sequences of BGL1:

Partial enzymatic proteolysis with *Staphylococcus aureus* V8 protease of purified BGL1 was conducted. The undigested protein and cleavage products were separated by SDS-PAGE, followed by electroblotting onto PVDF membranes and determination of the N-terminal sequence of the native protein and two of the cleavage products. Amino acid sequences obtained were as follows:

N-terminal native protein: Asp-Glu-Leu-Ala-Tyr-Ser-Pro-Pro-Tyr-Tyr-Pro-Ser-Pro-Trp-Ala-Asn-Gly-Gln-Gly-Asp (SEQ ID NO:10). Underlined portion represents SEQ ID NO:4.

Internal cleavage product - E1 polypeptide: Val-Leu-Lys-His-Lys-Asn-Gly-Val-Phe-Thr-Ala-Thr-Asp-Asn-Trp-Ala-Ile-Asp-Gln-Ile-Glu-Ala-Leu-Ala-Lys (SEQ ID NO: 11).

Internal cleavage product - E2 polypeptide: Gly-Ala-Thr-Asp-Gly-Ser-Ala-Gln-Pro-Ile-Leu-Pro-Ala-Gly-Gly-Gly-Pro-Gly-Gly-Asn-Pro (SEQ ID NO:12). Underlined portion represents SEQ ID NO:6.

FastA analysis (32) indicated that the N-terminal sequence, as well as the internal sequences, have sequence similarity with sequences of β-glucosidase from the yeast *Saccharomycopsis fibuligera* which belonging to Family 3 of the glycosyl hydrolases.

Isolation and characterization of bgl1 cDNA and genomic DNA:

In order to clone the *A. niger* β-glucosidase gene, degenerate primers were designed according to the sequence of digest fragments of the

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polypeptide. These oligonucleotides were used to amplify a cDNA fragment of the β-glucosidase gene by RT-PCR. A 1.2 kb probe was excised from the resultant 2.2 kb amplification product and was used to screen a genomic library, constructed in pYEUra3 yeast/*E. coli* shuttle vector. Positive clones were successfully subcloned and sequenced, resulting in full length bgl1 genomic sequence (SEQ ID NO:3, Figure 5a). Amplification primers were then generated, according to the genomic DNA sequence, corresponding to the N- and C-terminal of the mature protein. RT-PCR was thereafter used for amplifying the full length β-glucosidase cDNA sequence (SEQ ID NO:1, Figure 5a, GenBank Accession No. AJ132386). The cDNA sequence perfectly matched the DNA sequence of the combined exons. The open reading frame was found to encode a polypeptide with a predicted molecular weight of 92 kDa. The gene includes 7 exons intercepted by 6 introns (Figure 5b). Analysis of the DNA sequence upstream to the sequence encoding for the mature protein revealed a putative leader sequence, intercepted by an 82 bp intron.

Production of rBGL1 in E. coli:

Recombinant BGL1 was overexpressed in *E. coli*. No apparent β-glucosidase activity could be detected in the *E. coli* extracts, however SDS-PAGE analysis revealed a relatively intense protein band expressed at the expected molecular weight. Western blot analysis using rabbit polyclonal anti-native BGL1 antibodies (AniLab Biological Services, Tal-Sachar, Israel), positively identified the 90 kDa protein band (not shown). Further analysis revealed that the protein was accumulated in inclusion bodies. Several refolding experiments were conducted, however, these efforts to produce active protein from *E. coli* failed (not shown).

Expression of recombinant BGL1 in S. cerevisiae and P. pastoris:

Recombinant BGL1 was successfully expressed both in *S. cerevisiae* and *P. pastoris*. In *S. cerevisiae* a relatively low level of expression was found. The recombinant protein was detected by a Western blot analysis

(Figure 6a). The total protein extract of *S. cerevisiae* expressing bgl1 cDNA had a β-glucosidase activity of 1.9 units/mg protein. No β-glucosidase activity was detected in control *S. cerevisiae*, transformed with vector only, under the same assay conditions. However, no protein band corresponding to recombinant BGL1 could be detected by coomassie blue staining. *P. pastoris* transformed with bgl1 secreted relatively high levels of recombinant BGL1 to the medium (about 0.5 g/l) appearing as an almost pure protein in the culture supernatant (Figure 6b). This recombinant enzyme was highly active (124 units/mg protein) and without further purification, yielded specific activity similar to that of the pure native enzyme.

1H-NMR determination of stereochemical outcome:

1H-NMR spectra of a reaction mixture containing pNPGlc and BGL1 revealed that the beta anomer of glucose was formed first (H-1 = 4.95 ppm), with delayed appearance of the alpha anomer (H-1 5.59 ppm), the consequence of mutarotation (Figure 7). BGL1 is indeed, therefore, a retaining glycosidase, as has been observed for other family members (33, 34).

Inactivation and reactivation of A. niger β-glucosidase:

Enzyme was incubated in the presence of various concentrations of 2FGlcF and residual enzyme activity was monitored at different time intervals. Enzyme activity decreased in a time-dependent manner, according to pseudo-first order kinetics, allowing the determination of pseudo-first order rate constants: $K_i = 4.5\ min^{-1}$ and $K_I = 35.4$ mM, for inactivation at each inactivator concentration (0, 0.5, 1, 2, 4, and 6 mM, Figure 8).

Rates of reactivation of 2-deoxy-2-fluoroglucosyl-BGL1 were determined in the presence of different concentrations of linamarin by monitoring activity regain after 0, 10, 20 and 30 min (Figure 9). The regain of activity followed a first order process at each linamarin concentration.

Thermal stability of A. niger β-glucosidase:

Thermal stability of the recombinant enzyme was evaluated at different temperatures, presented as percent enzymatic activity relative to an enzyme

solution kept at 4 °C. Results obtained are summarized in Table 2 and illustrated in Figure 10. The purified enzyme exhibits high thermal stability, as majority (above 50 %) of the activity is maintained at a temperature ranging from 4-60 °C.

TABLE 2

Temp.°C	% activity
4	100
50	91.5
55	83.5
60	68
65	17.8

Expression of BGL1 in tobacco plants:

Agrobacterium mediated leaf disc transformation resulted in transgenic tobacco plants as was proved by PCR (Figure 12) for the presence of the transgene, Western blotting (Figures 13a-b) for presence of the protein and activity assays (Figures 14 and 15) for presence of protein activity. Table 3 below summarizes the results.

TABLE 3

Gene construct	BGL1	Cel1 + BGL1 + HDEL	Cel1 + BGL1
Number of Regenerates	33	14	27
PCR positive	29	9	23
Western Blot positive	4	9	18
Activity gel positive	0	9	18

Of the 29 PCR positive regenerates transformed with cDNA encoding BGL1, which fails to encode a signal peptide, only in 4 the BGL1 protein was detectable via Western blotting, however no BGL1 activity was measurable in any of which. The BGL1 was found smaller in molecular weight compared to wild type *A. niger* beta-glucosidase and of processed recombinant BGL1

containing a signal peptide. Its apparent size of about 95 kDa is very close to 92 kDa which is the calculated molecular weight of the un-glycosylated *A. niger* beta-glucosidase. This result coincides with the fact that a protein with no signal peptide is expected to be released from the ribosomes and remain in the cytoplast (42) un-glycosylated, as protein glycosylation is conducted in the lumen of the endoplasmic reticulum (43).

Of the 9 PCR positive regenerates transformed with a cDNA encoding the BGL1 and a Cel1 signal peptide and in addition encodes the HDEL ER retaining peptide, all plants expressed detectable amounts of BGL1 protein and activity.

Of the 23 PCR positive regenerates transformed with a cDNA which encodes the BGL1 protein and the Cel1 signal peptide but not the HDEL ER retaining peptide, 18 plants expressed detectable amounts of BGL1 protein and activity.

The effect of BGL1 on flavor compound evolution and composition in transgenic tobacco plants:

Extracts of transgenic plants (CB14 and CBT21 containing similar BGL1 activity, see Figure 15) were incubated for 9 hours at 37 °C, and flavor compounds were analyzed by SPMI-GC/MS. The results, which are summarized in Table 4 below, show that with the exception of oleyl alcohol, the concentration of different flavor compounds is increased in transgenic plants expressing active BGL1 compared with the control. Furthermore, it seems that compartmentalization of BGL1 in the ER (or for that matter, any other subcellular organelle), rather then its secretion to the apoplast, results in higher release of flavor compounds. It is likely that this is resulted from the localization many flavor compounds in the apoplast, thus, secretion of BGL1 to the apoplast cause *in vivo* release of flavor compounds, while compartmentalization of BGL1 in the ER results in release of flavor compounds only in the event of cell disruption and decompartmentalization.

TABLE 4

Retention Time (minutes)	Scan	Name	CB14	CBT 21
3.917	419	Hexanal	-[a]	-
4.749	508	3-methyl-pentanoic acid		-
4.863	520	2-Hexenal	-	+[b]
5.167	552	?	-	+
6.564	702	1-Heptanol	-	-
7.1	752	?	+	++[d]
8.085	865	2-ethyl-1-pexanol	-	+
8.132	870	Limonene	++	+
8.194	877	2-methyl-phenol	-	+
10.653	1139	Menthol	+	+
11.757	1258	Nerol	-	+
12.039	1288	6-Quinolinol	-	+
12.1	1294	2-butyl-1-octanol	-	+
13.0	1458	?	-	+
13.7	1466	?	-	+
14.091	1507	Vitispirane	-	+
14.094	1516	4-[2,6,6-trimethyl-1-cyclohexen-1-yl] 3-Buten-1-one	+	++
15.985	1710	?	-	-
19.327	2069	Oleyl alcohol	--[c]	--

CB14 - transgenic plant containing Cell signal peptide + BGL1; CBT 21 - transgenic plant containing Cell signal peptide + BGL1 + HDEL ER retaining peptide. a - "-" means no significant difference in concentration compared with wile type. b - "+" means significant increase compared with the wild type. c - "--" means significant decrease compared with the wild type. d - "++" means significant increase compared with a respective mark "+". ? - unknown compound.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. In addition, citation or identification of any reference in this application shall not be

construed as an admission that such reference is available as prior art to the present invention.

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<120> ASPEGILLUS NIGER BETA-GLUCOSIDASE GENE, PROTEIN AND USES THEREOF

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<213> Aspergillus niger

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35 40 45

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Gly Thr Gly Trp Glu Leu Glu Leu Cys Val Gly Gln Thr Gly Gly Val

65 70 75 80

Pro Arg Leu Gly Val Pro Gly Met Cys Leu Gln Asp Ser Pro Leu Gly

85 90 95

Val Arg Asp Ser Asp Tyr Asn Ser Ala Phe Pro Ala Gly Met Asn Val

100 105 110

Ala Ala Thr Trp Asp Lys Asn Leu Ala Tyr Leu Arg Gly Lys Ala Met

115 120 125

Gly Gln Glu Phe Ser Asp Lys Gly Ala Asp Ile Gln Leu Gly Pro Ala

130 135 140

Ala Gly Pro Leu Gly Arg Ser Pro Asp Gly Gly Arg Asn Trp Glu Gly

145 150 155 160

Phe Ser Pro Asp Pro Ala Leu Ser Gly Val Leu Phe Ala Glu Thr Ile

165 170 175

Lys Gly Ile Gln Asp Ala Gly Val Val Ala Thr Ala Lys His Tyr Ile

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Ala Tyr Glu Gln Glu His Phe Arg Gln Ala Pro Glu Ala Gln Gly Phe

195 200 205

Gly Phe Asn Ile Ser Glu Ser Gly Ser Ala Asn Leu Asp Asp Lys Thr

210 215 220

Met His Glu Leu Tyr Leu Trp Pro Phe Ala Asp Ala Ile Arg Ala Gly

225 230 235 240

Ala Gly Ala Val Met Cys Ser Tyr Asn Gln Ile Asn Asn Ser Tyr Gly

245 250 255

Cys Gln Asn Ser Tyr Thr Leu Asn Lys Leu Leu Lys Ala Glu Leu Gly

260 265 270

Phe Gln Gly Phe Val Met Ser Asp Trp Ala Ala His His Ala Gly Val

275 280 285

Ser Gly Ala Leu Ala Gly Leu Asp Met Ser Met Pro Gly Asp Val Asp

290 295 300

Tyr Asp Ser Gly Thr Ser Tyr Trp Gly Thr Asn Leu Thr Ile Ser Val

305 310 315 320

Leu Asn Gly Thr Val Pro Gln Trp Arg Val Asp Asp Met Ala Val Arg

325 330 335

Ile Met Ala Ala Tyr Tyr Lys Val Gly Arg Asp Arg Leu Trp Thr Pro

340 345 350

Pro Asn Phe Ser Ser Trp Thr Arg Asp Glu Tyr Gly Tyr Lys Tyr Tyr

355 360 365

Tyr Val Ser Glu Gly Pro Tyr Glu Lys Val Asn Gln Tyr Val Asn Val

370 375 380

Gln Arg Asn His Ser Glu Leu Ile Arg Arg Ile Gly Ala Asp Ser Thr

385 390 395 400

Val Leu Leu Lys Asn Asp Gly Ala Leu Pro Leu Thr Gly Lys Glu Arg

405 410 415

Leu Val Ala Leu Ile Gly Glu Asp Ala Gly Ser Asn Pro Tyr Gly Ala

420 425 430

Asn Gly Cys Ser Asp Arg Gly Cys Asp Asn Gly Thr Leu Ala Met Gly

435 440 445

Trp Gly Ser Gly Thr Ala Asn Phe Pro Tyr Leu Val Thr Pro Glu Gln

450 455 460

Ala Ile Ser Asn Glu Val Leu Lys His Lys Asn Gly Val Phe Thr Ala

465 470 475 480

Thr Asp Asn Trp Ala Ile Asp Gln Ile Glu Ala Leu Ala Lys Thr Ala

485 490 495

Ser Val Ser Leu Val Phe Val Asn Ala Asp Ser Gly Glu Gly Tyr Ile

500 505 510

Asn Val Asp Gly Asn Leu Gly Asp Arg Arg Asn Leu Thr Leu Trp Arg

515 520 525

Asn Gly Asp Asn Val Ile Lys Ala Ala Ala Ser Asn Cys Asn Asn Thr

530 535 540

Ile Val Val Ile His Ser Val Gly Pro Val Leu Val Asn Glu Trp Tyr

545 550 555 560

Asp Asn Pro Asn Val Thr Ala Ile Leu Trp Gly Gly Leu Pro Gly Gln

565 570 575

Glu Ser Gly Asn Ser Leu Ala Asp Val Leu Tyr Gly Arg Val Asn Pro

580 585 590

Gly Ala Lys Ser Pro Phe Thr Trp Gly Lys Thr Arg Glu Ala Tyr Gln

595 600 605

Asp Tyr Leu Val Thr Glu Pro Asn Asn Gly Asn Gly Ala Pro Gln Glu

610 615 620

Asp Phe Val Glu Gly Val Phe Ile Asp Tyr Arg Gly Phe Asp Lys Arg

625 630 635 640

Asn Glu Thr Pro Ile Tyr Glu Phe Gly Tyr Gly Leu Ser Tyr Thr Thr

645 650 655

Phe Asn Tyr Ser Asn Leu Glu Val Gln Val Leu Ser Ala Pro Ala Tyr

660 665 670

Glu Pro Ala Ser Gly Glu Thr Glu Ala Ala Pro Thr Phe Gly Glu Val

675 680 685

Gly Asn Ala Ser Asp Tyr Leu Tyr Pro Ser Gly Leu Leu Arg Ile Thr

690 695 700

Lys Phe Ile Tyr Pro Trp Leu Asn Gly Thr Asp Leu Glu Ala Ser Ser

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725 730 735

Ala Thr Asp Gly Ser Ala Gln Pro Ile Leu Pro Ala Gly Gly Gly Pro
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Gly Gly Asn Pro Arg Leu Tyr Asp Glu Leu Ile Arg Val Ser Val Thr
755 760 765

Ile Lys Asn Thr Gly Lys Val Ala Gly Asp Glu Val Pro Gln Leu Tyr
770 775 780

Val Ser Leu Gly Gly Pro Asn Glu Pro Lys Ile Val Leu Arg Gln Phe
785 790 795 800

Glu Arg Ile Thr Leu Gln Pro Ser Glu Glu Thr Lys Trp Ser Thr Thr
805 810 815

Leu Thr Arg Arg Asp Leu Ala Asn Trp Asn Val Glu Lys Gln Asp Trp
820 825 830

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<211> 3885

<212> DNA

<213> Aspergillus niger

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<213> Aspergillus niger

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<223> Staphylococcus aureus V8 protease cleavage product

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<223> Staphylococcus aureus V8 protease cleavage product

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<223> Staphylococcus aureus V8 protease cleavage product

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<210> 13

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<212> DNA

<213> Artificial sequence

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<223> Recombinant sequence expressing BGL1 in tobacco plants

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<211> 841

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<213> Artificial sequence

<220>

<223> Recombinant sequence expressing BGL1 in tobacco plants

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Ser Gln Met Thr Leu Asp Glu Lys Val Asn Leu Thr Thr Gly Thr Gly
35 40 45

Trp Glu Leu Glu Leu Cys Val Gly Gln Thr Gly Gly Val Pro Arg Leu
50 55 60

Gly Val Pro Gly Met Cys Leu Gln Asp Ser Pro Leu Gly Val Arg Asp
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Ser Asp Tyr Asn Ser Ala Phe Pro Ala Gly Met Asn Val Ala Ala Thr

85 90 95

Trp Asp Lys Asn Leu Ala Tyr Leu Arg Gly Lys Ala Met Gly Gln Glu

100 105 110

Phe Ser Asp Lys Gly Ala Asp Ile Gln Leu Gly Pro Ala Ala Gly Pro

115 120 125

Leu Gly Arg Ser Pro Asp Gly Gly Arg Asn Trp Glu Gly Phe Ser Pro

130 135 140

Asp Pro Ala Leu Ser Gly Val Leu Phe Ala Glu Thr Ile Lys Gly Ile

145 150 155 160

Gln Asp Ala Gly Val Val Ala Thr Ala Lys His Tyr Ile Ala Tyr Glu

165 170 175

Gln Glu His Phe Arg Gln Ala Pro Glu Ala Gln Gly Phe Gly Phe Asn

180 185 190

Ile Ser Glu Ser Gly Ser Ala Asn Leu Asp Asp Lys Thr Met His Glu

195 200 205

Leu Tyr Leu Trp Pro Phe Ala Asp Ala Ile Arg Ala Gly Ala Gly Ala

210 215 220

Val Met Cys Ser Tyr Asn Gln Ile Asn Asn Ser Tyr Gly Cys Gln Asn

225 230 235 240

Ser Tyr Thr Leu Asn Lys Leu Leu Lys Ala Glu Leu Gly Phe Gln Gly

245 250 255

Phe Val Met Ser Asp Trp Ala Ala His His Ala Gly Val Ser Gly Ala

260 265 270

Leu Ala Gly Leu Asp Met Ser Met Pro Gly Asp Val Asp Tyr Asp Ser

275 280 285

Gly Thr Ser Tyr Trp Gly Thr Asn Leu Thr Ile Ser Val Leu Asn Gly

290 295 300

Thr Val Pro Gln Trp Arg Val Asp Asp Met Ala Val Arg Ile Met Ala

305 310 315 320

Ala Tyr Tyr Lys Val Gly Arg Asp Arg Leu Trp Thr Pro Pro Asn Phe
325 330 335
Ser Ser Trp Thr Arg Asp Glu Tyr Gly Tyr Lys Tyr Tyr Tyr Val Ser
340 345 350
Glu Gly Pro Tyr Glu Lys Val Asn Gln Tyr Val Asn Val Gln Arg Asn
355 360 365
His Ser Glu Leu Ile Arg Arg Ile Gly Ala Asp Ser Thr Val Leu Leu
370 375 380
Lys Asn Asp Gly Ala Leu Pro Leu Thr Gly Lys Glu Arg Leu Val Ala
385 390 395 400
Leu Ile Gly Glu Asp Ala Gly Ser Asn Pro Tyr Gly Ala Asn Gly Cys
405 410 415
Ser Asp Arg Gly Cys Asp Asn Gly Thr Leu Ala Met Gly Trp Gly Ser
420 425 430
Gly Thr Ala Asn Phe Pro Tyr Leu Val Thr Pro Glu Gln Ala Ile Ser
435 440 445
Asn Glu Val Leu Lys His Lys Asn Gly Val Phe Thr Ala Thr Asp Asn
450 455 460
Trp Ala Ile Asp Gln Ile Glu Ala Leu Ala Lys Thr Ala Ser Val Ser
465 470 475 480
Leu Val Phe Val Asn Ala Asp Ser Gly Glu Gly Tyr Ile Asn Val Asp
485 490 495
Gly Asn Leu Gly Asp Arg Arg Asn Leu Thr Leu Trp Arg Asn Gly Asp
500 505 510
Asn Val Ile Lys Ala Ala Ala Ser Asn Cys Asn Asn Thr Ile Val Val
515 520 525
Ile His Ser Val Gly Pro Val Leu Val Asn Glu Trp Tyr Asp Asn Pro
530 535 540
Asn Val Thr Ala Ile Leu Trp Gly Gly Leu Pro Gly Gln Glu Ser Gly

545 550 555 560

Asn Ser Leu Ala Asp Val Leu Tyr Gly Arg Val Asn Pro Gly Ala Lys

565 570 575

Ser Pro Phe Thr Trp Gly Lys Thr Arg Glu Ala Tyr Gln Asp Tyr Leu

580 585 590

Val Thr Glu Pro Asn Asn Gly Asn Gly Ala Pro Gln Glu Asp Phe Val

595 600 605

Glu Gly Val Phe Ile Asp Tyr Arg Gly Phe Asp Lys Arg Asn Glu Thr

610 615 620

Pro Ile Tyr Glu Phe Gly Tyr Gly Leu Ser Tyr Thr Thr Phe Asn Tyr

625 630 635 640

Ser Asn Leu Glu Val Gln Val Leu Ser Ala Pro Ala Tyr Glu Pro Ala

645 650 655

Ser Gly Glu Thr Glu Ala Ala Pro Thr Phe Gly Glu Val Gly Asn Ala

660 665 670

Ser Asp Tyr Leu Tyr Pro Ser Gly Leu Leu Arg Ile Thr Lys Phe Ile

675 680 685

Tyr Pro Trp Leu Asn Gly Thr Asp Leu Glu Ala Ser Ser Gly Asp Ala

690 695 700

Ser Tyr Gly Gln Asp Ser Ser Asp Tyr Leu Pro Glu Gly Ala Thr Asp

705 710 715 720

Gly Ser Ala Gln Pro Ile Leu Pro Ala Gly Gly Gly Pro Gly Gly Asn

725 730 735

Pro Arg Leu Tyr Asp Glu Leu Ile Arg Val Ser Val Thr Ile Lys Asn

740 745 750

Thr Gly Lys Val Ala Gly Asp Glu Val Pro Gln Leu Tyr Val Ser Leu

755 760 765

Gly Gly Pro Asn Glu Pro Lys Ile Val Leu Arg Gln Phe Glu Arg Ile

770 775 780

Thr Leu Gln Pro Ser Glu Glu Thr Lys Trp Ser Thr Thr Leu Thr Arg
785 790 795 800

Arg Asp Leu Ala Asn Trp Asn Val Glu Lys Gln Asp Trp Glu Ile Thr
805 810 815

Ser Tyr Pro Lys Met Val Phe Val Gly Ser Ser Ser Arg Lys Leu Pro
820 825 830

Leu Arg Ala Ser Leu Pro Thr Val His
835 840

<210> 15

<211> 3329

<212> DNA

<213> Artificial sequence

<220>

<223> Recombinant sequence expressing BGL1 fused to Cel1

<400> 15

gaattcccga tcctatctgt cacttcatca aaaggacagt agaaaaggaa ggtggcacta 60

caaatgccat cattgcgata aaggaaaggc tatcgttcaa gatgcctctg ccgacagtgg 120

tcccaaagat ggacccccac ccacgaggag catcgtggaa aaagaagacg ttccaaccac 180

gtcttcaaag caagtggatt gatgtgatat ctccactgac gtaagggatg acgcacaatc 240

ccactatcct tcgcaagacc cttcctctat ataaggaagt tcatttcatt tggagaggac 300

aggcttcttg agatccttca acaattacca acaacaacaa acaacaaaca acattacaat 360

tactatttac aattacagtc gaggggatct atggcgcgaa aatccctaat tttccggtg 420

attttgctcg ccgttcttct cttctctccg ccgatttact ccgccggtca cgattaccgc 480

gacgctctcc gtaaatctag catggctgat gaattggcct actccccacc gtattaccca 540

tcccccttggg ccaatggcca gggcgactgg gcgcaggcat accagcgcgc tgttgatatt 600

gtctcgcaaa tgacattgga tgagaaggtc aatctgacca caggaactgg atgggaattg 660

gaactatgtg ttggtcagac tggcggtgtt ccccgattgg gagttccggg aatgtgttta 720

caggatagcc ctctgggcgt tcgcgactcc gactacaact ctgctttccc tgccggcatg 780

aacgtggctg cgacctggga caagaatctg gcataccttc gcggcaaggc tatgggtcag 840

gaatttagtg acaagggtgc cgatatccaa ttgggtccag ctgccggccc tctcggtaga 900

agtcccgacg gtggtcgtaa ctgggagggc ttctccccag accctgccct aagtggtgtg 960

ctctttgccg agaccatcaa gggtatccaa gatgctggtg tggttgcgac ggctaagcac 1020

tacattgctt acgagcaaga gcatttccgt caggcgcctg aagcccaagg ttttggattt 1080

aatatttccg agagtggaag tgcgaacctc gatgataaga ctatgcacga gctgtacctc 1140

tggcccttcg cggatgccat ccgtgcaggt gctggcgctg tgatgtgctc ctacaaccag 1200

atcaacaaca gttatggctg ccagaacagc tacactctga acaagctgct caaggccgag 1260

ctgggcttcc agggctttgt catgagtgat tgggctgctc accatgctgg tgtgagtggt 1320

gctttggcag gattggatat gtctatgcca ggagacgtcg actacgacag tggtacgtct 1380

tactggggta caaacttgac cattagcgtg ctcaacggaa cggtgcccca atggcgtgtt 1440

gatgacatgg ctgtccgcat catggccgcc tactacaagg tcggccgtga ccgtctgtgg 1500

actcctccca acttcagctc atggaccaga gatgaatacg gctacaagta ctactacgtg 1560

tcggagggac cgtacgagaa ggtcaaccag tacgtgaatg tgcaacgcaa ccacagcgaa 1620

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agtggtactg ccaacttccc atacctggtg acccccgagc aggccatctc aaacgaggtg 1860

cttaagcaca agaatggtgt attcaccgcc accgataact gggctatcga tcaaattgag 1920

gcgcttgcta agaccgccag tgtctctctt gtctttgtca acgccgactc tggtgagggt 1980

tacatcaatg tggacggaaa cctgggtgac cgcaggaacc tgaccctgtg gaggaacggc 2040

gataatgtga tcaaggctgc tgctagcaac tgcaacaaca caatcgttgt cattcactct 2100

gtcggaccag tcttggttaa cgagtggtac gacaacccca atgttaccgc tatcctctgg 2160

ggtggtttgc ccggtcagga gtctggcaac tctcttgccg acgtcctcta tggccgtgtc 2220

aaccccggtg ccaagtcgcc ctttacctgg ggcaagactc gtgaggccta ccaagactac 2280

ttggtcaccg agcccaacaa cggcaacgga gccgctcagg aagactttgt cgagggcgtc 2340

ttcattgact accgtggatt tgacaagcgc aacgagaccc cgatctacga gttcggctat 2400

ggtctgagct acaccacttt caactactcg aaccttgagg tgcaggtgct gagcgcccct 2460

gcatacgagc ctgcttcggg tgagaccgag gcagcgccaa ccttcggaga ggttggaaat 2520

gcgtcggatt acctctaccc cagcggattg ctgagaatta ccaagttcat ctacccctgg 2580

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aacaccggca aggttgctgg tgatgaagtt ccccaactgt atgtttcccc tggcggtccc 2820

aatgagccca agatcgtgct gcgtcaattc gagcgcatca cgctgcagcc gtcggaggag 2880

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gactgggaga ttacgtcgta tcccaagatg gtgtttgtcg gaagctcctc gcggaagctg 3000

ccgctccggg cgtctctgcc tactgttcac taacccgggc gagctcgaat tgatcgttca 3060

aacatttggc aataaagttt cttaagattg aatcctgttg ccggtcttgc gatgattatc 3120

atataatttc tgttgaatta cgttaagcat gtaataatta aacatgtaat gcatgacgtt 3180

atttatgaga tggggttttt atgattaaga gtccccgcaa ttatacattt taatacgcga 3240

tagaaaaaca aaatatagcg cccaaactaa ggataaaatt attcgcgccg cgggggggca 3300

ttctatggtt actagatctc tagaattcc 3329

<210> 16

<211> 880

<212> PRT

<213> Artificial sequence

<220>

<223> Recombinant sequence expressing BGL1 fused to Cel1

<400> 16

Met Ala Arg Lys Ser Leu Ile Phe Pro Val Ile Leu Leu Ala Val Leu

1 5 10 15

Leu Phe Ser Pro Pro Ile Tyr Ser Ala Gly His Asp Tyr Arg Asp Ala

20 25 30

Leu Arg Lys Ser Ser Met Ala Asp Glu Leu Ala Tyr Ser Pro Pro Tyr

35 40 45

Tyr Pro Ser Pro Trp Ala Asn Gly Gln Gly Asp Trp Ala Gln Ala Tyr

50 55 60

Gln Arg Ala Val Asp Ile Val Ser Gln Met Thr Leu Asp Glu Lys Val

65 70 75 80

Asn Leu Thr Thr Gly Thr Gly Trp Glu Leu Glu Leu Cys Val Gly Gln

85 90 95

Thr Gly Gly Val Pro Arg Leu Gly Val Pro Gly Met Cys Leu Gln Asp

100 105 110

Ser Pro Leu Gly Val Arg Asp Ser Asp Tyr Asn Ser Ala Phe Pro Ala

115 120 125

Gly Met Asn Val Ala Ala Thr Trp Asp Lys Asn Leu Ala Tyr Leu Arg

130 135 140

Gly Lys Ala Met Gly Gln Glu Phe Ser Asp Lys Gly Ala Asp Ile Gln

145 150 155 160

Leu Gly Pro Ala Ala Gly Pro Leu Gly Arg Ser Pro Asp Gly Gly Arg

165 170 175

Asn Trp Glu Gly Phe Ser Pro Asp Pro Ala Leu Ser Gly Val Leu Phe

180 185 190

Ala Glu Thr Ile Lys Gly Ile Gln Asp Ala Gly Val Val Ala Thr Ala

195 200 205

Lys His Tyr Ile Ala Tyr Glu Gln Glu His Phe Arg Gln Ala Pro Glu

210 215 220

Ala Gln Gly Phe Gly Phe Asn Ile Ser Glu Ser Gly Ser Ala Asn Leu

225 230 235 240

Asp Asp Lys Thr Met His Glu Leu Tyr Leu Trp Pro Phe Ala Asp Ala

245 250 255

Ile Arg Ala Gly Ala Gly Ala Val Met Cys Ser Tyr Asn Gln Ile Asn
260 265 270
Asn Ser Tyr Gly Cys Gln Asn Ser Tyr Thr Leu Asn Lys Leu Leu Lys
275 280 285
Ala Glu Leu Gly Phe Gln Gly Phe Val Met Ser Asp Trp Ala Ala His
290 295 300
His Ala Gly Val Ser Gly Ala Leu Ala Gly Leu Asp Met Ser Met Pro
305 310 315 320
Gly Asp Val Asp Tyr Asp Ser Gly Thr Ser Tyr Trp Gly Thr Asn Leu
325 330 335
Thr Ile Ser Val Leu Asn Gly Thr Val Pro Gln Trp Arg Val Asp Asp
340 345 350
Met Ala Val Arg Ile Met Ala Ala Tyr Tyr Lys Val Gly Arg Asp Arg
355 360 365
Leu Trp Thr Pro Pro Asn Phe Ser Ser Trp Thr Arg Asp Glu Tyr Gly
370 375 380
Tyr Lys Tyr Tyr Tyr Val Ser Glu Gly Pro Tyr Glu Lys Val Asn Gln
385 390 395 400
Tyr Val Asn Val Gln Arg Asn His Ser Glu Leu Ile Arg Arg Ile Gly
405 410 415
Ala Asp Ser Thr Val Leu Leu Lys Asn Asp Gly Ala Leu Pro Leu Thr
420 425 430
Gly Lys Glu Arg Leu Val Ala Leu Ile Gly Glu Asp Ala Gly Ser Asn
435 440 445
Pro Tyr Gly Ala Asn Gly Cys Ser Asp Arg Gly Cys Asp Asn Gly Thr
450 455 460
Leu Ala Met Gly Trp Gly Ser Gly Thr Ala Asn Phe Pro Tyr Leu Val
465 470 475 480
Thr Pro Glu Gln Ala Ile Ser Asn Glu Val Leu Lys His Lys Asn Gly

485 490 495

Val Phe Thr Ala Thr Asp Asn Trp Ala Ile Asp Gln Ile Glu Ala Leu

500 505 510

Ala Lys Thr Ala Ser Val Ser Leu Val Phe Val Asn Ala Asp Ser Gly

515 520 525

Glu Gly Tyr Ile Asn Val Asp Gly Asn Leu Gly Asp Arg Arg Asn Leu

530 535 540

Thr Leu Trp Arg Asn Gly Asp Asn Val Ile Lys Ala Ala Ala Ser Asn

545 550 555 560

Cys Asn Asn Thr Ile Val Val Ile His Ser Val Gly Pro Val Leu Val

565 570 575

Asn Glu Trp Tyr Asp Asn Pro Asn Val Thr Ala Ile Leu Trp Gly Gly

580 585 590

Leu Pro Gly Gln Glu Ser Gly Asn Ser Leu Ala Asp Val Leu Tyr Gly

595 600 605

Arg Val Asn Pro Gly Ala Lys Ser Pro Phe Thr Trp Gly Lys Thr Arg

610 615 620

Glu Ala Tyr Gln Asp Tyr Leu Val Thr Glu Pro Asn Asn Gly Asn Gly

625 630 635 640

Ala Pro Gln Glu Asp Phe Val Glu Gly Val Phe Ile Asp Tyr Arg Gly

645 650 655

Phe Asp Lys Arg Asn Glu Thr Pro Ile Tyr Glu Phe Gly Tyr Gly Leu

660 665 670

Ser Tyr Thr Thr Phe Asn Tyr Ser Asn Leu Glu Val Gln Val Leu Ser

675 680 685

Ala Pro Ala Tyr Glu Pro Ala Ser Gly Glu Thr Glu Ala Ala Pro Thr

690 695 700

Phe Gly Glu Val Gly Asn Ala Ser Asp Tyr Leu Tyr Pro Ser Gly Leu

705 710 715 720

Leu Arg Ile Thr Lys Phe Ile Tyr Pro Trp Leu Asn Gly Thr Asp Leu
725 730 735
Glu Ala Ser Ser Gly Asp Ala Ser Tyr Gly Gln Asp Ser Ser Asp Tyr
740 745 750
Leu Pro Glu Gly Ala Thr Asp Gly Ser Ala Gln Pro Ile Leu Pro Ala
755 760 765
Gly Gly Gly Pro Gly Gly Asn Pro Arg Leu Tyr Asp Glu Leu Ile Arg
770 775 780
Val Ser Val Thr Ile Lys Asn Thr Gly Lys Val Ala Gly Asp Glu Val
785 790 795 800
Pro Gln Leu Tyr Val Ser Leu Gly Gly Pro Asn Glu Pro Lys Ile Val
805 810 815
Leu Arg Gln Phe Glu Arg Ile Thr Leu Gln Pro Ser Glu Glu Thr Lys
820 825 830
Trp Ser Thr Thr Leu Thr Arg Arg Asp Leu Ala Asn Trp Asn Val Glu
835 840 845
Lys Gln Asp Trp Glu Ile Thr Ser Tyr Pro Lys Met Val Phe Val Gly
850 855 860
Ser Ser Ser Arg Lys Leu Pro Leu Arg Ala Ser Leu Pro Thr Val His
865 870 875 880

<210> 17

<211> 4

<212> PRT

<213> Artificial sequence

<220>

<223> ER retainig signal peptide

<400> 17

His Asp Glu Leu

1

<210> 18

<211> 3288

<212> DNA

<213> Artificial sequence

<220>

<223> Recombinant sequence expressing BGL1 fused to Cel1 and ER retaini
ng peptid

<400> 18

gaattcccga tcctatctgt cacttcatca aaaggacagt agaaaaggaa ggtggcacta 60

caaatgccat cattgcgata aaggaaaggc tatcgttcaa gatgcctctg ccgacagtgg 120

tcccaaagat ggacccccac ccacgaggag catcgtggaa aaagaagacg ttccaaccac 180

gtcttcaaag caagtggatt gatgtgatat ctccactgac gtaagggatg acgcacaatc 240

ccactatcct tcgcaagacc cttcctctat ataaggaagt tcatttcatt tggagaggac 300

aggcttcttg agatccttca acaattacca acaacaacaa acaacaaaca acattacaat 360

tactatttac aattacagtc gaggggatct atggcgcgaa aatccctaat tttcccggtg 420

attttgctcg ccgttcttct cttctctccg ccgatttact ccgccggtca cgattaccgc 480

gacgctctcc gtaaatctag catggctgat gaattggcct actccccacc gtattaccca 540

tcccccttggg ccaatggcca gggcgactgg gcgcaggcat accagcgcgc tgttgatatt 600

gtctcgcaaa tgacattgga tgagaaggtc aatctgacca caggaactgg atgggaattg 660

gaactatgtg ttggtcagac tggcggtgtt ccccgattgg gagttccggg aatgtgttta 720

caggatagcc ctctgggcgt tcgcgactcc gactacaact ctgctttccc tgccggcatg 780

aacgtggctg cgacctggga caagaatctg gcataccttc gcggcaaggc tatgggtcag 840

62bb

gaatttagtg acaagggtgc cgatatccaa ttgggtccag ctgccggccc tctcggtaga 900

agtcccgacg gtggtcgtaa ctgggagggc ttctccccag accctgccct aagtggtgtg 960

ctctttgccg agaccatcaa gggtatccaa gatgctggtg tggttgcgac ggctaagcac 1020

tacattgctt acgagcaaga gcatttccgt caggcgcctg aagcccaagg ttttggattt 1080

aatatttccg agagtggaag tgcgaacctc gatgataaga ctatgcacga gctgtacctc 1140

tggcccttcg cggatgccat ccgtgcaggt gctggcgctg tgatgtgctc ctacaaccag 1200

atcaacaaca gttatggctg ccagaacagc tacactctga acaagctgct caaggccgag 1260

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aaccccggtg ccaagtcgcc ctttacctgg ggcaagactc gtgaggccta ccaagactac 2280

ttggtcaccg agcccaacaa cggcaacgga gcccctcagg aagactttgt cgagggcgtc 2340

ttcattgact accgtggatt tgacaagcgc aacgagaccc cgatctacga gttcggctat 2400

ggtctgagct acaccacttt caactactcg aaccttgagg tgcaggtgct gagcgccgct 2460

gcatacgagc ctgcttcggg tgagaccgag gcagcgccaa ccttcggaga ggttggaaat 2520

gcgtcggatt acctctaccc cagcggattg ctgagaatta ccaagttcat ctacccctgg 2580

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gactgggaga ttacgtcgta tcccaagatg gtgtttgtcg gaagctcctc gcggaagctg 3000

ccgctccggg cgtctctgcc tactgttcat gatgaacttt aacccgggcg agctcgaatt 3060

gatcgttcaa acatttggca ataaagtttc ttaagattga gttaagcatg taataattaa 3120

acatgtaatg catgacgtta tttatgagat ggggttttta tgattaagag tccccgcaat 3180

tatacatttt aatacgcgat agaaaaacaa aatatagcgc ccaaactaag gataaaatta 3240

ttcgcgccgc gggggggcat tctatggtta ctagatctct agaattcc 3288

<210> 19

<211> 883

<212> PRT

<213> Artificial sequence

<220>

<223> Recombinant sequence expressing BGL1 fused to Cell and ER retaining peptid

<400> 19

Met Ala Arg Lys Ser Leu Ile Phe Pro Val Ile Leu Leu Ala Val Leu
1 5 10 15

Leu Phe Ser Pro Pro Ile Tyr Ser Ala Gly His Asp Tyr Arg Asp Ala
20 25 30

Leu Arg Lys Ser Ser Met Ala Asp Glu Leu Ala Tyr Ser Pro Pro Tyr
35 40 45

Tyr Pro Ser Pro Trp Ala Asn Gly Gln Gly Asp Trp Ala Gln Ala Tyr
50 55 60
Gln Arg Ala Val Asp Ile Val Ser Gln Met Thr Leu Asp Glu Lys Val
65 70 75 80
Asn Leu Thr Thr Gly Thr Gly Trp Glu Leu Glu Leu Cys Val Gly Gln
85 90 95
Thr Gly Gly Val Pro Arg Leu Gly Val Pro Gly Met Cys Leu Gln Asp
100 105 110
Ser Pro Leu Gly Val Arg Asp Ser Asp Tyr Asn Ser Ala Phe Pro Ala
115 120 125
Gly Met Asn Val Ala Ala Thr Trp Asp Lys Asn Leu Ala Tyr Leu Arg
130 135 140
Gly Lys Ala Met Gly Gln Glu Phe Ser Asp Lys Gly Ala Asp Ile Gln
145 150 155 160
Leu Gly Pro Ala Ala Gly Pro Leu Gly Arg Ser Pro Asp Gly Gly Arg
165 170 175
Asn Trp Glu Gly Phe Ser Pro Asp Pro Ala Leu Ser Gly Val Leu Phe
180 185 190
Ala Glu Thr Ile Lys Gly Ile Gln Asp Ala Gly Val Val Ala Thr Ala
195 200 205
Lys His Tyr Ile Ala Tyr Glu Gln Glu His Phe Arg Gln Ala Pro Glu
210 215 220
Ala Gln Gly Phe Gly Phe Asn Ile Ser Glu Ser Gly Ser Ala Asn Leu
225 230 235 240
Asp Asp Lys Thr Met His Glu Leu Tyr Leu Trp Pro Phe Ala Asp Ala
245 250 255
Ile Arg Ala Gly Ala Gly Ala Val Met Cys Ser Tyr Asn Gln Ile Asn
260 265 270
Asn Ser Tyr Gly Cys Gln Asn Ser Tyr Thr Leu Asn Lys Leu Leu Lys

275 280 285

Ala Glu Leu Gly Phe Gln Gly Phe Val Met Ser Asp Trp Ala Ala His

290 295 300

His Ala Gly Val Ser Gly Ala Leu Ala Gly Leu Asp Met Ser Met Pro

305 310 315 320

Gly Asp Val Asp Tyr Asp Ser Gly Thr Ser Tyr Trp Gly Thr Asn Leu

325 330 335

Thr Ile Ser Val Leu Asn Gly Thr Val Pro Gln Trp Arg Val Asp Asp

340 345 350

Met Ala Val Arg Ile Met Ala Ala Tyr Tyr Lys Val Gly Arg Asp Arg

355 360 365

Leu Trp Thr Pro Pro Asn Phe Ser Ser Trp Thr Arg Asp Glu Tyr Gly

370 375 380

Tyr Lys Tyr Tyr Tyr Val Ser Glu Gly Pro Tyr Glu Lys Val Asn Gln

385 390 395 400

Tyr Val Asn Val Gln Arg Asn His Ser Glu Leu Ile Arg Arg Ile Gly

405 410 415

Ala Asp Ser Thr Val Leu Leu Lys Asn Asp Gly Ala Leu Pro Leu Thr

420 425 430

Gly Lys Glu Arg Leu Val Ala Leu Ile Gly Glu Asp Ala Gly Ser Asn

435 440 445

Pro Tyr Gly Ala Asn Gly Cys Ser Asp Arg Gly Cys Asp Asn Gly Thr

450 455 460

Leu Ala Met Gly Trp Gly Ser Gly Thr Ala Asn Phe Pro Tyr Leu Val

465 470 475 480

Thr Pro Glu Gln Ala Ile Ser Asn Glu Val Leu Lys His Lys Asn Gly

485 490 495

Val Phe Thr Ala Thr Asp Asn Trp Ala Ile Asp Gln Ile Glu Ala Leu

500 505 510

Ala Lys Thr Ala Ser Val Ser Leu Val Phe Val Asn Ala Asp Ser Gly
515 520 525
Glu Gly Tyr Ile Asn Val Asp Gly Asn Leu Gly Asp Arg Arg Asn Leu
530 535 540
Thr Leu Trp Arg Asn Gly Asp Asn Val Ile Lys Ala Ala Ala Ser Asn
545 550 555 560
Cys Asn Asn Thr Ile Val Val Ile His Ser Val Gly Pro Val Leu Val
565 570 575
Asn Glu Trp Tyr Asp Asn Pro Asn Val Thr Ala Ile Leu Trp Gly Gly
580 585 590
Leu Pro Gly Gln Glu Ser Gly Asn Ser Leu Ala Asp Val Leu Tyr Gly
595 600 605
Arg Val Asn Pro Gly Ala Lys Ser Pro Phe Thr Trp Gly Lys Thr Arg
610 615 620
Glu Ala Tyr Gln Asp Tyr Leu Val Thr Glu Pro Asn Asn Gly Asn Gly
625 630 635 640
Ala Pro Gln Glu Asp Phe Val Glu Gly Val Phe Ile Asp Tyr Arg Gly
645 650 655
Phe Asp Lys Arg Asn Glu Thr Pro Ile Tyr Glu Phe Gly Tyr Gly Leu
660 665 670
Ser Tyr Thr Thr Phe Asn Tyr Ser Asn Leu Glu Val Gln Val Leu Ser
675 680 685
Ala Pro Ala Tyr Glu Pro Ala Ser Gly Glu Thr Glu Ala Ala Pro Thr
690 695 700
Phe Gly Glu Val Gly Asn Ala Ser Asp Tyr Leu Tyr Pro Ser Gly Leu
705 710 715 720
Leu Arg Ile Thr Lys Phe Ile Tyr Pro Trp Leu Asn Gly Thr Asp Leu
725 730 735
Glu Ala Ser Ser Gly Asp Ala Ser Tyr Gly Gln Asp Ser Ser Asp Tyr

740 745 750

Leu Pro Glu Gly Ala Thr Asp Gly Ser Ala Gln Pro Ile Leu Pro Ala

755 760 765

Gly Gly Gly Pro Gly Gly Asn Pro Arg Leu Tyr Asp Glu Leu Ile Arg

770 775 780

Val Ser Val Thr Ile Lys Asn Thr Gly Lys Val Ala Gly Asp Glu Val

785 790 795 800

Pro Gln Leu Tyr Val Ser Leu Gly Gly Pro Asn Glu Pro Lys Ile Val

805 810 815

Leu Arg Gln Phe Glu Arg Ile Thr Leu Gln Pro Ser Glu Glu Thr Lys

820 825 830

Trp Ser Thr Thr Leu Thr Arg Arg Asp Leu Ala Asn Trp Asn Val Glu

835 840 845

Lys Gln Asp Trp Glu Ile Thr Ser Tyr Pro Lys Met Val Phe Val Gly

850 855 860

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865 870 875 880

Asp Glu Leu

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<220>

<223> ER retaining signal peptide

<400> 24

Lys Asp Glu Leu

1

CLAIMS:

1. A method of increasing a level of at least one fermentation substance in a fermentation product, the method comprising introducing a nucleic acid construct including the polynucleotide sequence as set forth in SEQ ID NO: 1 or 3 into a yeast cell, said polynucleotide sequence encoding a polypeptide having a ß-glucosidase catalytic activity, and fermenting a glucose containing fermentation starting material by said yeast cell overexpressing said polypeptide such that said polypeptide releases at least one fermentation substance from said glucose containing fermentation starting material, thereby increasing the level of said at least one fermentation substance in the fermentation product, relative to the level of said at least one fermentation substance produced by a yeast cell not overexpressing said polypeptide.

2. The method of claim 1, wherein said polypeptide is as set forth in SEQ ID NO: 2 having said β-glucosidase catalytic activity.

3. A method of increasing a level of at least one aroma substance in a plant derived product, the method comprising introducing a nucleic acid construct including the polynucleotide sequence as set forth in SEQ ID NO: 1 or 3 into a yeast cell, said polynucleotide sequence encoding a polypeptide having a ß-glucosidase catalytic activity, and incubating a glucose containing plant starting material with said yeast cell overexpressing said polypeptide such that said polypeptide releases at least one aroma substance from said glucose containing plant starting material, thereby increasing the level of said at least one aroma substance in the plant derived product, relative to the level of said at least one aroma substance produced by a yeast cell not overexpressing said polypeptide.

4. The method of claim 3, wherein said plant derived product is a fermentation product.

5. The method of claim 3, wherein said polypeptide is as set forth in SEQ ID NO: 2 having said β-glucosidase catalytic activity.

6. A method of increasing a level of free glucose in a fermentation product, the method comprising introducing a nucleic acid construct including the polynucleotide sequence as set forth in SEQ ID NO: 1 or 3 into a cell, said polynucleotide sequence encoding a polypeptide having a ß-glucosidase catalytic activity, and fermenting a glucose containing fermentation starting material with the cell overexpressing said polypeptide such that said polypeptide releases free glucose from said glucose containing fermentation starting material, thereby increasing said level of free glucose in the fermentation product, relative to the level of free glucose produced by a yeast cell not overexpressing said polypeptide.

7. The method of claim 6, wherein said cell is selected from the group consisting of a prokaryotic cell and a eukaryotic cell.

8. The method of claim 7, wherein said prokaryotic cell is *E. coli*.

9. The method of claim 7, wherein said eukaryotic cell is selected from the group consisting of a yeast cell, a fungus cell, a plant cell and an animal cell.

10. The method of claim 6, wherein said polypeptide is as set forth in SEQ ID NO: 2 having said β-glucosidase catalytic activity.

11. A method of increasing a level of free glucose in a plant, the method comprising introducing a nucleic acid construct including the polynucleotide sequence as set forth in SEQ ID NO: 1 or 3 into a plant cell, said polynucleotide sequence encoding a polypeptide having a ß-glucosidase catalytic activity, and overexpressing said polypeptide in said plant cell, thereby increasing the level of the free glucose in the plant, relative to the level of free glucose produced by a plant cell not overexpressing said polypeptide.

12. The method of claim 11, wherein said polypeptide is as set forth in SEQ ID NO:2 having said β-glucosidase catalytic activity.

13. A method of producing an alcohol, the method comprising introducing a nucleic acid construct including the polynucleotide sequence as set forth in SEQ ID NO: 1 or 3 into a cell, said polynucleotide sequence encoding a polypeptide having a ß-glucosidase catalytic activity, and fermenting a glucose containing fermentation starting material by the cell overexpressing said polypeptide such that said polypeptide releases the alcohol from said glucose containing fermentation starting material in said cell, thereby producing the alcohol.

14. The method of claim 13, wherein said cell is selected from the group consisting of a prokaryotic cell and a eukaryotic cell.

15. The method of claim 14, wherein said prokaryotic cell is *E. coli*.

16. The method of claim 14, wherein said eukaryotic cell is selected from the group consisting of a yeast cell, a fungus cell, a plant cell and an animal cell.

17. The method of claim 13, wherein said polypeptide is as set forth in SEQ ID NO: 2 having said β-glucosidase catalytic activity.

18. A method of producing an aroma spreading plant, the method comprising introducing into a plant a nucleic acid construct including a polynucleotide encoding a polypeptide having a ß-glucosidase catalytic activity, said ß-glucosidase catalytic activity being capable of generating volatile compounds in said plant, and overexpressing said polypeptide in said plant such that existing aroma producing compounds of said plant are converted into a volatile form, thereby producing the aroma spreading plant.

19. The method of claim 18, wherein overexpressing said nucleic acid construct is performed in a tissue specific manner.

20. The method of claim 18, wherein said polypeptide is as set forth in SEQ ID NO: 2 having said β-glucosidase catalytic activity.

21. The method of claim 18, wherein said polynucleotide is as set forth in SEQ ID NO: 1 or SEQ ID NO:3.

22. The method of claim 18, wherein overexpressing said nucleic acid construct is limited to at least one tissue selected from the group consisting of flower, fruit, seed, root, stem, pollen and leaves.

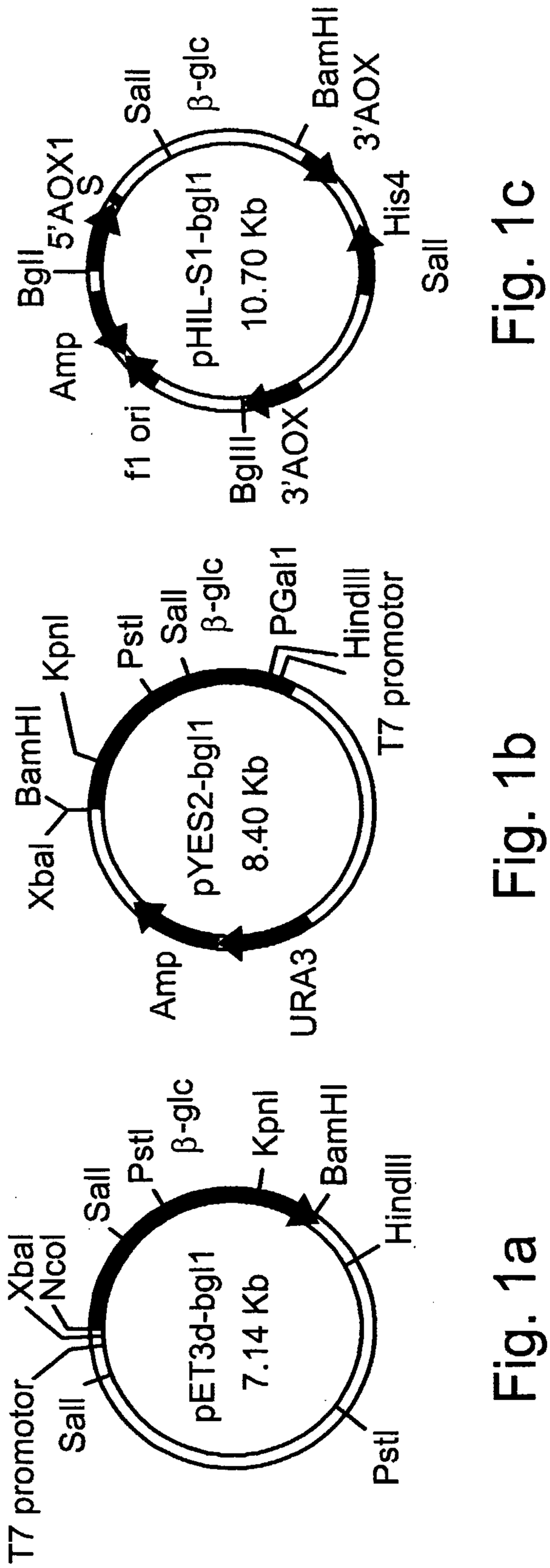
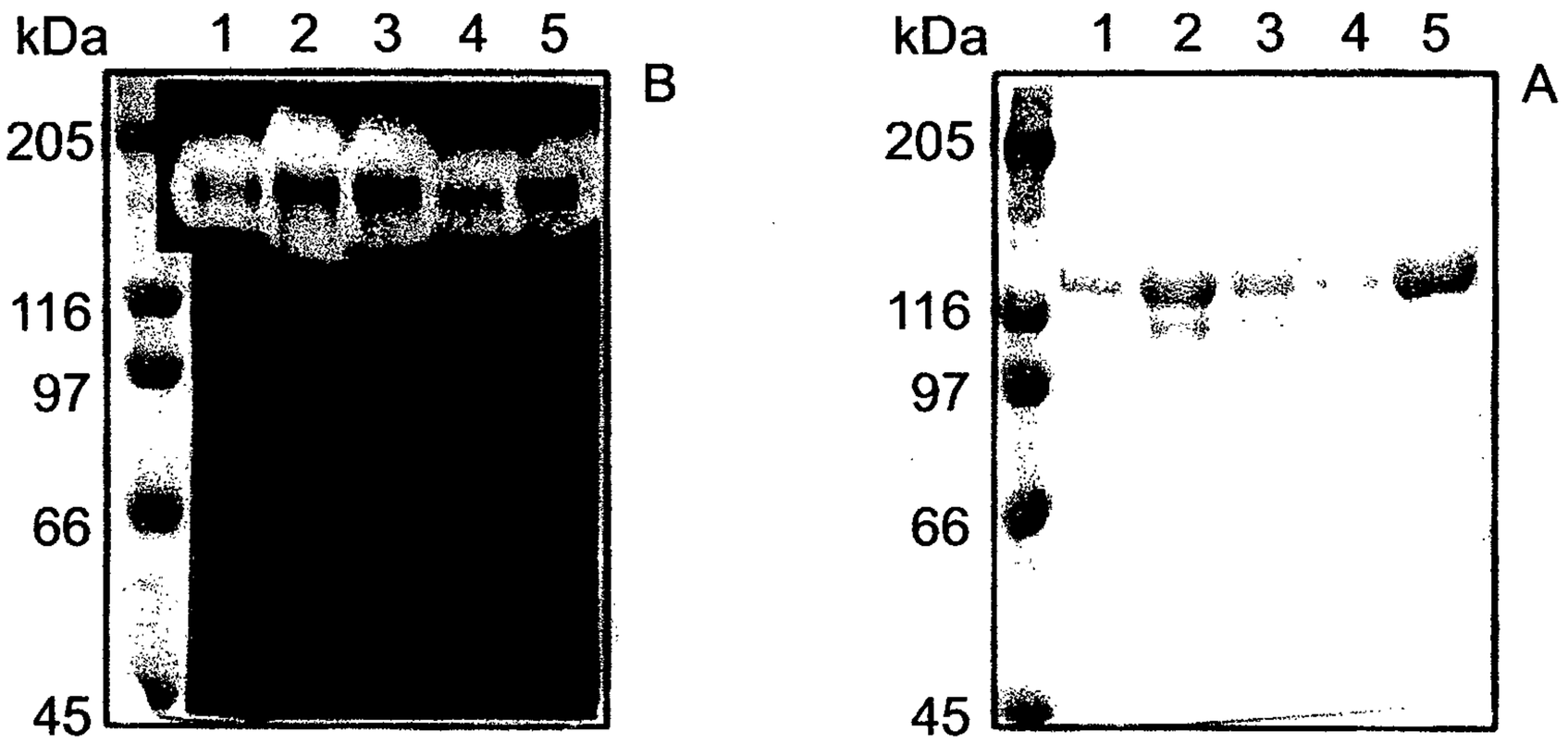


Fig. 1c

Fig. 1b

Fig. 1a


kDa
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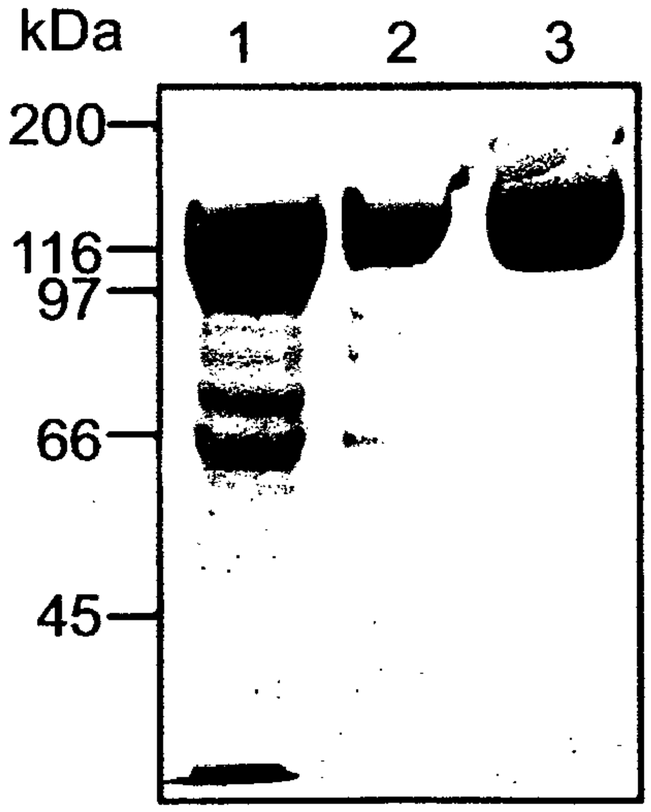
B
205
116
97
66
45
kDa
1 2 3 4 5
A
205
116
97
66
45

Fig. 2


kDa
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200
116
97
66
45

Fig. 3

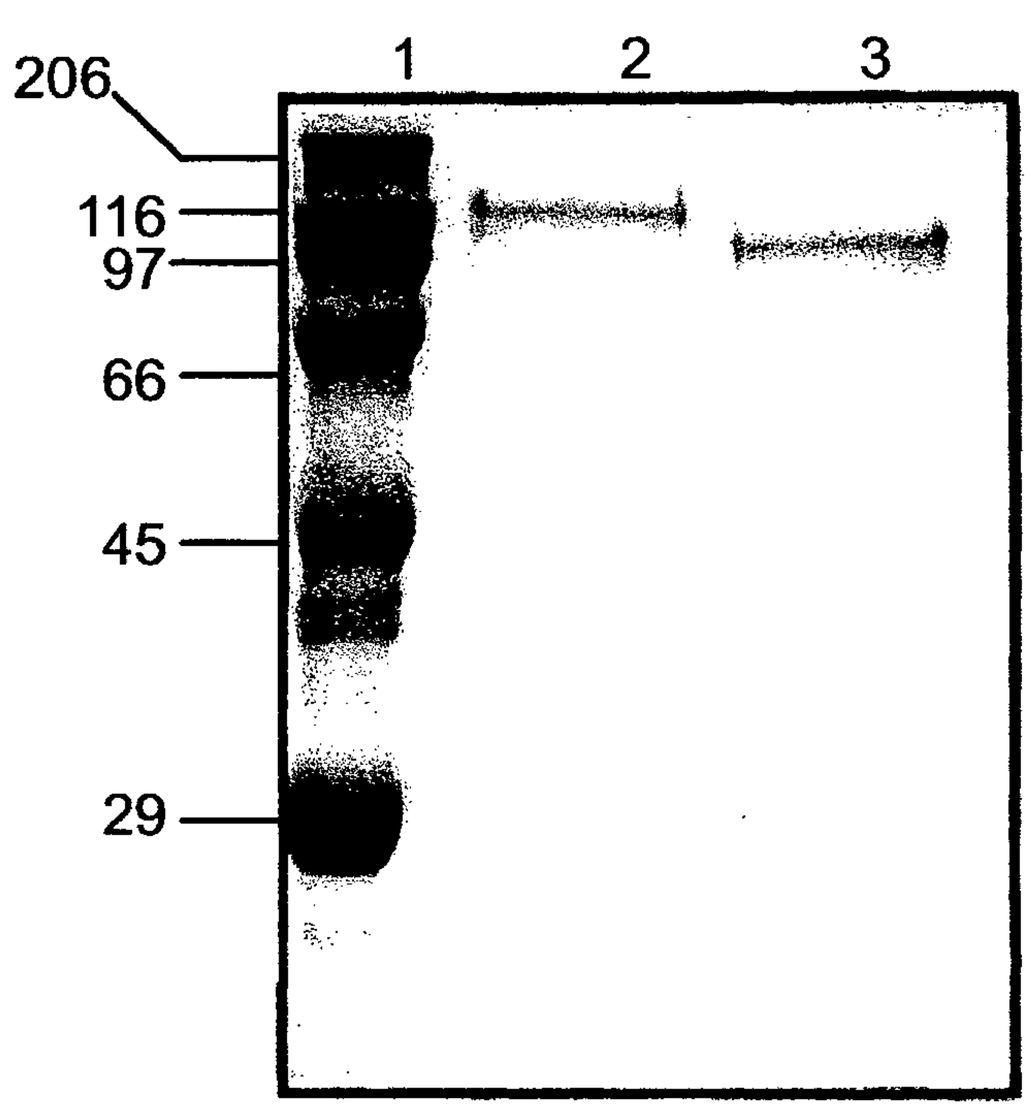

Fig. 4

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121  ATTCGCATGCCGTGGCGGACGGTCACTTTGTGGCGCTCAAACTATTTAATATGGCCCAGC  180

181  TCCCCTTTCTCTCGCTGTTTTCGTTTCTGTCCTCCCTAAACCTCCAGTCTCTCCATTGGA  240

241  CAGGTGTTGCACGGTTGCTCACCTGGTTTGTTTTGCTCCCCCTTTGGGCGACCTTGCCAT  300

301  CATGAGGTTCACTTTGATCGAGGCGGTGGCTCTGACTGCCGTCTCGCTGGCCAGCGCTGT  360
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                    Signal Peptide

361  ACGTGCCGTTACTTTGTCCTGAGAATTGCAATTGTGCTTAATTAGATTCATTTGTTTGTT  420
                    Intron#1

421  TCATCATCGCTGACAATGGTCTTTTCATAGGATGAATTGGCCTACTCCCCACCGTATTAC  480
                                   AspGluLeuAlaTyrSerProProTyrTyr

481  CCATCCCCTTGGGCCAATGGCCAGGGCGACTGGGCGCAGGCATACCAGCGCGCTGTTGAT  540
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541  ATTGTCTCGCAAATGACATTGGATGAGAAGGTCAATCTGACCACAGGAACTGGGTAGGGC  600
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601  TTACATGGCGCAATCTGTATGCTCCGGCTAACAACTTCTACATGGGAATTGGAACTATGT  660
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661  GTTGGTCAGACTGGCGGTGTTCCCCGGTAGGTTTGAAAATATTGTCGAGACAGGGGACAT  720
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721  TATTGATTAACGGTGACAGATTGGGAGTTCCGGGAATGTGTTTACAGGATAGCCCTCTGG  780
                                LeuGlyValProGlyMetCysLeuGlnAspSerProLeuG

781  GCGTTCGCGACTGTAAGCCATCTGCTGTTGTTAGGCTTCGATGCTCTTACTGACACGGCG  840
     lyValArgAspS                                Intron#4

841  CAGCCGACTACAACTCTGCTTTCCCTGCCGGCATGAACGTGGCTGCAACCTGGGACAAGA  900
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901  ATCTGGCATACCTTCGCGGCAAGGCTATGGGTCAGGAATTTAGTGACAAGGGTGCCGATA  960
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961  TCCAATTGGGTCCAGCTGCCGGCCCTCTCGGTAGAAGTCCCGACGGTGGTCGTAACTGGG 1020
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1021 AGGGCTTCTCCCCAGACCCTGCCCTAAGTGGTGTGCTCTTTGCCGAGACCATCAAGGGTA 1080
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Fig. 5a

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1501 GCGTGCTCAACGGAACGGTGCCCCAATGGCGTGTTGATGACATGGCTGTCCGCATCATGG  1560
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1681 ACCAGTACGTGAATGTGCAACGCAACCACAGCGAACTGATTCGCCGCATTGGAGCGGACA  1740
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     erThrValLeuLeuLysAsnAspGlyAlaLeuProLeuThrGlyLysGluArgLeuValA

1801 CGCTTATCGGAGAAGATGCGGGCTCCAACCCTTATGGTGCCAACGGCTGCAGTGACCGTG  1860
     laLeuIleGlyGluAspAlaGlySerAsnProTyrGlyAlaAsnGlyCysSerAspArgG

1861 GATGCGACAATGGAACATTGGCGATGGGCTGGGGAAGTGGTACTGCCAACTTCCCATACC  1920
     lyCysAspAsnGlyThrLeuAlaMetGlyTrpGlySerGlyThrAlaAsnPheProTyrL

1921 TGGTGACCCCCGAGCAGGCCATCTCAAACGAGGTGCTTAAGCACAAGAATGGTGTATTCA  1980
     euValThrProGluGlnAlaIleSerAsnGluValLeuLysHisLysAsnGlyValPheT

1981 CCGCCACCGATAACTGGGCTATCGATCAGATTGAGGCGCTTGCTAAGACCGCCAGGTAAG  2040
     hrAlaThrAspAsnTrpAlaIleAspGlnIleGluAlaLeuAlaLysThrAlaArg

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2161 ACCGCAGGAACCTGACCCTGTGGAGGAACCGCGATAATGTGATCAAGGCTGCTGCTAGCA  2220
     spArgArgAsnLeuThrLeuTrpArgAsnArgAspAsnValIleLysAlaAlaAlaSerA

2281 ACCACAACCCCAATGTTACCGCTATCCTCTGGGGTGGTTTGCCCGGTCAGGAGTCTGGCA  2340
     snHisAsnProAsnValThrAlaIleLeuTrpGlyGlyLeuProGlyGlnGluSerGlyA

2341 ACTCTCTTGCCGACGTCCTCTATGGCCGTGTCAACCCCGGTGCCAAGTCGCCCTTTACCT  2400
     snSerLeuAlaAspValLeuTyrGlyArgValAsnProGlyAlaLysSerProPheThrT

2401 GGGGCAAGACTCGTGAGGCCTACCAAGACTACTTGGTCACCGAGCCCAACAACGGCAACG  2460
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2461 GAGCCCCTCAGGAAGACTTTGTCGAGGGCGTCTTCATTGACTACCGTGGATTTGACAAGC  2520
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2581 CGAACCTTGAGGTGCAGGTGCTGAGCGCCCCTGCATACGAGCCTGCTTCGGGTGAGACCG  2640
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2701 TGCAGAGAATTACCAAGTTCATCTACCCCTGGCTCAACGGTACCGATCTCGAGGCATCTT  2760
     luGlnArgIleThrLysPheIleTyrProTrpLeuAsnGlyThrAspLeuGluAlaSerS

2761 CCGGGGATGCTAGCTACGGGCAGGACTCCTCCGACTATCTTCCCGAGGGAGCCACCGATG  2820
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```

Fig. 5a (continued)

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2821 GCTCTGCGCAACCGATCCTGCCTGCCGGTGGCGGTCCTGGCGGCAACCCTCGCCTGTACG  2880
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2881 ACGAGCTCATCCGCGTGTCAGTGACCATCAAGAACACCGGCAAGGTTGCTGGTGATGAAG  2940
     spGluLeuIleArgValSerValThrIleLysAsnThrGlyLysValAlaGlyAspGluV

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     alProGlnLeu                    Intron#6

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       TyrValSerLeuGlyGlyProAsnGluProLysIleValLeuArgGlnPheGluArgI

3061 TCACGCTGCAGCCGTCGGAGGAGACGAAGTGGAGCACGACTCTGACGCGCCGTGACCTTG  3120
     leThrLeuGlnProSerGluGluThrLysTrpSerThrThrLeuThrArgArgAspLeuA

3121 CAAACTGGAATGTTGAGAAGCAGGACTGGGAGATTACGTCGTATCCCAAGATGGTGTTTG  3180
     laAsnTrpAsnValGluLysGlnAspTrpGluIleThrSerTyrProLysMetValPheV

3181 TCGGAAGCTCCTCGCGGAAGCTGCCGCTCCGGGCGTCTCTGCCTACTGTTCACTAAATAG  3240
     alGlySerSerSerArgLysLeuProLeuArgAlaSerLeuProThrValHis***

3241 CTCTCAAATGGTATACCATGATGGCCGTGGTATATGAATTAATGATTTATGCCAACAGCA  3300

3301 AGACCACTGTAGATGTAGATGTAGAATGAGTATTGCGTAGTAGCGTGTAGATGATGATAC  3360

3361 AAGCGATCCGACACATGGTAGGAAGAGTGGCGCTAGTTGGGGCGGAAACCAAGCGACGTC  3420

3421 ATCCGCTGCCGACTTCGCCAGTCTTTCTTCTTTTCCTCTTCAGCCTTCTTCCTCCGCTTA  3480

3481 ATCCAGCAACCATTGCCAATTGCCTCTACAACAACTAATTGCCATAATACTCTACTCCTA  3540

3541 TTCAATATATACACCACAATCTCGACATAATCACACAAGCCTGAACACACGAGCAACCAT  3600

3601 GCCCTCTCCCGATCCTCCAGCCCCAGCGATACGACCCTTCCAACCACCCATAACAGCGCT  3660

3661 CCTCATCTACCCAGCGACCCTAATCGTGGGATCACTCTTCTCCGTCCTCTCTCCCACCGC  3720

3721 ACAAGGCACACGCGACGACGGCTCCAGCACCCTCCACCCACACGTCGAGCCCCTAGCCCC  3780

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Fig. 5a (continued)

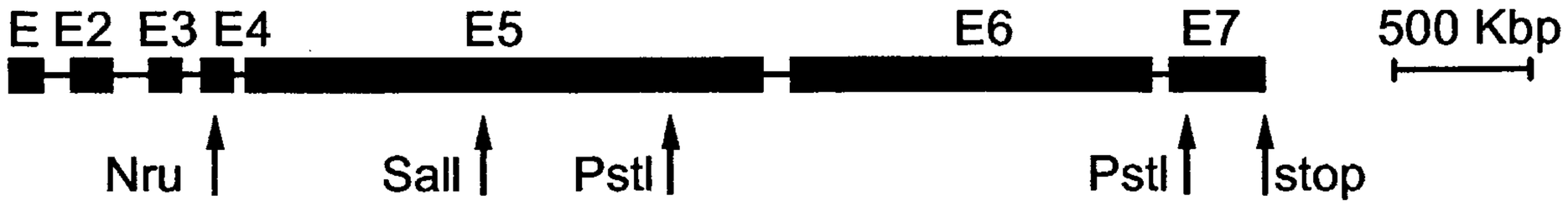
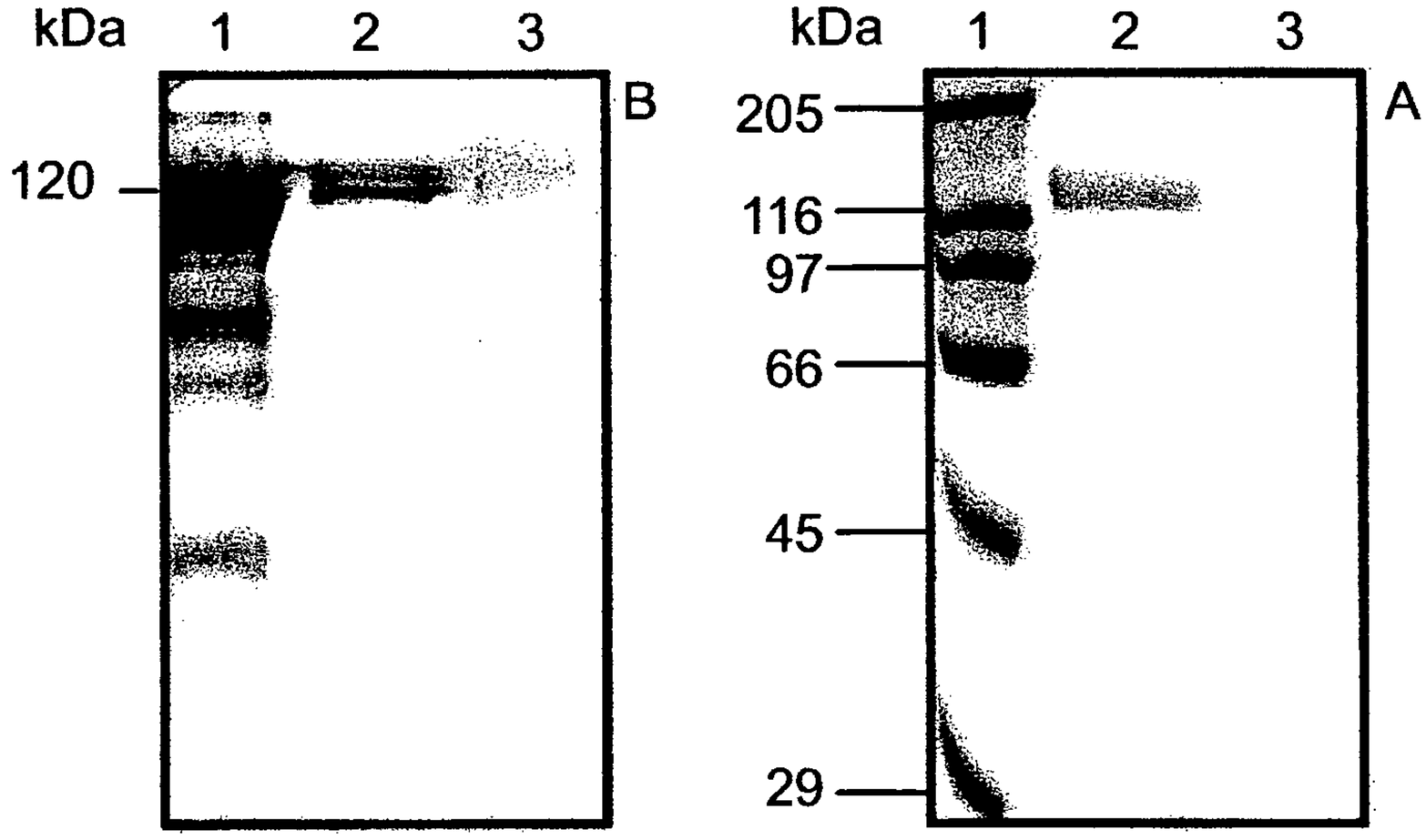


Fig. 5b



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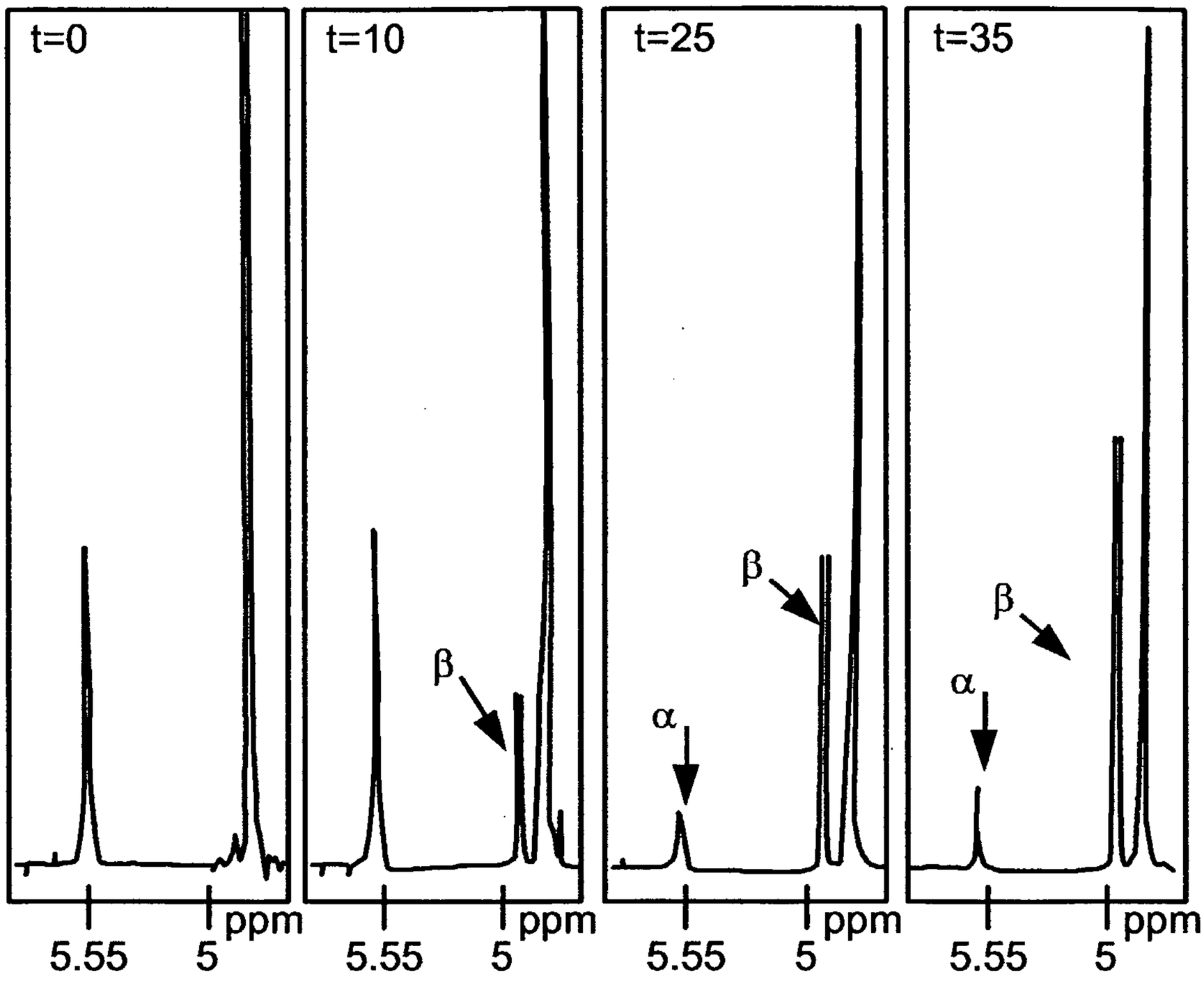

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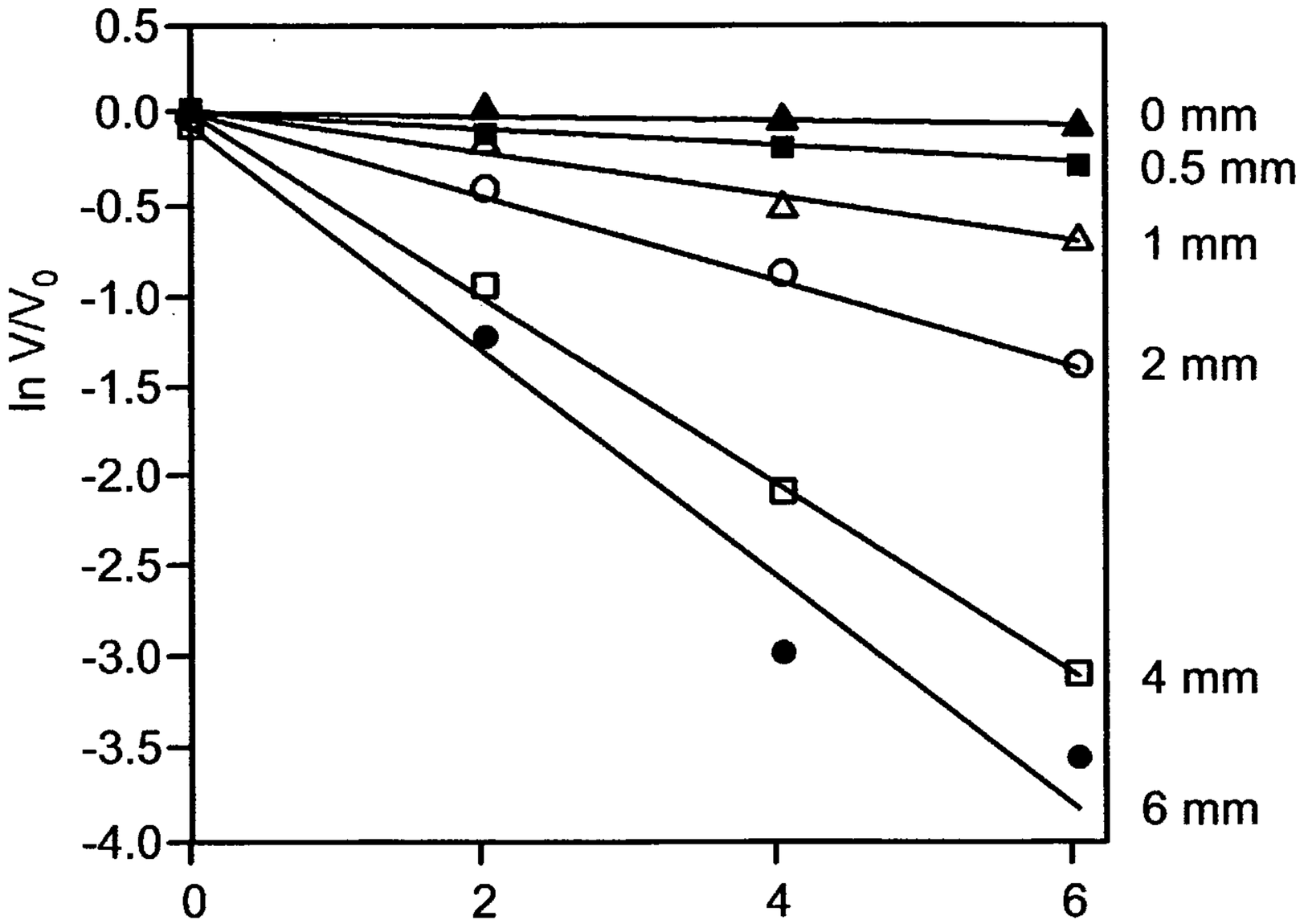
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-3.5
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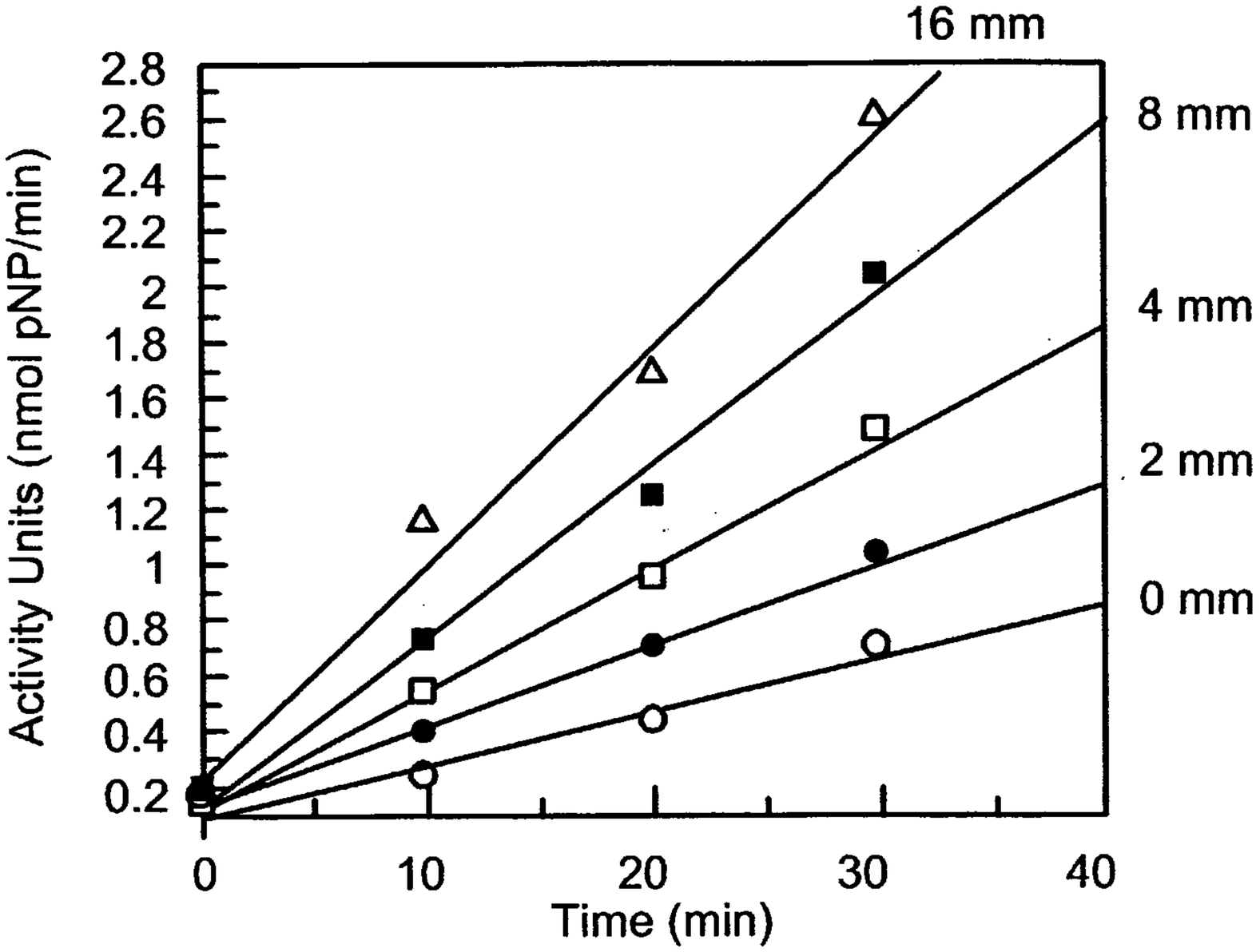
ln V/V0
0 mm
0.5 mm
1 mm
2 mm
4 mm
6 mm
0
2
4
6
Time (min)

Fig. 8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8 mm
4 mm
2 mm
0 mm
Activity Units (nmol pNP/min)
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2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10
20
30
40
Time (min)

Fig. 9

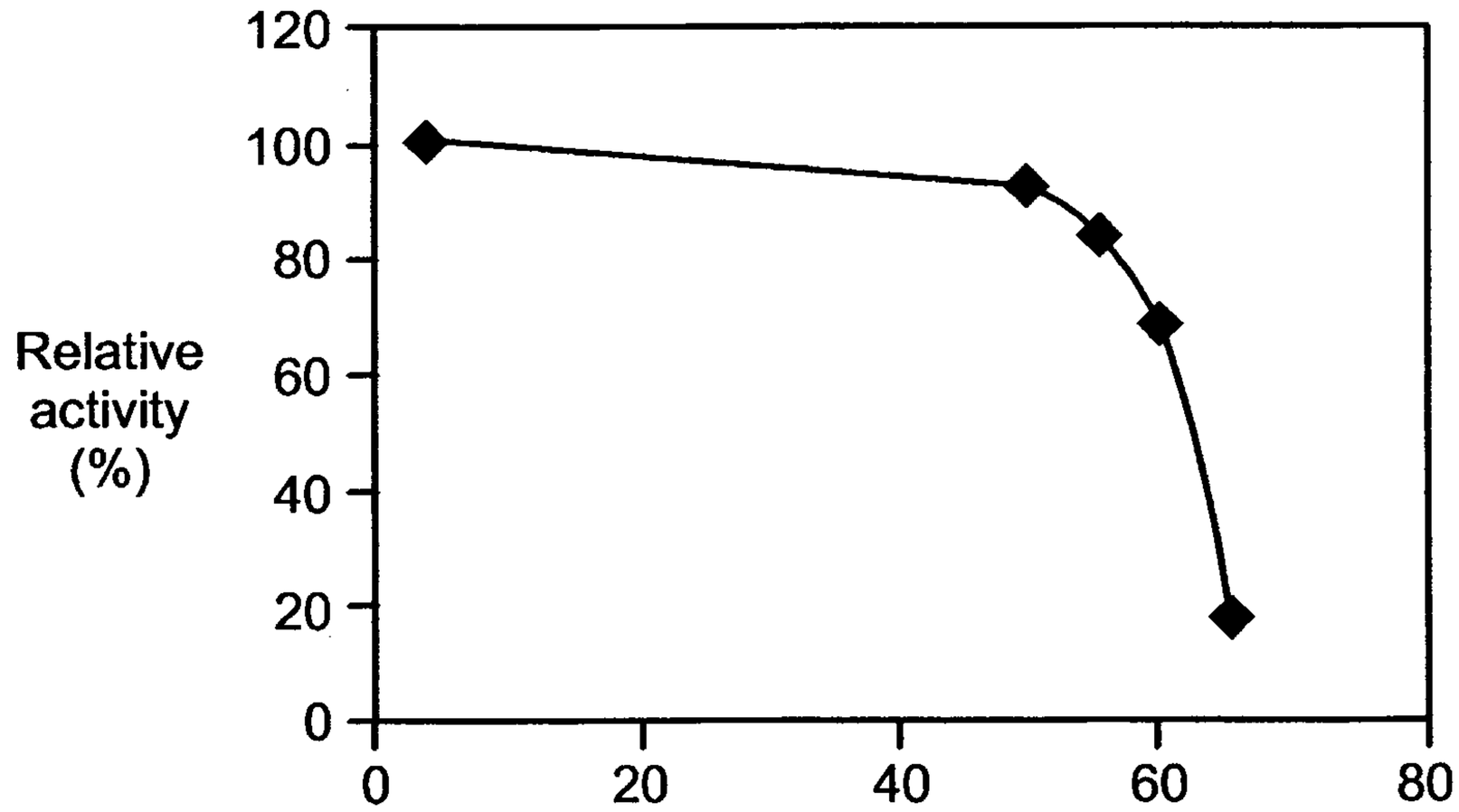

Relative activity (%)
120
100
80
60
40
20
0
0
20
40
60
80
Temp. (C)

Fig. 10

Fig. 11a 35SΩ + bg11

Fig. 11b 35SΩ + Ce11 + bg11

Fig. 11c 35SΩ + Ce11 + bg11 + HDEL

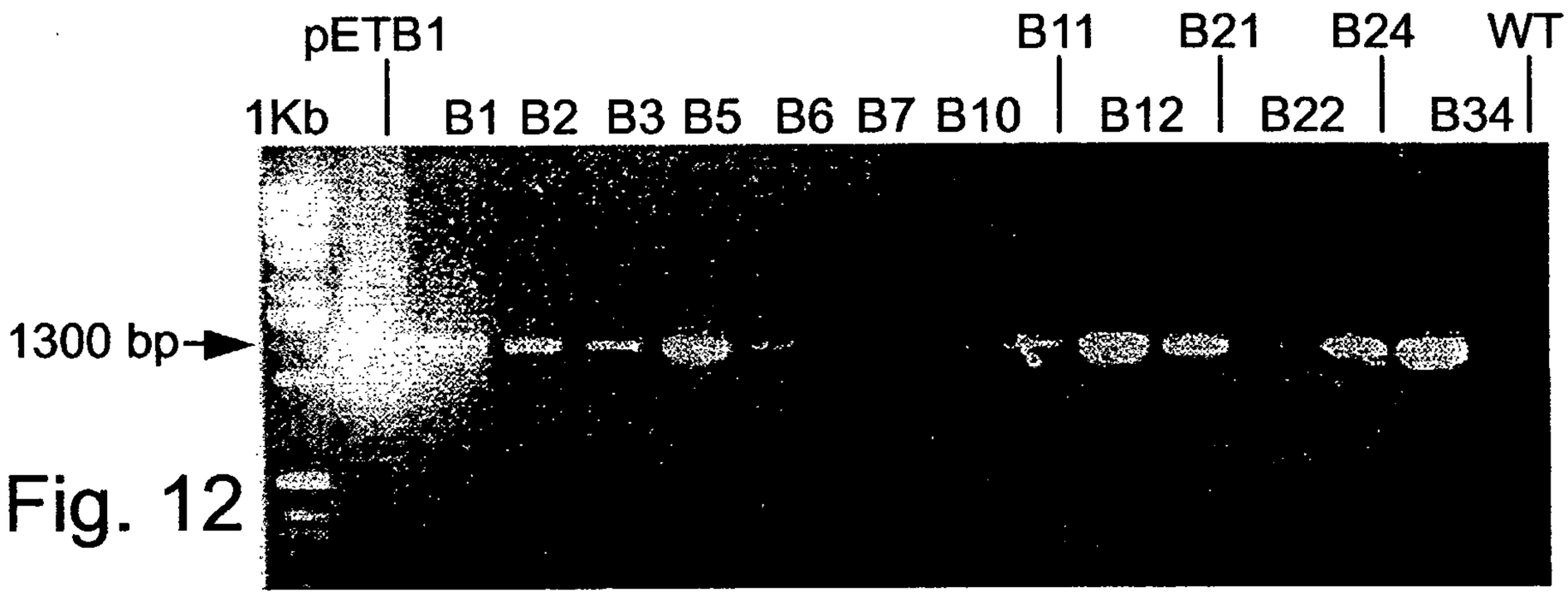


Fig. 12

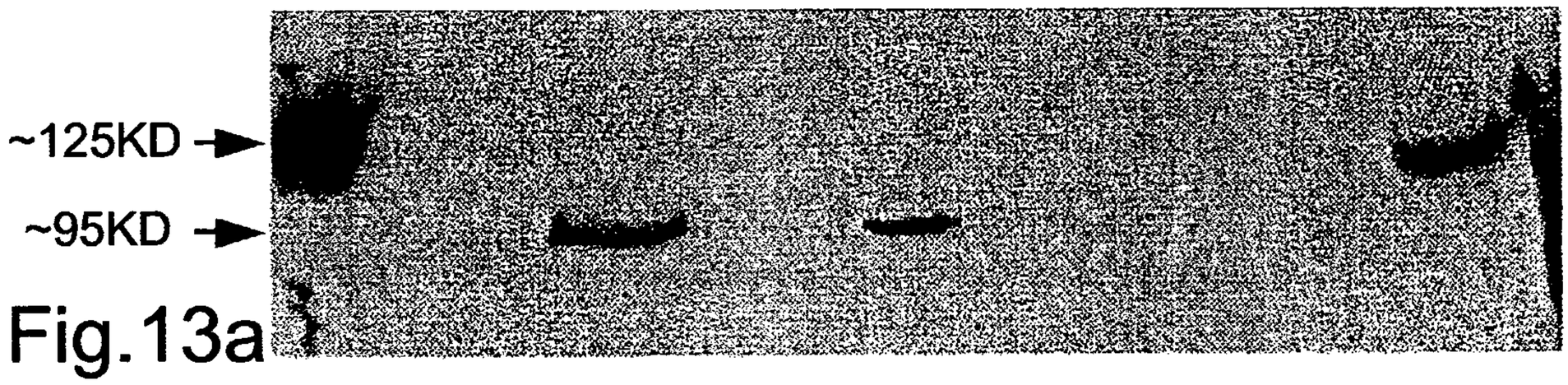


Fig.13a

Fig. 13b WT CB10 CB12 Blank CBT1 CBT3 CBT7 CBT8 AnGlu

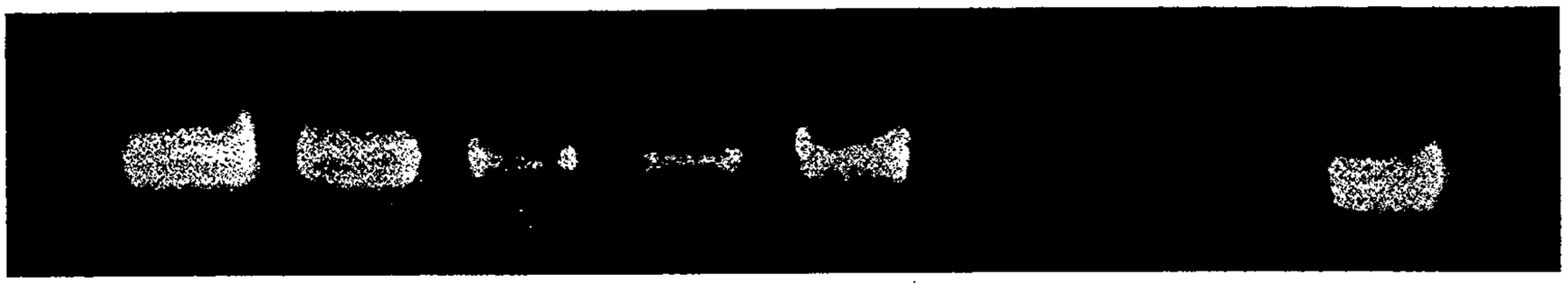
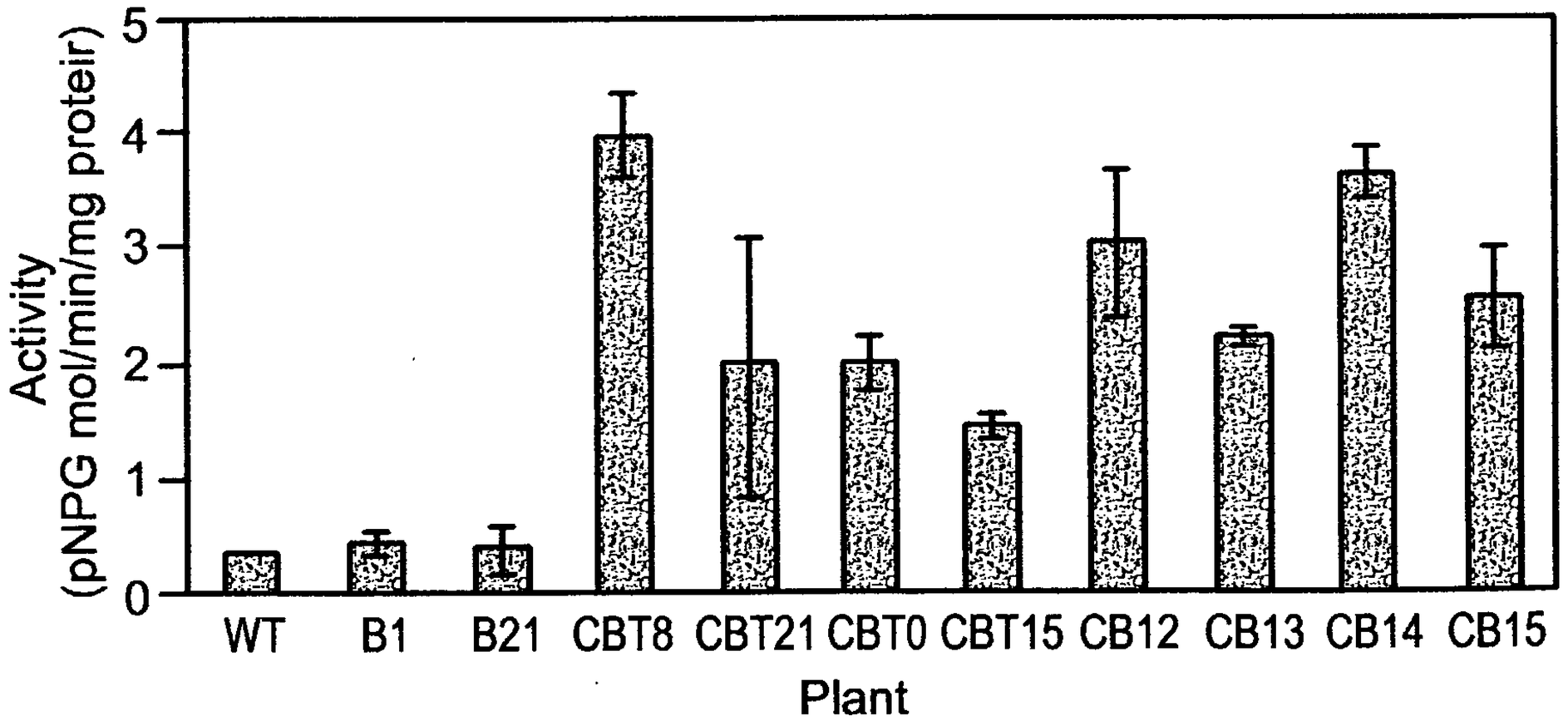

WT
CB10
CB11
CBT3
CBT8
CBT15
B1
B34
An Glu

Fig. 14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(pNPG mol/min/mg proteir)
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1
2
3
4
5
WT
B1
B21
CBT8
CBT21
CBT0
CBT15
CB12
CB13
CB14
CB15
Plant

Fig. 15