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(54) Title: PLANTS WITH ELEVATED LEVELS OF GLUCAN

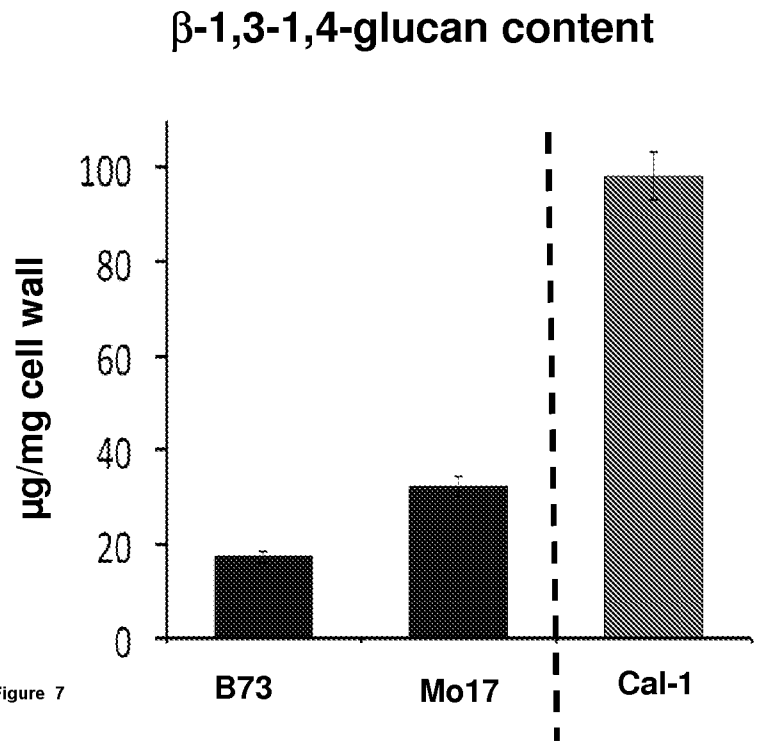

Figure 7

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(57) Abstract: The present disclosure relates to mutations in licheninase genes encoding polypeptides with decreased licheninase activity, which when expressed in plants results in elevated levels of glucan in the plants. In particular, the disclosure relates to licheninase nucleic acids and polypeptides related to glucan accumulation in plants, plants with reduced expression of a licheninase nucleic acid, and methods related to the generation of plants with increased glucan content in the cell walls of leaf tissue.

PLANTS WITH ELEVATED LEVELS OF GLUCAN

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 61/492,769, filed June 2, 2011, which is hereby incorporated by reference, in its entirety.

SUBMISSION OF SEQUENCE LISTING AS ASCII TEXT FILE

[0002] The content of the following submission on ASCII text file is incorporated herein by reference in its entirety: a computer readable form (CRF) of the Sequence Listing (file name: 416272009740SeqList.txt, date recorded: June 1, 2012, size: 445 KB).

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0003] This invention was made with government support under awarded Contract No. DE-SC0004822, awarded by the United States Department of Energy to The University of California at Berkeley. The government has certain rights in this invention.

FIELD

[0004] The present disclosure relates to mutations in licheninase genes encoding polypeptides with decreased licheninase activity, which when expressed in plants results in elevated levels of glucan in the plants. In particular, the disclosure relates to licheninase nucleic acids and polypeptides related to glucan accumulation in plants, plants with reduced expression of a licheninase nucleic acid, and methods related to the generation of plants with increased glucan content in the cell walls of leaf tissue.

BACKGROUND

[0005] Members of the grasses, such as wheat, rice and maize, represent some of the major economically relevant crops. According to the Food and Agriculture Organization, the world production of grasses in 2008 was 2.5 billion metric tons (FAOSTAT: Food and Agriculture Organization of the United Nations, Rome Italy website). In addition to their nutritional importance, grasses have recently attracted attention as potential second

generation bioenergy crops due to their potential to produce large quantities of biomass in short times with little agricultural input from growers(Heaton, EA, et al., *Global Change Biology* 14, 2000–14, 2008).

[0006] The grasses are noteworthy for their complex cell wall structure that is distinct from that of dicotyledons such as trees. One profound difference is that heteroxylans constitute the major hemicellulose in their primary cell wall. The cell walls of grasses also have less pectic polysaccharides compared to other higher plants. Another major difference in the primary cell wall is the presence of (1,3;1,4)-β-D-glucans, a polysaccharide that is absent outside the Poales in higher plants. The unique cell wall structure of grasses such as maize has potential to provide large quantities sugars that can be used as feedstocks for the production of biofuels such as ethanol. For example, the glucose containing components of cell walls of maize can be used in the production of ethanol.

[0007] There is a need to develop plants, such as grasses, with improved cell wall characteristics such as increased levels of glucose containing polymers.

BRIEF SUMMARY

[0008] In order to meet the above needs, the present disclosure provides non-naturally occurring mutant plants having elevated levels of glucan, resulting from a mutation in at least one licheninase gene, and methods of producing and using such plants.

[0009] Accordingly, certain aspects of the present disclosure relate to a non-naturally occurring mutant plant containing a mutation in at least one licheninase gene, where the mutant plant has elevated levels of glucan, compared to the levels of glucan in a corresponding plant lacking the mutation in the at least one licheninase gene.

[0010] In certain embodiments, the at least one licheninase gene encodes a polypeptide containing consensus sequence SEQ ID NO: 9. In certain embodiments, the at least one licheninase gene contains a nucleic acid sequence selected from: (a) SEQ ID NO: 3 or 7; (b) a homolog of SEQ ID NO: 3 or 7; (c) a paralog of SEQ ID NO: 3 or 7; and (d) an ortholog of SEQ ID NO: 3 or 7. In certain embodiments that may be combined with any of the preceding embodiments, the at least one mutant licheninase gene encodes a polypeptide sequence having a Glu to Ly substitution at position 262 of SEQ ID NO: 4, a Glu to Ly substitution at a

position analogous to position 262 of SEQ ID NO: 4, a Glu to Lys substitution at position 242 of SEQ ID NO: 8, or a Glu to Ly substitution at a position analogous to position 242 of SEQ ID NO: 8. In certain embodiments that may be combined with any of the preceding embodiments, the plant has at least a 20% increase in levels of glucan as compared to a corresponding plant lacking the mutation. In certain embodiments that may be combined with any of the preceding embodiments, the plant further contains a mutation in at least one additional licheninase gene. In certain embodiments, the at least one additional licheninase gene encodes a polypeptide containing consensus sequence SEQ ID NO: 9. In certain embodiments that may be combined with any of the preceding embodiments, the at least one licheninase gene or the at least one additional licheninase gene contains a partial deletion or a complete deletion of the gene. In certain embodiments that may be combined with any of the preceding embodiments, the plant further contains a mutation in at least one *bm1* gene, a homolog thereof, a paralog thereof, or an ortholog thereof, where the plant exhibits an increased saccharification yield compared to the saccharification yield in a corresponding plant lacking the mutation in the at least one licheninase gene and the mutation in at least one *bm1* gene. In certain embodiments that may be combined with any of the preceding embodiments, the plant further contains a mutation in at least one *bm3* gene, a homolog thereof, a paralog thereof, or an ortholog thereof, where the plant exhibits an increased saccharification yield compared to the saccharification yield in a corresponding plant lacking the mutation in the at least one licheninase gene and the mutation in at least one *bm3* gene. Other aspects of the present disclosure relate to a seed of a plant of any of the preceding embodiments.

[0011] Other aspects of the present disclosure relate to a plant containing an RNAi-inducing vector, where the vector generates RNAi against a licheninase gene.

[0012] In certain embodiments, the licheninase gene encodes a polypeptide containing consensus sequence SEQ ID NO: 9. In certain embodiments that may be combined with any of the preceding embodiments, the licheninase gene contains a nucleic acid sequence selected from: (a) SEQ ID NO: 3 or 7; (b) a homolog of SEQ ID NO: 3 or 7; (c) a paralog of SEQ ID NO: 3 or 7; and (d) an ortholog of SEQ ID NO: 3 or 7. In certain embodiments that may be combined with any of the preceding embodiments, the plant further contains one or more additional RNAi-inducing vectors, where the vectors generate RNAi against one or more additional licheninase genes. In certain embodiments, the one or more additional licheninase

genes encode a polypeptide containing consensus sequence SEQ ID NO: 9. In certain embodiments that may be combined with any of the preceding embodiments, the plant further contains an additional RNAi-inducing vector, where the additional vector generates RNAi against a *bm1* gene, a homolog thereof, a paralog thereof, or an ortholog thereof, where the plant exhibits an increased saccharification yield compared to the saccharification yield in a corresponding plant lacking the vectors generating RNAi against a licheninase genes and a *bm1* gene. In certain embodiments that may be combined with any of the preceding embodiments, the plant further contains an additional RNAi-inducing vector, where the additional vector generates RNAi against a *bm3* gene, a homolog thereof, a paralog thereof, or an ortholog thereof, where the plant exhibits an increased saccharification yield compared to the saccharification yield in a corresponding plant lacking the vectors generating RNAi against a licheninase genes and a *bm3* gene. In certain embodiments that may be combined with any of the preceding embodiments, the RNAi-inducing vector or one or more additional RNAi-inducing vectors are stably transformed in the plant. Other aspects of the present disclosure relate to a seed of a plant of any of the preceding embodiments.

[0013] Other aspects of the present disclosure relate to a plant having reduced expression of at least one licheninase gene encoding a polypeptide containing consensus sequence SEQ ID NO: 9, where the plant has elevated levels of glucan compared to the levels of glucan in a corresponding plant lacking the reduced expression of the at least one licheninase gene.

[0014] In certain embodiments, the polypeptide contains an amino acid sequence selected from: (a) SEQ ID NO 4 or 8; (b) a homolog of SEQ ID NO: 4 or 8; (c) a paralog of SEQ ID NO: 4 or 8; and (d) an ortholog of SEQ ID NO: 4 or 8. In certain embodiments that may be combined with any of the preceding embodiments, the plant further contains reduced expression of at least one additional licheninase gene encoding a polypeptide containing consensus sequence SEQ ID NO: 9. In certain embodiments that may be combined with any of the preceding embodiments, the plant further contains reduced expression of at least one *bm1* gene, a homolog thereof, a paralog thereof, or an ortholog thereof, where the plant exhibits an increased saccharification yield compared to the saccharification yield in a corresponding plant having reduced expression of the at least one licheninase gene and reduced expression of the at least one *bm1* gene. In certain embodiments that may be combined with any of the preceding embodiments, the plant further contains reduced expression of at least one *bm3* gene, a homolog thereof, a paralog thereof, or an ortholog

thereof, where the plant exhibits an increased saccharification yield compared to the saccharification yield in a corresponding plant having reduced expression of the at least one licheninase gene and reduced expression of the at least one *bm3* gene. In certain embodiments that may be combined with any of the preceding embodiments, the reduced expression of the at least one licheninase gene, the at least one additional licheninase gene, the at least one *bm1* gene, and/or the at least one *bm3* gene is a result of RNAi, antisense RNA, T-DNA insertion, transposon insertion, or TILLING. Other aspects of the present disclosure relate to a seed of a plant of any of the preceding embodiments.

[0015] In certain embodiments that may be combined with any of the preceding embodiments, the plant is selected from corn (*Zea mays)*, barley (*Hordeum vulgare*), rice (*Oryza sativa*), sorghum (*Sorghum bicolor*), foxtail millet (*Setaria italica*), sugar cane (*Saccharum spp.*), wheat (*Triticum spp.*), soy (*Glysine sp.*), cotton (*Gossypium sp.*), sugar beet (*Beta vulgaris*), sunflower (*Helianthus sp.*), miscanthus (*Miscanthus sp.*), giant miscanthus (*Miscanthus giganteus*), rape (*Brassica napus*), grass (*Poaceae sp.*), switchgrass (*Panicum virgatum*), giant reed (*Arundo donax*), reed canary grass (*Phalaris arundinacea*), sericea lespedeza (*Lespedeza cuneata*), millet *(Panicum miliaceum*), ryegrass (*Lolium sp.*), timothy-grass (*Phleum sp.*), kochia (*Kochia sp.*), kenaf (*Hibiscus cannabinus*), bahiagrass (*Paspalum sp.*), bermudagrass (*Cynodon dactylon*), pangolagrass (*Digitaria decumbens*), bluestem grass (*Andropogon sp.*), indiangrass (*Sorghastrum sp.*), bromegrass (*Bromus sp.*), elephant grass (*Pennisetum purpureum*), jatropha (*Jatropha sp.*), alfalfa (*Medicago sp.*), clover (*Trifolium*), sunn hemp (*Crotalaria juncea*), fescue (*Festuca sp.*), orchard grass (*Dactylis sp.*), purple false brome (*Brachypodium distachyon*), sesame (*Sesamum indicum*), poplar (*Populus trichocarpa*), spruce (*Picea sp.*), pine (*Pinaceae spp.*), willow (*Salix sp.*), eucalyptus (*Eucalyptus sp.*), castor oil plant (*Ricinus communis*), and palm tree (*Arecaceae sp.*).

[0016] Other aspects of the present disclosure relate to a method of increasing levels of glucan in a plant, by reducing the expression in a plant of at least one licheninase gene.

[0017] In certain embodiments, the method further includes reducing the expression of at least one additional licheninase gene. In certain embodiments that may be combined with any of the preceding embodiments, the plant has at least a 20% increase in levels of glucan as compared to a corresponding plant lacking the reduced expression.

[0018] Other aspects of the present disclosure relate to a method of increasing the amount of glucose generated from biomass in a saccharification procedure, by: (a) obtaining biomass from a plant having reduced expression of at least one licheninase gene; and (b) subjecting the biomass to an enzymatic or chemical saccharification procedure, where an increased amount of glucose is generated from the plant having reduced expression of a licheninase gene, as compared to the amount of glucose generated from a corresponding plant lacking the reduced expression.

[0019] In certain embodiments, the plant further contains reduced expression in at least one additional licheninase gene. In certain embodiments that may be combined with any of the preceding embodiments, the amount of glucose generated is increased by at least 20%, as compared to the amount of glucose generated from a corresponding plant lacking the reduced expression. In certain embodiments that may be combined with any of the preceding embodiments, the plant further contains reduced expression of at least one *bm1* gene, a homolog thereof, a paralog thereof, or an ortholog thereof. In certain embodiments that may be combined with any of the preceding embodiments, the plant further contains reduced expression of at least one *bm3* gene, a homolog thereof, a paralog thereof, or an ortholog thereof. In certain embodiments that may be combined with any of the preceding embodiments, the amount of glucose generated is increased by at least 40%, as compared to the amount of glucose generated from a corresponding plant lacking the reduced expression of at least one licheninase gene and the at least one *bm1* gene or the at least one *bm3* gene.

[0020] Other aspects of the present disclosure relate to a method of increasing the yield of fermentation product from a fermentation reaction, by: (a) obtaining biomass from a plant having reduced expression of at least one licheninase gene; (b) subjecting the biomass to an enzymatic or chemical saccharification procedure; and (c) incubating the degraded biomass with a fermentative organism under conditions suitable to yield a fermentation product, where an increased yield of fermentation product from the fermentation reaction is obtained, as compared to the yield of fermentation product obtained from a fermentation reaction using degraded biomass from a corresponding plant lacking the reduced expression.

[0021] In certain embodiments, the plant further contains reduced expression in at least one additional licheninase gene. In certain embodiments that may be combined with any of the preceding embodiments, the plant further contains reduced expression of at least one *bm1*

gene, a homolog thereof, a paralog thereof, or an ortholog thereof. In certain embodiments that may be combined with any of the preceding embodiments, the plant further contains reduced expression of at least one *bm3* gene, a homolog thereof, a paralog thereof, or an ortholog thereof. In certain embodiments that may be combined with any of the preceding embodiments, the at least one licheninase gene encodes a polypeptide containing consensus sequence SEQ ID NO: 9. In certain embodiments that may be combined with any of the preceding embodiments, the at least one licheninase gene contains a nucleic acid sequence is selected from: (a) SEQ ID NO: 3 or 7; (b) a homolog of SEQ ID NO: 3 or 7; (c) a paralog of SEQ ID NO: 3 or 7; and (d) an ortholog of SEQ ID NO: 3 or 7. In certain embodiments that may be combined with any of the preceding embodiments, the reduced expression of the at least one licheninase gene or the at least one additional licheninase gene is a result of mutagenesis of the gene. In certain embodiments that may be combined with any of the preceding embodiments, the reduced expression of the at least one *bm1* gene or the at least one *bm3* gene is a result of mutagenesis of the gene. In certain embodiments that may be combined with any of the preceding embodiments, the mutagenesis of the gene is by TILLING, T-DNA insertion, or transposon insertion. In certain embodiments that may be combined with any of the preceding embodiments, the mutagenesis of the gene results in a partial deletion or a complete deletion of the gene. In certain embodiments that may be combined with any of the preceding embodiments, the reduced expression of the at least one licheninase gene or the at least one additional licheninase gene is a result of RNAi or antisense RNA. In certain embodiments that may be combined with any of the preceding embodiments, the reduced expression of the at least one *bm1* gene or the at least one *bm3* gene is a result of RNAi or antisense RNA.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawings will be provided by the office upon request and payment of the necessary fee.

[0023] Figure 1A depicts a protein model of Cal-1 T01. Figure 1B depicts a protein model of Cal-1 T02.

[0024] Figure 2 depicts the nucleic acid sequence and amino acid sequence of Cal-1 T01 licheninase from the Cal-1 maize mutant and wild-type A619 maize. Figure 2A shows the

nucleic acid sequence of Cal-1 T01 from the Cal-1 maize mutant (SEQ ID NO: 1); Figure 2B shows the amino acid sequence of Cal-1 T01 from the Cal-1 maize mutant (SEQ ID NO: 2); Figure 2C shows the nucleic acid sequence of Cal-1 T01 from A619 maize (SEQ ID NO: 3); and Figure 2D shows the amino acid sequence of Cal-1 T01 from A619 maize (SEQ ID NO: 4). The underlined portion of Figure 2D shows the GH17 domain of Cal-1 T01. The highlighted regions show the location of the point mutation and corresponding amino acid substitution.

[0025] Figure 3 depicts the nucleic acid sequence and amino acid sequence of Cal-1 T02 licheninase from the Cal-1 maize mutant and wild-type A619 maize. Figure 3A shows the nucleic acid sequence of Cal-1 T02 from the Cal-1 maize mutant (SEQ ID NO: 5); Figure 3B shows the amino acid sequence of Cal-1 T02 from the Cal-1 maize mutant (SEQ ID NO: 6); Figure 3C shows the nucleic acid sequence of Cal-1 T02 from A619 maize (SEQ ID NO: 7); and Figure 3D shows the amino acid sequence of Cal-1 T02 from A619 maize (SEQ ID NO: 8). The underlined portion of Figure 3D shows the GH17 domain of Cal-1 T01.The highlighted regions show the location of the point mutation and corresponding amino acid substitution.

[0026] Figure 4 depicts an alignment of the amino acid sequence of the mutant Cal-1 T01 licheninase (SEQ ID NO: 2) with the amino acid sequences of GRMZM2G137535 P01 licheninase from the maize database (SEQ ID NO: 13), the wild-type Cal-1 T01 licheninase (SEQ ID NO: 4); a barley licheninase(SEQ ID NO: 15); and a consensus sequence (SEQ ID NO: 16). Boxed regions depict catalytic amino acid residues.

[0027] Figure 5 depicts an alignment of the amino acid sequence of the mutant Cal-1 T02 licheninase (SEQ ID NO: 6) with the amino acid sequences of GRMZM2G137535 P02 licheninase from the maize database (SEQ ID NO: 14), the wild-type Cal-1 T02 licheninase (SEQ ID NO: 8); a barley licheninase (SEQ ID NO: 15); and a consensus sequence (SEQ ID NO: 17). Boxed regions depict catalytic amino acid residues.

[0028] Figure 6A diagrammatically depicts the amount of monosaccharides by weight of the hemicellulosic fraction, produced by young Cal-1 maize mutant seedlings. Figure 6B diagrammatically depicts the amount of monosaccharides by weight of the hemicellulosic fraction, produced by adult Cal-1 maize mutant leaves. Figure 6C diagrammatically depicts the amount of monosaccharides by weight of the hemicellulosic fraction, produced by

senesced leaf material. Figure 6D diagrammatically depicts the amount of monosaccharides by weight of the hemicellulosic fraction, produced by senesced stem material.

[0029] Figure 7 diagrammatically depicts the cell wall β-1,3-1,4-glucan content of the Cal-1 maize mutant.

[0030] Figure 8A diagrammatically depicts the monosaccharide composition, by weight, of a hemicellulose extract (extraction by 4 molar potassium hydroxide) derived maize seedlings from wild-type (Mo17) and Cal-1/Mo17 inbred lines, indicating that the high glucan content is present in the hemicellulosic fraction. Figure 8B diagrammatically depicts the glycosidic linkage composition, by mol %, of that 4M potassium hydroxide fraction, indicating that the high glucan content is due to an increase of mixed linked β-1,3-1,4-glucan compared to wild-type maize.

[0031] Figure 9A diagrammatically depicts the monosaccharide composition of the total hydrolysate of the residue after 4M potassium hydroxide extraction. The main component is glucose representing cellulose. Hence, no difference in cellulose-content is observed. Figure 9B diagrammatically depicts the lignin content of the Cal-1 maize mutant and wild-type maize (in mass %).

[0032] Figure 10A diagrammatically depicts the amount of glucose, by weight, produced by leaf material from the Cal-1 maize mutant during saccharification of wall material with a mixture of wall degrading enzymes. Figure 10B diagrammatically depicts the saccharification yield (glucose) derived from senesced leaves from the Cal-1 maize mutant. Figure 10C diagrammatically depicts the saccharification yield (glucose) derived from senesced stem from the Cal-1 maize mutant.

[0033] Figure 11 depicts the kernel yield and biomass of the Cal-1 maize mutant. Figure 11A diagrammatically depicts the total grain weight (kg). Figure 11B diagrammatically depicts the total dry biomass weight (kg). Figure 11C diagrammatically depicts the percent grain moisture. Figure 11D diagrammatically depicts the percent biomass moisture.

[0034] Figure 12 diagrammatically depicts the activity of the purified Cal-1 licheninase protein.

[0035] Figure 13A diagrammatically depicts the hemicellulosic glucan content of the Cal-1 maize mutant crossed with the bm1 maize mutant and crossed with the bm3 maize mutant. Figure 13B diagrammatically depicts the saccharification yield of the Cal-1 maize mutant crossed with the bm1 maize mutant and crossed with the bm3 maize mutant.

[0036] Figure 14 depicts an amino acid sequence alignment of the GH117 domains of 66 maize proteins having at least 40% amino acid sequence identity with the Cal-1 T01 licheninase (the sequences correspond to SEQ ID NOS: 18-82, in the order as listed except for the amino acid sequences of GRMZM2G137535, which are SEQ ID NOS: 4 and 8).

[0037] Figure 15 depicts a phylogenetic tree of the 66 maize proteins having a GH17 domain and at least 40% amino acid sequence identity with the Cal-1 T01 licheninase.

[0038] Figure 16 depicts an amino acid sequence alignment of the GH117 domains of 77 proteins from grass species *Zea mays, Oryza sativa, Sorghum bicolor, Brachypodium distachyon* and *Setaria italic* having at least 40% amino acid sequence identity with the Cal-1 T01 licheninase (the sequences correspond to SEQ ID NOS: 83-164, in the order as listed except for the amino acid sequences of GRMZM2G137535, which are SEQ ID NOS: 4 and 8).

[0039] Figure 17 depicts a phylogenetic tree of the 77 proteins from grass species *Zea mays, Oryza sativa, Sorghum bicolor, Brachypodium distachyon* and *Setaria italic* having a GH17 domain and at least 40% amino acid sequence identity with the Cal-1 T01 licheninase.

DETAILED DESCRIPTION

[0040] The present disclosure relates to non-naturally occurring mutant plants having elevated levels of glucan, and is based, in part, on the discovery that non-transgenic and non-naturally occurring mutant maize plants containing a mutation in the candy leaf-1 (Cal-1) licheninase gene have elevated levels of glucan, as compared to the levels of glucan in wild-type maize plants lacking the mutation. The Cal-1 mutation was determined to be a point mutation encoding an amino substitution of an active site glutamic acid of the licheninase polypeptide, which resulted in a decrease in licheninase activity. This decrease in licheninase activity resulted in elevated levels of β-glucan in the leaf and stem tissues of the mutant

plants. Advantageously, mutant plants having elevated levels of glucan provide greater amounts of fermentable glucose, resulting in higher yields of biofuels.

[0041] As used herein, "glucan" refers to a polysaccharide of D-glucose monomers linked by glycosidic bonds. Examples of glucans include, without limitation, α-glucans, such as dextran (α-1,6-glucan with α-1,3-branches) and glycogen (α-1,4- and α-1,6-glucan); and β-glucans, such as lichenin (β-1,3- and β-1,4-glucan) and cellulose (β-1,4-glucan). In certain embodiments, the present disclosure relates to maize having elevated levels of glucans, such as lichenin. In general, the primary cell walls of grasses, such as maize, contain glucans.

[0042] Certain aspects of the present disclosure relate to using the genetic information (*i.e.*, the nucleotide sequence and structure and sequence of the encoded polypeptide) of genes involved in the production of glucan, the regulation of glucan, and/or the regulation of genes involved in the production of glucan to produce plants having elevated levels of glucan. For example, the genetic information of such glucan-related genes may be used to identify regions of the genes that may be modified (*e.g.*, mutated) to produce elevated levels of glucan in plants, and to identify homologous, paralogous, and orthologous glucan-related genes suitable for use in producing plants with elevated levels of glucan. Plants having elevated levels of glucan may be produced by mutating a glucan-related gene in the plant, by reducing or inhibiting expression of a glucan-related gene in the plant, or by reducing or inhibiting the expression or activity of the polypeptide encoded by a glucan-related gene. Either known glucan-related genes or novel glucan-related genes may be used. In the case of novel glucan-related genes, the genes may be identified, for example, by mutagenizing plants and screening for mutants having elevated levels of glucan. Methods of mutagenizing plants are well known in the art and described herein. Mutants having elevated levels of glucan can then be analyzed to identify the gene mutation resulting in the glucan phenotype. Methods of identifying gene mutations are well known in the art and described herein.

[0043] In one particular example, licheninase genes may be used to produce plants having elevated levels of glucan. Licheninase genes encode polypeptides involved in the degradation of the β-glucan lichenin. As used herein, "licheninase" or "polypeptides with licheninase activity", refers to a polypeptide having E.C. 3.2.1.73 activity, which catalyzes the hydrolysis of 1,4-β-D-glucosidic linkages in β-D-glucans containing 1,3- and 1,4-bonds. As used here, a "licheninase" includes, without limitation, licheninases, lichenases, endo-β-

1,3-1,4 glucanases, 1,3-1,4-β-D-glucan 4-glucanohydrolases, and mixed linkage β-glucanases.

[0044] Accordingly, certain aspects of the present disclosure re relate to a non-naturally occurring mutant plant having elevated levels of glucan compared to the levels of glucan in a corresponding plant lacking the mutation, where the mutant plant contains a mutation in at least one licheninase gene. Other embodiments of the present disclosure relate to a plant having reduced expression of at least one licheninase gene, where the plant has elevated levels of glucan compared to a corresponding plant lacking the reduced expression of the at least one may be reduced to elevate the levels of glucan in plants. In other embodiments, the plants having reduced expression in at least one licheninase gene produce more glucan-released glucose than plants lacking the reduced expression of the at least one licheninase gene. In still other embodiments, biomass derived from plants having reduced expression of at least one licheninase gene provide an increased yield of a fermentation product in a fermentation reaction compared to biomass derived from plants lacking the reduced expression of the at least one licheninase gene.

[0045] Other embodiments of the present disclosure relate to methods of producing plants with elevated levels of glucan by reducing expression of at least one licheninase gene in the plant, as well as methods of using such plants, *e.g.*, to increase biofuel yield from plant material. In certain embodiments, the yield of a fermentation product from a fermentation reaction is generally increased with increased levels of glucan in the plant. To obtain sugars, such as glucose, for the fermentation reaction, one or both of enzymatic or chemical degradation of glucan from plant material can be used. The degradation and fermentation of glucan from the plant can be performed in one reaction mixture or using separate reaction mixtures. Plant material from a plant having reduced expression of at least one licheninase gene, *e.g.*, cell wall material from leaves, shoots, stems, etc., can be degraded either enzymatically or chemically in one reaction and the degradation products then fermented in a separate reaction mixture. In other aspects, the degradation reaction and the fermentation reaction are conducted in the same reaction mixture such that the degradation products generated from enzymatic or chemical degradation of the plant biomass is fermented in the same mixture in which the biomass is degraded.

[0046] An "increased yield" from a fermentation reaction can thus arise from an increase in the overall amount of product obtained from a reaction.

Plants with Elevated Levels of Glucan

[0047] Certain aspects of the present disclosure relate to plants having elevated levels of glucan. In certain embodiments, the elevated levels of glucan are the result of a non-naturally occurring mutation in at least one licheninase gene. In other embodiments, the elevated levels of glucan are the result of reduced expression of at least one licheninase gene. In still other embodiments, the elevated levels of glucan are the result of reduced expression of at least one licheninase polypeptide. In further embodiments, the elevated levels of glucan are the result of reduced licheninase activity in at least one licheninase polypeptide.

[0048] As used herein, "elevated" level of glucan refers to increased levels of glucan in a modified plant as compared to the level of glucan in a corresponding non-modified plant. As used herein, a "non-modified" plant refers to a plant that has not been modified in regards to the trait at issue (*e.g.*, in this case, expression levels of at least one licheninase gene).

[0049] As used herein, "non naturally-occurring mutation" refers to plants that have been subjected to mutagenesis. Mutagenesis may be accomplished by any method of mutagenesis disclosed herein or any method known in the art. Examples include, without limitation, chemical mutagenesis and radiation mutagenesis.

[0050] As used herein, "reduced expression" of a licheninase gene or polypeptide refers to a modified plant having levels of expression that are reduced as compared to the levels of expression in a corresponding non-modified plant. As used herein, "reduced licheninase activity" in a licheninase polypeptide refers to a modified plant having levels of licheninase activity in a licheninase polypeptide that are reduced as compared to the levels of licheninase activity in the licheninase polypeptide in a corresponding non-modified plant.

[0051] In some embodiments, plants having an elevated levels of glucan contain levels of glucan that are elevated by at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 100%, at least 200%, at least 300%, at least 400%, at least 500%, at least 600%, at least 700%, at least

800%, at least 900%, at least 1,000%, or more compared to the levels of glucan in a corresponding non-modified plant.

[0052] In other embodiments, plants having elevated levels of glucan contain reduced expression of at least one licheninase gene, where the level of expression is about 5 % less, 10% less, 15% less, 20% less, 25% less, 30% less, 35% less, 40% less, 45% less, 50% less, 55% less, 60% less, 65% less, 70% less, 75% less, 80% less, 85% less, 90% less, 95% less, 100% less, 125% less, 150% less, 175% less, 200% less, 300% less, 400% less, 500% less, 600% less, 700% less, 800% less, 900% less, 1000% less, or a greater percentage less than the level of expression of the at least one licheninase gene in a corresponding non-modified plant.

[0053] In still other embodiments, plants having elevated levels of glucan contain reduced expression of at least one licheninase polypeptide, where the level of expression is about 5 % less, 10% less, 15% less, 20% less, 25% less, 30% less, 35% less, 40% less, 45% less, 50% less, 55% less, 60% less, 65% less, 70% less, 75% less, 80% less, 85% less, 90% less, 95% less, 100% less, 125% less, 150% less, 175% less, 200% less, 300% less, 400% less, 500% less, 600% less, 700% less, 800% less, 900% less, 1000% less, or a greater percentage less than the level of expression of the at least one licheninase polypeptide in a corresponding non-modified plant.

[0054] In further embodiments, plants having elevated levels of glucan contain reduced licheninase activity in at least one licheninase polypeptide, where the level of licheninase activity is about 5 % less, 10% less, 15% less, 20% less, 25% less, 30% less, 35% less, 40% less, 45% less, 50% less, 55% less, 60% less, 65% less, 70% less, 75% less, 80% less, 85% less, 90% less, 95% less, 100% less, 125% less, 150% less, 175% less, 200% less, 300% less, 400% less, 500% less, 600% less, 700% less, 800% less, 900% less, 1000% less, or a greater percentage less than the level of licheninase activity in the at least one licheninase polypeptide in a corresponding non-modified plant.

[0055] In other embodiments, plants having elevated levels of glucan have been modified to alter the level of one or more polypeptides that affect the levels of glucan in the plant. In yet other embodiments, plants having elevated levels of glucan have been modified to alter the expression of one or more genes encoding one or more polypeptides that affect the levels of glucan.

[0056] The present disclosure also includes offspring of plants that have been modified to have elevated levels of glucan. The present disclosure further includes seeds, cuttings, rhizomes, runners, plant cells, and tissues of plants that have been modified to have elevated levels of glucan.

Plant types

[0057] As disclosed herein, various types of plants may be modified to produce plants having elevated levels of glucan. Suitable plants that may be modified include both monocotyledonous plants and dicotyledonous plants. Examples of suitable plants that may be modified to produce plants having elevated levels of glucan include, without limitation, maize (*Zea mays)*, barley (*Hordeum vulgare*), rice (*Oryza sativa*), sorghum (*Sorghum bicolor*), foxtail millet (*Setaria italica*), sugar cane (*Saccharum spp.*), wheat (*Triticum spp.*), soy (*Glysine sp.*), cotton (*Gossypium sp.*), sugar beet (*Beta vulgaris*), sunflower (*Helianthus sp.*), miscanthus (*Miscanthus sp.*), giant miscanthus (*Miscanthus giganteus*), switchgrass (*Panicum virgatum*), grass (*Poaceae sp.*), rape (*Brassica napus*), giant reed (*Arundo donax*), reed canary grass (*Phalaris arundinacea*), sericea lespedeza (*Lespedeza cuneata*), millet (*Panicum miliaceum*), ryegrass (*Lolium sp.*), timothy-grass (*Phleum sp.*), kochia (*Kochia sp.*), kenaf (*Hibiscus cannabinus*), bahiagrass (*Paspalum sp.*), bermudagrass (*Cynodon dactylon*), pangolagrass (*Digitaria decumbens*), bluestem grass (*Andropogon sp.*), indiangrass (*Sorghastrum sp.*), bromegrass (*Bromus sp.*), elephant grass (*Pennisetum purpureum*), jatropha (*Jatropha sp.*), alfalfa (*Medicago sp.*), clover (*Trifolium*), sunn hemp (*Crotalaria juncea*), fescue (*Festuca sp.*), orchard grass (*Dactylis sp.*), purple false brome (*Brachypodium distachyon*), sesame (*Sesamum indicum*), poplar (*Populus trichocarpa*), spruce (*Picea sp.*), pine (*Pinaceae spp.*), willow (*Salix sp.*), eucalyptus (*Eucalyptus sp.*), castor oil plant (*Ricinus communis*), and palm tree (*Arecaceae sp.*).

[0058] In certain preferred embodiments, plants that may be modified to produce plants having elevated levels of glucan are grasses, such as maize, wheat, rice, sorghum, and switchgrass. In some embodiments, the plants of the present disclosure are used as feedstocks for biofuel production and/or the production of commodity chemicals. In other embodiments, plants of the disclosure are used for, without limitation, food, cosmetic, or pharmaceutical production.

Suitable Licheninase Polypeptides

[0059] Other aspects of the present disclosure relate to plants having elevated levels of glucan, where the elevated glucan levels are the result of at least one modified licheninase polypeptide having reduced licheninase activity.

[0060] As used herein, a "polypeptide" is an amino acid sequence including a plurality of consecutive polymerized amino acid residues (*e.g.*, at least about 15 consecutive polymerized amino acid residues). As used herein, "polypeptide" refers to an amino acid sequence, oligopeptide, peptide, protein, or portions thereof.

[0061] Suitable polypeptides of the present disclosure that may be used to produce plant with elevated levels of glucan include, without limitation, licheninase polypeptides that have been modified or inhibited to reduce their licheninase activity compared to the licheninase activity of a corresponding licheninase polypeptide that lacks such a modification. Examples of modified polypeptides include, without limitation, polypeptides containing one or more insertions, duplications, amplifications, truncations, deletions, or amino acid substitutions that reduce the licheninase activity of the polypeptide as compared to the licheninase activity in a corresponding licheninase polypeptide that lacks such a modification, or that inhibit the licheninase activity. Methods of generating and identifying polypeptides with one or more modifications are well known in the art.

[0062] As used herein, gene expression, polypeptide expression, or polypeptide activity that has been "inhibited", refers to expression or activity that is below the detection level of any known method of detecting gene or polypeptide expression, or of detecting polypeptide activity.

[0063] As disclosed herein, licheninase polypeptides modulate the levels of glucan in plants by hydrolyzing 1,4-β-D-glucosidic linkages in (1,3;1,4)-β-glucans. Licheninase polypeptides of the present disclosure are members of glycosylhydrolase family 17 (GH17) family of glycosylhydrolases. GH17 polypeptides contain a conserved GH17 domain that is unique to members of the GH17 family of polypeptides. A consensus sequence of the GH17 domain is set forth below (SEQ ID NO: 9):

X-X-XX-X-X-X-X-X-X-[I/L/V/H/A/F]-G-[V/I/A]-[N/T/S/C]-[Y/N/I/W/H]-G-X-[V/Q/M/S/I/L/T/N/R/A]-[A/S/G/M/V]-X-[N/H/D/T/S]-[L/P/Q/R/I]-[P/L/I/A]-X-[P/L//H/A/K/S/T]-X-X-[V/A/M/S/P/L/K/I]-[V/A/I/M/S/P/L/T]-X-[L/Q/R/K/E/D/M/I/F]-[L/V/M/G/Y/C/A/I]-[R/L/K/Q/A/E/S/V/L/T]-X-

[S/D/G/A/R/K/L/Q]-X-X-[I/V/F/A]-X-[K/R/A/L/V/Y/D/S/G/N/M/H]-[V/A/M/L]-
[R/K/T]-[L/S/M/I/T]-[Y/F/I/L]-[D/E/N/L/A/W/H/F/S/G]-[A/T/P/S/V]-
[D/M/E/V/N/Q]-X-X-[V/A/P/T/I/L/F/M]-[L/M/P/V/I]-X-[A/S]-[L/F/V/A]-
[A/V/G/R/S]-[G/D/H/K/N/R/A]-X-X-[T/S/A/P]-[G/S/D/R/N]-[I/V/L/W]-X-
[V/A/L/F]-[V/M/T/A/I/D]-[V/L/A/P/I]-[G/A/D/S/M/T]-[V/I/A/L/T/E/F]-
[P/T/L/G/A]-[N/D]-X-X-X-[L/R/A/D/E/G/S/K/I]-X-X-X-[A/P/DS/R/T/G/I/L/M]-
[A/D/Y/S/G/R/Q/T/V/N]-[S/A/G/D/Y/P/V/M/Q/R/T/N]-X-X-X-X-X-X-[A/V/S]-
X-X-[W/C/L]-[V/A/L]-X-X-[N/L/A/Y/T/S/H/R]-[V/I/L]-X-[P/A/R/K/T/S]-
[Y/V/N/H/A/F/T/S]-X-[P/L/F/N/S/G/Q/D]-[A/D/K/R/S/Q/V]-X-X-X-X-X-X-
[I/C/L/F/V/S/T]-X-X-[V/I/L/M]-[A/C/N/V/T/S]-[V/L/A/G]-[G/D/N]-
[N/P/A/E/S/D]-[E/S/V]-[V/A/F/I/L/T]-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-
X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-
X-X-X-X-X-X-[L/T/I/V]-[L/F/V/I/A/M]-[P/Q/G/D]-A-[M/L/V/I]-
[R/Q/K/T/E/A/S/L]-[N/S/T/A/C/R/Y]-[L/I/V/M/A]-[H/Q/R/E/D/N/S/A/Y/L]-X-
[A/S/G]-[L/I/V/A]-X-X-[A/L/H/R/V/S/G/N/E]-[G/N/S/R/H/A]-
[L/I/F/H/V/F/M/D]-X-X-X-X-X-[V/I/A/T]-[K/H/P/R/T/E/N/A]-[V/A/L/C/I/F]-
[S/T/V/G/F]-[T/V/C/S]-X-[V/L/C/H/I/N]-[S/N/A/K/R/Q/T/Y/P]-X-X-[V/I/A/D]-
[L/Y/I/V/T/F/M]-[A/M/N/D/S/E/Q/G/T/R/L/V]-X-[S/P/Q/T/A]-X-X-X-X-
[P/V/Q/I]-[P/S]-[S/A/Q]-[A/Q/G/R/D/S/T/N]-[G/Q/E/A/S/C]-X-[F/W/T/S]-
[R/C/D/V/G/E/A/S/N/T/H]-X-X-[L/P/I/V/L/S/Y/A/D/E]-X-X-X-X-[M/L/V/I]-X-
[P/D/E/S/T/Q/Y/R]-[L/M/I/V]-[L/V/A/I]-X-[F/Y/L/H]-[L/F/H]-
[A/N/H/L/S/Q/E/D/V/R]-X-[T/N/S/H/K/R/I/V/A]-[G/D/R/N/Q/S]-[A/G/S/T/R]-
[P/V/A/C/Y/F]-[L/F/Y/V]-[L/T/V/M/F/Y/P/W/L]-[V/I/A/C/L/I]-[N/S/D]-
[I/H/A/V/L/C/P]-[Y/L]-[P/T]-[Y/R/F/C/W]-[F/S/L/Y]-[A/S/T/V/D]-
[Y/P/H/W/Q/L]-X-X-X-X-X-X-X-X-X-X-X-[I/S/F/V/M/L/E/S/A]-X-
[L/V/F/Q/M/I]-[D/E/N/A/S/G/P]-[Y/F/N]-[A/S/V/C]-[L/F/Y/I/T/V]-[F/L/G/S]-X-
[P/G/S/A/M/V]-X-X-X-X-X-X-X-X-X-X-X-X-X-X-[V/G/A/S/N/K/H/T/Y/R/M]-
[V/W/P/I/S/T/R/L/A/Q/M/Y]-[D/V/Q/I/L/T]-X-X-[T/S/H/N/G/A]-
[G/R/N/S/P/A/E/K]-[L/V/I/A/M/F/N/Y]-X-Y-[T/S/Y/Q/N/G/D/H/A/P]-[N/D/S]-
[M/V/A/L]-[F/L]-[D/Y/H/A/V]-[A/G/T/Q/E]-[Q/N/T/I/M/L/V]-
[V/F/Y/H/A/M/L]-D-[A/T/S/C]-[V/L/F/T/I/A]-[Y/V/I/H/R/F/T/K]-
[A/S/H/W/L/V/I/F/Y/T]-[A/S]-[L/M/V/I/A]-X-X-[L/V/A/H/N/I/E/M/K]-[G/N]-X-
X-X-X-X-X-X-X-X-X-X-X-X-X-[V/M/L/I/P]-X-[V/I/L/A]-[V/M/I/A/H/R/T/K/L]-
[V/I/L]-[S/G/T/A]-E-[T/V/A/I/S]-G-[W/H/C]-[P/A]-[S/T/N/Y/H]-X-[G/D/C/A]-
X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-
[E/D/N/A/H/Q/Y]-X-[G/H/Y/N/S/A/Q/V/E/D]-[A/E/G/V]-[T/K/N/S/G]-X-
[E/A/S/K/Q/T/G/D/R/H]-[N/Y/F/L/M/A/E]-[A/S]-X-X-[Y/F]-[N/Y/V/S/D/I]-X-
[N/G/K/Y]-[L/F/I/V/A/M]-[I/L/F/M/V/A/R]-[R/Q/D/T/N/E/L/A/K/M/S]-X-
[V/L/M/I/A/Q/C]-X-X-[G/N/S/R/D/Q/L/E]-X-X-X-G-T-P-X-[R/H/K/A/T/M]-
[P/K/T/S]-[G/N/Q/R/D/K/H/A/S]-X-X-X-X-X-X-X-[Y/F/I/M/S]-[I/L/V/M]-
[F/Y]-[A/G/S/D/E]-[L/M/T]-[F/L/V/I/Y]-[N/D]-E-[D/E/N]-X-[K/R]-X-X-X-
[G/P/D/E/A]-X-X-[S/F/Q/E/T/V/I/A]-[E/N/H/K/R]-[R/Q/N/K/A]-X-[W/F/Y]-G-
[L/I/V/M]-[F/L/M/Y]-X-[P/Y/F/A/G/K/T/M]-X-[D/N/S]-[G/M/K/R/Q/E/L]-
[T/Q/R/K/S/L/H/E/A/V]-[P/A/K/H/R/E/L/M/S/I]-[V/K/A/I/S/N/T]-[Y/F]-X-
[L/M/I/V/F]-X-X

[0064] In the above domain and all other domains provided herein, the accepted IUPAC single letter amino acid abbreviation is employed.

[0065] Accordingly, in certain embodiments, suitable polypeptides that may be modified to produce a plat with elevated levels of glucan contain the consensus sequence set forth in SEQ ID NO: 9.

[0066] Additionally, suitable polypeptides that may be modified to produce plants with elevated levels of glucan include the polypeptides encoded by the Cal-1 T01 and Cal-1 T02 licheninase genes. The amino acid sequence of the polypeptide encoded by Cal-1 T01 is set forth in SEQ ID NO: 4 and the amino acid sequence of the polypeptide encoded by Cal-1 T02 is set forth in SEQ ID NO: 8. In certain embodiments, suitable polypeptides contain an amino acid sequence having at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or higher percent identity to the sequence of SEQ ID NO: 4. In other embodiments, suitable polypeptides contain an amino acid sequence having at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or higher percent identity to the sequence of SEQ ID NO: 8. In further embodiments, suitable polypeptides contain at least 10, at least 12, at least 14, at least 16, at least 18, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100 , at least 125, at least 150, at least 175, at least 200, at least 225, at least 250, at least 275, at least 300, or more consecutive amino acids of SEQ ID NOs: 4 or 8.

[0067] Other suitable polypeptides that may be modified to produce plants with elevated levels of glucan include homologs, paralogs, and/or orthologs of the polypeptides encoded by the Cal-1 T01 and Cal-1 T02 licheninase genes. Methods for identifying polypeptides that are homologs, paralogs, and/or orthologs of a polypeptide of interest are well known to one of skill in the art, as described herein. Examples of suitable polypeptides that are homologous, paralogous, and/or orthologous to the polypeptides encoded by Cal-1 P0T and Cal-1 T02 include, without limitation, homologous, paralogous, and/or orthologous licheninase polypeptides from maize (*Zea mays*), barley (*Hordeum vulgare*), rice (*Oryza sativa*), sorghum (*Sorghum bicolor*), foxtail millet (*Setaria italica*), sugar cane (*Saccharum spp.*), wheat (*Triticum spp.*), soy (*Glysine sp.*), cotton (*Gossypium sp.*), sugar beet (*Beta*

vulgaris), sunflower (*Helianthus sp.*), miscanthus (*Miscanthus sp.*), giant miscanthus (*Miscanthus giganteus*), switchgrass (*Panicum virgatum*), grass (*Poaceae sp.*), rape (*Brassica napus*), giant reed (*Arundo donax*), reed canary grass (*Phalaris arundinacea*), sericea lespedeza (*Lespedeza cuneata*), millet (*Panicum miliaceum*), ryegrass (*Lolium sp.*), timothy-grass (*Phleum sp.*), kochia (*Kochia sp.*), kenaf (*Hibiscus cannabinus*), bahiagrass (*Paspalum sp.*), bermudagrass (*Cynodon dactylon*), pangolagrass (*Digitaria decumbens*), bluestem grass (*Andropogon sp.*), indiangrass (*Sorghastrum sp.*), bromegrass (*Bromus sp.*), elephant grass (*Pennisetum purpureum*), jatropha (*Jatropha sp.*), alfalfa (*Medicago sp.*), clover (*Trifolium*), sunn hemp (*Crotalaria juncea*), fescue (*Festuca sp.*), orchard grass (*Dactylis sp.*), purple false brome (*Brachypodium distachyon*), sesame (*Sesamum indicum*), poplar (*Populus trichocarpa*), spruce (*Picea sp.*), pine (*Pinaceae spp.*), willow (*Salix sp.*), eucalyptus (*Eucalyptus sp.*), castor oil plant (*Ricinus communis*), and palm tree (*Arecaceae sp.*).

[0068] Suitable polypeptides that may also be modified to produce plants with elevated levels of glucan include maize polypeptides that are homologous to the licheninase polypeptides encoded by Cal-1 T01 and Cal-1 T02. For example, suitable polypeptides include, without limitation, the polypeptides encoded by the genes listed in Table 1.

Table 1

Gene ID	Organism of Origin
ACl59612.1_FG007	*Zea mays*
GRMZM2G020898	*Zea mays*
GRMZM2G078566	*Zea mays*
GRMZM2G083599	*Zea mays*
GRMZM2G005798	*Zea mays*
GRMZM2G310739	*Zea mays*
AC217887.3_FG004	*Zea mays*
GRMZM2G097207	*Zea mays*
GRMZM2G152638	*Zea mays*
GRMZM2G335111	*Zea mays*
GRMZM2G014723	*Zea mays*
GRMZM2G137535	*Zea mays*
GRMZM2G041961	*Zea mays*
GRMZM2G019185	*Zea mays*
GRMZM2G088951	*Zea mays*
GRMZM2G380561	*Zea mays*
GRMZM2G591605	*Zea mays*
GRMZM2G061403	*Zea mays*
GRMZM2G125032	*Zea mays*
GRMZM2G433365	*Zea mays*
GRMZM2G062600	*Zea mays*

Gene ID	Organism of Origin
GRMZM2G065585	*Zea mays*
GRMZM2G123107	*Zea mays*
GRMZM2G000959	*Zea mays*
GRMZM2G179354	*Zea mays*
GRMZM2G458164	*Zea mays*
GRMZM2G046459	*Zea mays*
GRMZM2G114140	*Zea mays*
GRMZM2G454550	*Zea mays*
GRMZM2G431039	*Zea mays*
GRMZM2G005082	*Zea mays*
GRMZM2G008627	*Zea mays*
GRMZM2G117872	*Zea mays*
GRMZM2G042870	*Zea mays*
GRMZM2G019619	*Zea mays*
GRMZM2G127117	*Zea mays*
GRMZM5G805609	*Zea mays*
GRMZM2G076584	*Zea mays*
GRMZM2G030850	*Zea mays*
GRMZM2G172537	*Zea mays*
GRMZM2G478892	*Zea mays*
GRMZM2G148400	*Zea mays*
GRMZM2G012758	*Zea mays*
GRMZM2G096591	*Zea mays*
GRMZM2G046101	*Zea mays*
GRMZM2G064202	*Zea mays*
GRMZM2G111143	*Zea mays*
GRMZM2G111324	*Zea mays*
GRMZM5G824920	*Zea mays*
GRMZM2G325008	*Zea mays*

[0069] Other suitable polypeptides that may be modified to produce plants with elevated levels of glucan, include *Oryza sativa, Sorghum bicolor, Brachypodium distachyon* and *Setaria italica* polypeptides that are homologous to the licheninase polypeptides encoded by Cal-1 T01 and Cal-1 T02. For example, suitable polypeptides include, without limitation, the polypeptides encoded by the genes listed in Table 2

Table 2

Gene ID	Organism of Origin
Bradi2q27140.1	*Brachypodium distachyon*
Bradi2q27140.2	*Brachypodium distachyon*
Sb09g018730.1	*Sorghum bicolor*
Sb09g018730.3	*Sorghum bicolor*
Sb09g018730.4	*Sorghum bicolor*

Gene ID	Organism of Origin
Sb09g018730.2	*Sorghum bicolor*
Si022614m	*Setaria italic*
Si022791m	*Setaria italic*
Si022794m	*Setaria italic*
Si022731m	*Setaria italic*
LOC_Os05g31140.1	*Oryza sativa*
LOC_Os05g31140.2	*Oryza sativa*
LOC_Os05g31140.3	*Oryza sativa*
Sb09g018750.1	*Sorghum bicolor*
Si022606m	*Setaria italic*
Si028122m	*Setaria italic*
Bradi2g60500.1	*Brachypodium distachyon*
Sb03q045480.1	*Sorghum bicolor*
Si002273m	*Setaria italic*
LOC_Os02g53200.1	*Oryza sativa*
LOC_Os02g53200.2	*Oryza sativa*
Si017035m	*Setaria italic*
Sb02g030930.1	*Sorghum bicolor*
LOC_Os09g36280.1	*Oryza sativa*
Sb09g024320.1	*Sorghum bicolor*
Si022492m	*Setaria italic*
LOC_Os05g41610.1	*Oryza sativa*
Sb03g037270.1	*Sorghum bicolor*
Si002065m	*Setaria italic*
LOC_Os01g58730.1	*Oryza sativa*
Sb03g045450.1	*Sorghum bicolor*
Sb03g045460.1	*Sorghum bicolor*
Si002182m	*Setaria italic*
LOC_Os01g71340.1	*Oryza sativa*
LOC_Os01g71400.1	*Oryza sativa*
LOC_Os01g71650.1	*Oryza sativa*
LOC_Os01g71930.1	*Oryza sativa*
Sb08g019670.1	*Sorghum bicolor*
Si022625m	*Setaria italic*
LOC_Os01q71410.1	*Oryza sativa*
LOC_Os01g51570.1	*Oryza sativa*
LOC_Os01g71350.1	*Oryza sativa*
Bradi2g60490.1	*Brachypodium distachyon*
Sb03g045490.1	*Sorghum bicolor*
Si004560m	*Setaria italic*
LOC_Os01g71380.1	*Oryza sativa*
LOC_Os01q71670.1	*Oryza sativa*
Sb03g045510.1	*Sorghum bicolor*
Si003802m	*Setaria italic*
Si005124m	*Setaria italic*
LOC_Os01g71680.1	*Oryza sativa*
Bradi2g60560.1	*Brachypodium distachyon*

Gene ID	Organism of Origin
Sb03g045520.1	*Sorghum bicolor*
Si000491m	*Setaria italic*
LOC_Os01g71690.2	*Oryza sativa*
LOC_Os01g71690.3	*Oryza sativa*
LOC_Os01g71810.1	*Oryza sativa*
LOC_Os01g71820.1	*Oryza sativa*
LOC_Os01g71830.1	*Oryza sativa*
LOC_Os01g71860.1	*Oryza sativa*
Sb03g045630.1	*Sorghum bicolor*
Si002306m	*Setaria italic*
Sb09g025890.1	*Sorghum bicolor*
Si024558m	*Setaria italic*

Modified polypeptides

[0070] In certain embodiments, licheninase polypeptides of the present disclosure are modified to reduce or inhibit the licheninase activity of the polypeptide, which when expressed in plant results in a plant with elevated levels of glucan. Licheninase polypeptide of the present disclosure may be modified to contain one or more amino acid substitutions at active site residues. For example, the polypeptide encoded by Cal-1 T01 may contain an amino acid substitution at one or both of its active site residues (*e.g.*, Glu 262 and Glu 318). In some embodiments, a licheninase polypeptide of the present disclosure contains an amino acid substitution at a position that is analogous to position 262 of SEQ ID NO: 4, the amino acid sequence of the polypeptide encoded by Cal-1 T01 (*i.e.*, at a position corresponding to position 262 in a homolog, ortholog, or paralog of Cal-1 T01). In other embodiments, the amino acid sequence of the polypeptide encoded by Cal-1 T01contains an amino acid substitution at position 262. In still other embodiments, a licheninase polypeptide of the present disclosure contains an amino acid substitution at a position that is analogous to position 318 of SEQ ID NO: 4 (*i.e.*, at a position corresponding to position 318 in a homolog, ortholog, or paralog of Cal-1 T01). In yet other embodiments, the amino acid sequence of the polypeptide encoded by Cal-1 T01 contains an amino acid substitution at position 318. In yet other embodiments, a licheninase polypeptide of the present disclosure contains an amino acid substitution at a position that is analogous to position 242 of SEQ ID NO: 8, the amino acid sequence of the polypeptide encoded by Cal-1 T02 (*i.e.*, at a position corresponding to position 242 in a homolog, ortholog, or paralog of Cal-1 T02). In still other embodiments, the amino acid sequence of the polypeptide encoded by Cal-1 T02 contains an amino acid

substitution at position 242. In further embodiments, a licheninase polypeptide of the present disclosure contains an amino acid substitution at a position that is analogous to position 298 of SEQ ID NO: 8 (*i.e.*, at a position corresponding to position 298 in a homolog, ortholog, or paralog of Cal-1 T02). In other embodiments, the amino acid sequence of the polypeptide encoded by Cal-1 T02 contains an amino acid substitution at position 298. The amino acid substitution may be any substitution that reduces or inhibits the licheninase activity of the polypeptide. In certain preferred embodiments, the amino acid substitution is a glutamic acid (Glu) to lysine (Lys) substitution.

[0071] In other embodiments, licheninase polypeptides of the present disclosure contain one or more amino acid substitutions in regions other than the active site that reduce or inhibit the licheninase activity of the polypeptide.

[0072] In further embodiments, licheninase polypeptides of the present disclosure are modified to contain an insertion, duplication, amplification, truncation, or deletion that results in reduced or inhibited licheninase activity.

Suitable Licheninase Polynucleotides

[0073] Further aspects of the present disclosure relate to plants having elevated levels of glucan, where the elevated glucan levels are the result of at least one modified licheninase polynucleotide encoding a polypeptide having reduced licheninase activity.

[0074] Polynucleotides that encode a polypeptide are also referred to herein as "genes". Methods for determining the relationship between a polypeptide and a polynucleotide that encodes the polypeptide are well known in the art. Similarly, methods of determining the polypeptide sequence encoded by a nucleic acid sequence are well known in the art.

[0075] As used herein, the terms "polynucleotide", "nucleic acid sequence", "nucleotide sequence", "nucleic acid", and variations thereof shall be generic to polydeoxyribonucleotides (containing 2-deoxy-D-ribose), to polyribonucleotides (containing D-ribose), to any other type of polynucleotide that is an N-glycoside of a purine or pyrimidine base, and to other polymers containing non-nucleotidic backbones, provided that the polymers contain nucleobases in a configuration that allows for base pairing and base stacking, as found in DNA and RNA. Thus, these terms include known types of nucleic acid

sequence modifications, for example, substitution of one or more of the naturally occurring nucleotides with an analog, and inter-nucleotide modifications. As used herein, the symbols for nucleotides and polynucleotides are those recommended by the IUPAC-IUB Commission of Biochemical Nomenclature.

[0076] Suitable polynucleotides of the present disclosure that may be modified to produce plants with elevated levels of glucan include, without limitation, licheninase genes that have been mutated to have reduced expression compared to the expression of a corresponding licheninase gene that lacks such a modification, or to inhibit expression of the licheninase gene. Examples of suitable mutations include, without limitation, point mutations, nonsense mutations, truncation mutations, missense mutations, substitution mutations, frameshift mutations, loss-of-function mutations, deletion mutations, insertion mutations, duplication mutations, amplification mutations, translocation mutations, or inversion mutations. Methods of generating and identifying polynucleotide with one or more mutations are well known in the art, and include, without limitation, nucleic acid sequencing, polymerase chain reaction, and hybridization.

[0077] Other suitable polynucleotides of the present disclosure affect the expression of a licheninase gene. In some embodiments, polynucleotides that affect the expression of a licheninase gene reduce or inhibit gene expression. In other embodiments, polynucleotides that reduce or inhibit gene expression have a sequence that is identical to the sequence of the licheninase gene to be affected. In other embodiments, polynucleotides that reduce or inhibit gene expression have a sequence that is 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 96% or more, 97% or more, 98% or more, or 99% or more identical to the sequence of the licheninase gene to be affected. In other embodiments, polynucleotides that reduce or inhibit gene expression have a sequence that is identical to a fragment of the sequence of the licheninase gene to be affected. In other embodiments, polynucleotides that reduce or inhibit gene expression have a sequence that is 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 96% or more, 97% or more, 98% or more, or 99% or more identical to a fragment of the sequence of the licheninase gene to be affected. In other embodiments, polynucleotides that reduce or inhibit gene expression have a sequence that is identical to a complement of the sequence of the licheninase gene to be affected. In other embodiments, polynucleotides that reduce or inhibit gene expression have a sequence that is 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 96% or more, 97% or

more, 98% or more, or 99% or more identical to a complement of the sequence of the licheninase gene to be affected. In other embodiments, polynucleotides that reduce or inhibit gene expression have a sequence that is identical to a fragment of the complement of the sequence of the licheninase gene to be affected. In other embodiments, polynucleotides that reduce or inhibit gene expression have a sequence that is 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 96% or more, 97% or more, 98% or more, or 99% or more identical to a fragment of the complement of the sequence of the licheninase gene to be affected.

[0078] Examples of suitable polynucleotides that may be modified to produce a plant with elevated levels of glucan include, without limitation, polynucleotides encoding a polypeptide containing the consensus sequence set forth in SEQ ID NO: 9.

[0079] Additionally, suitable polynucleotides that may be modified to produce plants with elevated levels of glucan include the Cal-1 T01 and Cal-1 T02 licheninase genes. The nucleic acid sequence of Cal-1 T01 is set forth in SEQ ID NO: 3 and the nucleic acid sequence of Cal-1 T02 is set forth in SEQ ID NO: 7. In certain embodiments, suitable polynucleotides contain a nucleic acid sequence that is at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or higher percent identity to the sequence of SEQ ID NO: 3. In other embodiments, suitable polynucleotides contain a nucleic acid sequence that is at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or higher percent identity to the sequence of SEQ ID NO: 7.

[0080] In further embodiments, suitable polynucleotides encode SEQ ID NO: 4 or SEQ ID NO: 8. In other embodiments, suitable polynucleotides encode polypeptides containing an amino acid sequence having at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or higher percent identity to the sequence of SEQ ID NO: 4. In still other embodiments, suitable polynucleotides encode polypeptides containing

an amino acid sequence having at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or higher percent identity to the sequence of SEQ ID NO: 8. In further embodiments, suitable polynucleotides encode polypeptides having at least 10, at least 12, at least 14, at least 16, at least 18, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100 , at least 125, at least 150, at least 175, at least 200, at least 225, at least 250, at least 275, at least 300, or more consecutive amino acids of SEQ ID NOs: 4 or 8.

[0081] Other suitable polynucleotides that may be modified to produce plants with elevated levels of glucan include homologs, paralogs, and/or orthologs of the Cal-1 T01 and Cal-1 T02 licheninase genes. Methods for identifying polynucleotides that are homologs, paralogs, and/or orthologs of a polynucleotide of interest are well known to one of skill in the art, as described herein.

[0082] Examples of suitable polynucleotides that are homologous, paralogous, or orthologous to Cal-1 T01 and Cal-1 T02 include without limitation, homologous, paralogous, or orthologous polynucleotides from maize (*Zea mays*), barley (*Hordeum vulgare*), rice (*Oryza sativa*), sorghum (*Sorghum bicolor*), foxtail millet (*Setaria italica*), sugar cane (*Saccharum spp.*), wheat (*Triticum spp.*), soy (*Glysine sp.*), cotton (*Gossypium sp.*), sugar beet (*Beta vulgaris*), sunflower (*Helianthus sp.*), miscanthus (*Miscanthus sp.*), giant miscanthus (*Miscanthus giganteus*), switchgrass (*Panicum virgatum*), grass (*Poaceae sp.*), rape (*Brassica napus*), giant reed (*Arundo donax*), reed canary grass (*Phalaris arundinacea*), sericea lespedeza (*Lespedeza cuneata*), millet (*Panicum miliaceum*), ryegrass (*Lolium sp.*), timothy-grass (*Phleum sp.*), kochia (*Kochia sp.*), kenaf (*Hibiscus cannabinus*), bahiagrass (*Paspalum sp.*), bermudagrass (*Cynodon dactylon*), pangolagrass (*Digitaria decumbens*), bluestem grass (*Andropogon sp.*), indiangrass (*Sorghastrum sp.*), bromegrass (*Bromus sp.*), elephant grass (*Pennisetum purpureum*), jatropha (*Jatropha sp.*), alfalfa (*Medicago sp.*), clover (*Trifolium*), sunn hemp (*Crotalaria juncea*), fescue (*Festuca sp.*), orchard grass (*Dactylis sp.*), purple false brome (*Brachypodium distachyon*), sesame (*Sesamum indicum*), poplar (*Populus trichocarpa*), spruce (*Picea sp.*), pine (*Pinaceae spp.*), willow (*Salix sp.*), eucalyptus (*Eucalyptus sp.*), castor oil plant (*Ricinus communis*), and palm tree (*Arecaceae sp.*).

[0083] In other embodiments, suitable polynucleotides that may be modified to produce plants with elevated levels of glucan include maize polynucleotides that are homologous to Cal-1 T01 and Cal-1 T02 include. For example, suitable polynucleotides include, without limitation, the genes listed in Table 1. Other suitable polynucleotides include *Oryza sativa, Sorghum bicolor, Brachypodium distachyon* and *Setaria italica* polynucleotides that are homologous to Cal-1 T01 and Cal-1 T02. For example, suitable polynucleotides include, without limitation, the genes listed in Table 2.

[0084] Suitable polynucleotides that may be modified to produce plants with elevated levels of glucan further include fragments of polynucleotides that encode licheninase polypeptides, polynucleotides that are complementary to polynucleotides that encode licheninase polypeptides, and fragments of polynucleotides that are complementary to polynucleotides that encode licheninase polypeptides.

Mutated polynucleotides

[0085] In certain embodiments, licheninase genes of the present disclosure are modified to reduce or inhibit the licheninase activity of the encoded polypeptide. Licheninase genes of the present disclosure may be modified to contain one or more mutations that encode licheninase polypeptides with reduced or inhibited licheninase activity. For example, Cal-1 T01 may be mutated to encode an amino acid substitution at one or both of its active site residues (*i.e.*, Glu 262 and Glu 318). In some embodiments, a mutated polynucleotide encodes a licheninase polypeptide containing an amino acid substitution at a position that is analogous to position 262 of SEQ ID NO: 4, the amino acid sequence of the polypeptide encoded by Cal-1 T01 (*i.e.*, at a position corresponding to position 262 in a homolog, ortholog, or paralog of Cal-1 T01). In other embodiments, a mutated polynucleotide encodes a Cal-1 T01 polypeptide containing an amino acid substitution at position 262. In still other embodiments, a mutated polynucleotide encodes a licheninase polypeptide containing an amino acid substitution at a position that is analogous to position 318 of SEQ ID NO: 4 (*i.e.*, at a position corresponding to position 318 in a homolog, ortholog, or paralog of Cal-1 T01).

[0086] In yet other embodiments, a mutated polynucleotide encodes a Cal-1 T01 polypeptide containing an amino acid substitution at position 318. In yet other embodiments, a mutated polynucleotide encodes a licheninase polypeptide containing an amino acid substitution at a position that is analogous to position 242 of SEQ ID NO: 8, the amino acid

sequence of the polypeptide encoded by Cal-1 T02 (*i.e.*, at a position corresponding to position 242 in a homolog, ortholog, or paralog of Cal-1 T02). In still other embodiments, a mutated polynucleotide encodes a Cal-1 T02 polypeptide containing an amino acid substitution at position 242. In further embodiments, a mutated polynucleotide encodes a licheninase polypeptide containing an amino acid substitution at a position that is analogous to position 298 of SEQ ID NO: 8 (*i.e.*, at a position corresponding to position 298 in a homolog, ortholog, or paralog of Cal-1 T02). In other embodiments, a mutated polynucleotide encodes a Cal-1 T02 polypeptide containing an amino acid substitution at position 298. The amino acid substitution may be any substitution that reduces or inhibits the licheninase activity of the encoded licheninase polypeptide. In certain preferred embodiments, the amino acid substitution is a glutamate (Glu) to lysine (Lys) substitution.

[0087] In further embodiments, the mutated polynucleotide encodes a licheninase polypeptide that contains one or more amino acid substitutions in regions other than the active site that reduce or inhibit the licheninase activity of the polypeptide. In still further embodiments, the mutated polynucleotides encode a truncated licheninase polypeptide having reduced or inhibited licheninase activity. Preferably, the truncated polypeptide lacks licheninase activity.

[0088] In yet further embodiments, the mutated polynucleotide contains a duplication, amplification, translocation, or inversion that reduces expression of the encoded polypeptide or that encodes a licheninase polypeptide that has reduced or inhibited licheninase activity. In other embodiments, the mutated polynucleotide contains a loss-of-function mutation and encodes a licheninase polypeptide that has reduced or inhibited licheninase activity.

Brown Midrib (BM) Polynucleotides

[0089] Certain embodiments of the present disclosure relate to plants exhibiting an increased saccharification yield, where the increased saccharification yield is the result of the combination of at least one modified licheninase polynucleotide encoding a polypeptide having reduced licheninase activity and at least one modified *brown midrib* (*bm*) polynucleotide encoding a polypeptide having reduced activity.

[0090] As used herein *brown midrib* (*bm*) genes are genes involved in lignin biosynthesis. Examples of *bm* genes include, without limitation, the maize *bm1* gene, the

maize *bm2* gene, the maize *bm3* gene, the maize *bm4* gene, homologs thereof, paralogs thereof, and orthologs thereof. Generally, reduced expression of at least one *bm* gene results in reduced and altered lignin content.

[0091] As used herein "saccharification yield" refers to the amount of oligosaccharides and/or monosaccharides produced by the saccharification of biomass derived from a plant of the present disclosure, or part thereof. Saccharification refers to the degradation of complex carbohydrates, such as starch, cellulose, and other plant polysaccharides, into simple sugars, such as oligosaccharides and/or monosaccharides. Any method of biomass saccharification known in the art may be used.

[0092] In certain preferred embodiments, plants of the present disclosure having reduced licheninase activity further having reduced expression of at least one *bm* gene. Preferably, the at least one *bm* gene is a *bm1* gene or a *bm3* gene. Without wishing to be bound by theory, it is believed that the *bm1* gene encodes cinnamyl alcohol dehydrogenase (CAD) and that the *bm3* gene encodes caffeic acid O-methyltransferase (COMT). Advantageously, it has been surprisingly shown that when a plant having reduced expression of a licheninase gene is crossed with a plant having reduced expression of either the *bm1* gene or the *bm3* gene, the resulting progeny produce a significantly higher saccharification yield than either parental plant alone (Fig. 13).

[0093] Accordingly, in certain embodiments, a mutant plant having a mutation in at least one licheninase gene further contains a mutation in at least one *bm1* gene or at least one *bm3* gene, where the plant having such mutations exhibit an increased saccharification yield compared to the saccharification yield in a corresponding plant lacking the mutation in the at least one licheninase gene and the mutation in at least one *bm1* gene or at least one *bm3* gene. Methods of generating plants with mutations in at least two genes are well known in the art and include those disclosed herein. Methods for measuring saccharification are also well known in the art.

[0094] In other embodiments, a plant containing an RNAi-inducing vector, where the vector generates RNAi against a licheninase gene, further contains an additional RNAi-inducing vector where the additional vector generates RNAi against a *bm1* gene or a *bm3* gene, and where the plant exhibits an increased saccharification yield compared to the

saccharification yield in a corresponding plant lacking the vectors generating RNAi against a licheninase genes and a *bm1* gene or *bm3* gene.

[0095] In other embodiments, a plant having reduced expression of at least one licheninase gene further contains reduced expression of at least one *bm1* gene or at least one *bm3* gene, where the plant exhibits an increased saccharification yield compared to the saccharification yield in a corresponding plant having reduced expression of the at least one licheninase gene and reduced expression of the at least one *bm1* gene or *bm3* gene. Any suitable method disclosed herein may be used to reduce expression of the at least one licheninase gene and the at least one *bm1* gene or at least one *bm3* gene.

[0096] Methods of measuring the saccharification yield of a plant are well known in the art and include those disclosed herein. In certain embodiments, the saccharification yield of a plant having reduced expression of a licheninase gene and reduced expression of a *bm* gene is increased by at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 100%, at least 125%, at least 150%, at least 175%, at least 200%, at least 225%, at least 250%, at least 275%, at least 300%, at least 325%, at least 350%, at least 375%, at least 400%, at least 425%, at least 450%, at least 475%, at least 500%, at least 525%, at least 550%, at least 575%, or more, as compared to the saccharification yield of a corresponding plant lacking reduced expression of a licheninase gene and reduced expression of a *bm* gene. In certain embodiments, an increased saccharification yield results in an increased amounts of glucose.

Methods of Decreasing Gene Expression / Polypeptide Levels in a Plant

[0097] Further aspects of the present disclosure relate to producing plants with elevated levels of glucan by decreasing the expression of at least one licheninase gene in the plant. As used herein, "decreasing" the level of expression of a gene includes reducing or inhibiting the expression of a gene. The level of expression of a gene may be assessed by measuring the level of mRNA encoded by the gene, and/or by measuring the level or activity of the polypeptide encoded by the gene.

[0098] Gene expression can be decreased using any number of techniques well known in the art. For example, gene expression may by decreased by genetically modifying the genome

of a plant through, for example, homologous recombination to replace the wild-type version of a gene of interest with a modified version that has reduced or inhibited expression. Methods of genetically modifying plants are well known in the art.

[0099] Another method of decreasing expression is through sense suppression (also known as co-suppression). Introduction of expression cassettes in which a nucleic acid is configured in the sense orientation with respect to the promoter has been shown to be an effective means by which to block the transcription of target genes. For an example of the use of this method to modulate expression of endogenous genes see, Napoli et al, *The Plant Cell* 2:279-289, 1990; Flavell, *Proc. Natl. Acad. Sci, USA* 91:3490-3496, 1994; Kooter and Moi, *Current Opin. Biol.* 4:166-171, 1993; and U.S. Patents Nos. 5,034,323, 5,231,020, and 5,283,184.

[0100] Generally, where inhibition of expression is desired, some transcription of the introduced sequence occurs. The effect may occur where the introduced sequence contains no coding sequence *per se*, but only intron or untranslated sequences homologous to sequences present in the primary transcript of the endogenous sequence. The introduced sequence generally will be substantially identical to the endogenous sequence intended to be repressed. This minimal identity will typically be greater than about 65%, but a higher identity can exert a more effective repression of expression of the endogenous sequences. In some embodiments, sequences with substantially greater identity are used, *e.g.*, at least about 80%, at least about 95%, or 100% identity are used. As with antisense regulation, further discussed below, the effect can be designed and tested to apply to any other proteins within a similar family of genes exhibiting homology or substantial homology.

[0101] For sense suppression, the introduced sequence in the expression cassette, needing less than absolute identity, also need not be full length, relative to either the primary transcription product or fully processed rnRNA. Furthermore, the introduced sequence need not have the same intron or exon pattern, and identity of non-coding segments will be equally effective. In some embodiments, a sequence of the size ranges noted above for antisense regulation is used, *i.e.*, 30-40, or at least about 20, 50, 100, 200, 500, or more nucleotides.

RNAi

[0102] Endogenous gene expression may also be decreased by means of RNA interference (RNAi) (and indeed co-suppression can be considered a type of RNAi), which uses a double-stranded RNA having a sequence identical or similar to the sequence of the target gene. As used herein RNAi, includes the use of micro RNA, such as artificial miRNA to suppress expression of a gene. RNAi is the phenomenon in which when a double-stranded RNA having a sequence identical or similar to that of the target gene is introduced into a cell, the expressions of both the inserted exogenous gene and target endogenous gene are suppressed. The double-stranded RNA may be formed from two separate complementary RNAs or may be a single RNA with internally complementary sequences that form a double-stranded RNA. Although complete details of the mechanism of RNAi are still unknown, it is considered that the introduced double-stranded RNA is initially cleaved into small fragments, which then serve as indexes of the target gene in some manner, thereby degrading the target gene. RNAi is known to be also effective in plants (see, *e.g.*, Chuang, C. F. & Meyerowitz, E. M., *Proc. Natl. Acad. Sd. USA* 97: 4985, 2000; Waterhouse et al, *Proc. Natl. Acad. Sci. USA* 95:13959-13964, 1998; Tabara et al. *Science* 282:430-431, 1998; Matthew, *Comp Fund Genom* 5: 240-244, 2004; Lu, et al, *Nucleic Acids Res.* 32(21):171, 2004).

[0103] Thus, in some embodiments, reduction or inhibition of gene expression is achieved using RNAi techniques. For example, to achieve reduction or inhibition of the expression of a DNA encoding a protein using RNAi, a double-stranded RNA having the sequence of a DNA encoding the protein, or a substantially similar sequence thereof (including those engineered not to translate the protein) or fragment thereof, is introduced into a plant of interest. As used herein, RNAi and dsRNA both refer to gene-specific silencing that is induced by the introduction of a double-stranded RNA molecule, see *e.g.*, U.S. Pat. Nos. 6,506,559 and 6,573,099, and includes reference to a molecule that has a region that is double-stranded, *e.g.*, a short hairpin RNA molecule. The resulting plants may then be screened for a phenotype associated with the reduced expression of the target gene, *e.g.*, elevated glucan, and/or by monitoring steady-state RNA levels for transcripts encoding the protein. Although the genes used for RNAi need not be completely identical to the target gene, they may be at least 70%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more identical to the target gene sequence. See, *e.g.*, U.S. Patent Application Publication No. 2004/0029283. The constructs encoding an RNA molecule with a stem-loop

structure that is unrelated to the target gene and that is positioned distally to a sequence specific for the gene of interest may also be used to inhibit target gene expression. See, *e.g.*, U.S. Patent Application Publication No. 2003/0221211.

[0104] The RNAi polynucleotides may encompass the full-length target RNA or may correspond to a fragment of the target RNA. In some cases, the fragment will have fewer than 100, 200, 300, 400, or 500 nucleotides corresponding to the target sequence. In addition, in some aspects, these fragments are at least, *e.g.*, 50, 100, 150, 200, or more nucleotides in length. Interfering RNAs may be designed based on short duplexes (*i.e.*, short regions of double-stranded sequences). Typically, the short duplex is at least about 15, 20, or 25-50 nucleotides in length (*e.g.*, each complementary sequence of the double stranded RNA is 15-50 nucleotides in length), often about 20-30 nucleotides, *e.g.*, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length. In some cases, fragments for use in RNAi will correspond to regions of a target protein that do not occur in other proteins in the organism or that have little similarity to other transcripts in the organism, *e.g.*, selected by comparison to sequences in analyzing publicly-available sequence databases. Similarly, RNAi fragments may be selected for similarity or identity with a conserved sequence of a gene family of interest, such as those described herein, so that the RNAi targets multiple different gene transcripts containing the conserved sequence.

[0105] RNAi may be introduced into a cell as part of a larger DNA construct. Often, such constructs allow stable expression of the RNAi in cells after introduction, *e.g.*, by integration of the construct into the host genome. Thus, expression vectors that continually express RNAi in cells transfected with the vectors may be employed for this disclosure. For example, vectors that express small hairpin or stem-loop structure RNAs, or precursors to microRNA, which get processed in vivo into small RNAi molecules capable of carrying out gene-specific silencing (Brummelkamp et al, *Science* 296:550-553, 2002; and Paddison, et al., *Genes & Dev.* 16:948-958, 2002) can be used. Post-transcriptional gene silencing by double-stranded RNA is discussed in further detail by Hammond et al., *Nature Rev Gen* 2: 110-119, 2001; Fire et al., *Nature* 391: 806-811, 1998; and Timmons and Fire, *Nature* 395: 854, 1998.

[0106] Methods for selection and design of sequences that generate RNAi are well known in the art (*e.g.,* U.S. Pat. Nos. 6,506,559; 6,511,824; and 6,489,127).

[0107] One of skill in the art will recognize that using technology based on specific nucleic acid sequences (*e.g.*, antisense or sense suppression technology), families of homologous genes can be suppressed with a single sense or antisense, discussed below, transcript. For instance, if a sense or antisense transcript is designed to have a sequence that is conserved among a family of genes, then multiple members of a gene family can be suppressed. Conversely, if the goal is to only suppress one member of a homologous gene family, then the sense or antisense transcript should be targeted to sequences with the most variation between family members.

[0108] The term "target gene" or "target sequences", refers to a gene targeted for reduced expression. In one format, one or more different genes can be inhibited using the same interfering RNA. For example, some or all licheninase genes in a plant may be targeted by using an RNAi that is designed to a conserved region of the licheninase gene. In other aspects, an individual licheninase gene may be targeted by using an RNAi that is specific for that gene.

Antisense and ribozyme suppression

[0109] A reduction or inhibition of gene expression in a plant of a target gene may also be obtained by introducing into plants antisense constructs based on a target gene nucleic acid sequence. For antisense suppression, a target sequence is arranged in reverse orientation relative to the promoter sequence in the expression vector. The introduced sequence need not be a full length cDNA or gene, and need not be identical to the target cDNA or a gene found in the plant variety to be transformed. Generally, however, where the introduced sequence is of shorter length, a higher degree of homology to the native target sequence is used to achieve effective antisense suppression. In some aspects, the introduced antisense sequence in the vector will be at least 30 nucleotides in length, and improved antisense suppression will typically be observed as the length of the antisense sequence increases. In some aspects, the length of the antisense sequence in the vector will be greater than 100 nucleotides. Transcription of an antisense construct as described results in the production of RNA molecules that are the reverse complement of mRNA molecules transcribed from an endogenous target gene. Suppression of a target gene expression can also be achieved using a ribozyme. The production and use of ribozymes are disclosed in U.S. Pat. Nos. 4,987,071 and 5,543,508.

Mutagenesis

[0110] In other embodiments, mutagenesis approaches may be used to disrupt or "knockout" the expression of a target gene using either chemical or insertional mutagenesis, or irradiation. In certain embodiments, the mutagenesis results in a partial deletion of the target gene. In other embodiments, the mutagenesis results in a complete deletion of the target gene.

[0111] One method of mutagenesis and mutant identification is known as TILLING (for "Targeting Induced Local Lesions in Genomes"). In this method, mutations are induced in the seed of a plant of interest, for example, using ethane methyl sulfonate (EMS) treatment (Hoffman, *Mutation Research* 75(1): 63-129, 1980) or fast neutron bombardment (Li et al., *Plant Journal* 27(3):235-242, 2001). The resulting plants are grown and self-fertilized, and the progeny are assessed. For example, the plants may be assed using PCR to identify whether a mutated plant has a mutation in a target gene, *e.g.*, that reduces expression of a target gene, or by evaluating whether the plant has increased levels of cell wall glucan content in a part of the plant that expressed the target gene, such as leaf tissue. TILLING can identify mutations that may alter the expression of specific genes or the activity of proteins encoded by these genes (see, Colbert et al. *Plant Physiol* 126:480-484, 2001; McCallum et al. *Nature Biotechnology* 18:455-457, 2000).

[0112] Another method for reducing or inhibiting the expression of a target gene is by insertion mutagenesis using the T-DNA of *Agrobacterium tumefaciens*, or transposons (see Winkler et al., *Methods Mol. Biol.* 82:129-136, 1989, and Martienssen *Proc. Natl. Acad. Sci.* 95:2021-2026, 1998). After generating the insertion mutants, the mutants can be screened to identify those containing the insertion in a target gene. Mutants containing a single mutation event at the desired gene may be crossed to generate homozygous plants for the mutation (see, Koncz et al. *Methods in Arabidopsis Research.* World Scientific, 1992).

[0113] Another method to disrupt a target gene is by use of the cre-lox system (for example, as described in U.S. Pat. No. 5,658,772).

[0114] In some aspects, the disclosure includes mutation of at least one licheninase gene. Examples of genes that may be disrupted by mutagenesis include, without limitation, Cal-1

T01 (SEQ ID NO: 3), Cal-1 T02 (SEQ ID NO: 7), and homologs, paralogs, or orthologs thereof..

Plants having multiple target genes inhibited

[0115] Expression of at least two target genes may be reduced or inhibited in a plant as described herein. As explained above, such plants can be generated by performing a molecular manipulation that targets multiple related gene targets in a plant, *e.g.*, using an RNAi to a conserved region to inactivate all of the target genes. Such plants can also be obtained by breeding plants each having individual mutations that inactivate different target genes to obtain progeny plants that are inactivated in all of the desired target genes. For example, to obtain a maize plant in which two target genes are inactivated, one of skill can target the genes using RNAi developed to a region that is conserved in both of the maize target genes, or target the genes individually, and breed the resulting mutant plants.

Expression of target gene inhibitors

[0116] Expression cassettes containing polynucleotides that encode target gene expression inhibitors, *e.g.*, an antisense or siRNA, can be constructed using methods well known in the art. Constructs include regulatory elements, including promoters and other sequences for expression and selection of cells that express the construct. Typically, plant transformation vectors include one or more cloned plant coding sequences (genomic or cDNA) under the transcriptional control of 5' and 3' regulatory sequences and a dominant selectable marker. Such plant transformation vectors typically also contain a promoter (*e.g.*, a regulatory region controlling inducible or constitutive, environmentally-or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, an RNA processing signal (such as intron splice sites), a transcription termination site, and/or a polyadenylation signal.

[0117] Examples of constitutive plant promoters which may be useful for expressing a target gene sequence include: the cauliflower mosaic virus (CaMV) 35S promoter, which confers constitutive, high-level expression in most plant tissues (see, *e.g.*, Odel et al., *Nature* 313:810, 1985); the nopaline synthase promoter (An et al., *Plant Physiol.* 88:547, 1988); and the octopine synthase promoter (Fromm et al., *Plant Cell* 1:977, 1989).

[0118] Additional constitutive regulatory elements including those for efficient expression in monocots also are known in the art, for example, the pEmu promoter and promoters based on the rice Actin-1 5' region (Last et al., *Theor. Appl. Genet.* 81:581, 1991; Mcelroy et al., *Mol Gen. Genet.* 231:150, 1991; and Mcelroy et al., *Plant Cell* 2:163, 1990). Chimeric regulatory elements, which combine elements from different genes, also can be useful for ectopically expressing a nucleic acid molecule encoding an INDl polynucleotide (Comai et al., *Plant Mol Biol.* 15:373, 1990).

[0119] Other examples of constitutive promoters include the 1'- or T- promoter derived from T-DNA of *Agrobacterium tumafaciens* (see, *e.g.*, O'Grady, *Plant Mol. Biol* 29:99-108, 1995); actin promoters, such as the *Arabidopsis* actin gene promoter (see, *e.g.*, Huang, *Plant Mol. Biol.* 33:125-139, 1997); alcohol dehydrogenase (Adh) gene promoters (see, *e.g.*, Millar, *Plant Mol Biol.* 31:897-904, 1996); ACTIl from *Arabidopsis* (Huang et al., *Plant Mol. Biol* 33:125-139, 1996), CatS from *Arabidopsis* (GenBank No. U43147, Zhong et al., *Mol Gen. Genet.* 251:196-203, 1996), the gene encoding stearoyl-acyl carrier protein desaturase from *Brassica napus* (Genbank No. X74782, Solocombe et al., *Plant Physiol.* 104:1167-1176, 1994), GPcI from maize (GenBank No. X15596, Martinez et al., *J. Mol. Biol* 208:551-565, 1989), Gpc2 from maize (GenBank No. U45855, Manjunath et al., *Plant Mol Biol.* 33:97-112, 1997), and other transcription initiation regions from various plant genes known in the art. See also Holtorf *Plant Mol Biol.* 29:637-646, 1995.

[0120] A variety of plant gene promoters that regulate gene expression in response to various environmental, hormonal, chemical, developmental signals, and in a tissue-active manner are known in the art. Examples of environmental conditions that may affect transcription by inducible promoters include anaerobic conditions, elevated temperature, drought, or the presence of light. Examples of environmental promoters include drought-inducible promoter of maize; the cold, drought, and high salt inducible promoter from potato (Kirch, *Plant Mol. Biol.* 33:897-909, 1997). Plant promoters that are inducible upon exposure to plant hormones, such as auxins, may also be employed. For example, the invention can use the auxin response elements El promoter fragment (AuxREs) in the soybean (Glycine max L.) (Liu, *Plant Physiol.* 115:397-407, 1997); the auxin-responsive *Arabidopsis* GST6 promoter (also responsive to salicylic acid and hydrogen peroxide) (Chen, *Plant J.* 10: 955-966, 1996); the auxin-inducible parC promoter from tobacco (Sakai, 37:906-913, 1996); a plant biotin response element (Streit, *Mol. Plant Microbe Interact.* 10:933-937, 1997); and,

the promoter responsive to the stress hormone abscisic acid (Sheen, *Science* 274:1900-1902, 1996).

[0121] Plant promoters which are inducible upon exposure to chemicals reagents that can be applied to the plant, such as herbicides or antibiotics, may also be used in vectors as described herein. For example, the maize In2 2 promoter, activated by benzenesulfonamide herbicide safeners, can be used; application of different herbicide safeners induces distinct gene expression patterns, including expression in the root, hydathodes, and the shoot apical meristem. Other promoters, *e.g.*, a tetracycline inducible promoter; a salicylic acid responsive element promoter, promoters containing copper-inducible regulatory elements; promoters containing ecdysone inducible regulatory elements; heat shock inducible promoters, a nitrate-inducible promoter, or a light-inducible promoter may also be used.

[0122] In some aspects, the plant promoter may direct expression of a polynucleotide of the disclosure in a specific tissue (tissue-specific promoters), such as a leaf or a stem. Tissue specific promoters are transcriptional control elements that are only active in particular cells or tissues at specific times during plant development, such as in vegetative tissues or reproductive tissues. Examples of tissue-specific promoters include promoters that initiate transcription primarily in certain tissues, such as vegetative tissues, *e.g.*, roots or leaves, or reproductive tissues, such as fruit, ovules, seeds, pollen, pistols, flowers, or any embryonic tissue. Other examples are promoters that direct expression specifically to cells and tissues with secondary cell wall deposition, such as xylem and fibers.

[0123] Plant expression vectors may also include RNA processing signals that may be positioned within, upstream or downstream of the coding sequence. In addition, the expression vectors may include additional regulatory sequences from the 3 '-untranslated region of plant genes, *e.g.*, a 3' terminator region to increase mRNA stability of the mRNA, such as the PI-II terminator region of potato or the octopine or nopaline synthase 3' terminator regions.

[0124] Plant expression vectors routinely also include dominant selectable marker genes to allow for the ready selection of transformants. Such genes include those encoding antibiotic resistance genes (*e.g.*, resistance to hygromycin, kanamycin, bleomycin, G418, streptomycin or spectinomycin), herbicide resistance genes (*e.g.*, phosphinothricin acetyltransferase), and genes encoding positive selection enzymes (*e.g.,* mannose isomerase).

[0125] Once an expression cassette containing a polynucleotide encoding an inhibitor of the expression of a target gene, *e.g.*, an antisense or siRNA, has been constructed, standard techniques may be used to introduce the polynucleotide into a plant in order to modify the target gene activity and accordingly, the levels of glucan in the plant or plant part in which the target gene is expressed. See protocols described in Ammirato et al., *Handbook of Plant Cell Culture-Crop Species*. Macmillan Publ. Co, 1984; Shimamoto et al., *Nature* 338:274-276, 1989; Fromm et al., *Bio/Technology* 8:833-839, 1990; and Vasil et al., *Bio/Technology* 8:429-434, 1990.

[0126] Transformation and regeneration of plants is known in the art, and the selection of the most appropriate transformation technique will be determined by the practitioner. Suitable methods may include, but are not limited to: electroporation of plant protoplasts; liposome-mediated transformation; polyethylene glycol (PEG) mediated transformation; transformation using viruses; micro-injection of plant cells; micro-projectile bombardment of plant cells; vacuum infiltration; and *Agrobacterium tumeficiens* mediated transformation. Transformation means introducing a nucleic acid sequence in a plant in a manner to cause stable or transient expression of the sequence. Examples of these methods in various plants include: U.S. Pat. Nos. 5,571,706; 5,677,175; 5,510,471; 5,750,386; 5,597,945; 5,589,615; 5,750,871; 5,268,526; 5,780,708; 5,538,880; 5,773,269; 5,736,369; and 5,610,042.

[0127] Following transformation, plants are preferably selected using a dominant selectable marker incorporated into the transformation vector. Typically, such a marker will confer antibiotic or herbicide resistance on the transformed plants or the ability to grow on a specific substrate, and selection of transformants can be accomplished by exposing the plants to appropriate concentrations of the antibiotic, herbicide, or substrate.

Sequence Homologs / Orthologs / Paralogs

[0128] As used herein, "homologs" are polypeptide or polynucleotide sequences that share a significant degree of sequence identity or similarity. Sequences that are homologs are referred to as being "homologous" to each other. Homologs include sequences that are orthologs or paralogs.

[0129] As used herein, "orthologs" are evolutionarily related polypeptide or polynucleotide sequences in different species that have similar sequences and functions, and

that develop through a speciation event. Sequences that are orthologs are referred to as being "orthologous" to each other.

[0130] As used herein, "paralogs" are evolutionarily related polypeptide or polynucleotide sequences in the same organism that have similar sequences and functions, and that develop through a gene duplication event. Sequences that are paralogs are referred to as being "paralogous" to each other.

Methods of Identification of Homologous Sequences / Sequence Identity and Similarity

[0131] Several different methods are known to those of skill in the art for identifying homologous sequences, including phylogenetic methods, sequence similarity analysis, and hybridization methods.

Phylogenetic methods

[0132] Phylogenetic trees may be created for a gene family by using a program such as CLUSTAL (Thompson et al. *Nucleic Acids Res.* 22: 4673-4680, 1994; Higgins et al. *Methods Enzymol* 266: 383-402,1996) or MEGA (Tamura et al. *Mol. Biol. & Evo.* 24:1596-1599,2007). Once an initial tree for genes from one species is created, potential orthologous sequences can be placed in the phylogenetic tree and their relationships to genes from the species of interest can be determined. Evolutionary relationships may also be inferred using the Neighbor-Joining method (Saitou & Nei, *Mol. Biol. & Evo.* 4:406-425,1987). Homologous sequences may also be identified by a reciprocal BLAST strategy. Evolutionary distances may be computed using the Poisson correction method (Zuckerkandl & Pauling, pp. 97-166 in *Evolving Genes and Proteins*, edited by V. Bryson and H.J. Vogel. Academic Press, New York, 1965).

[0133] In addition, evolutionary information may be used to predict gene function. Functional predictions of genes can be greatly improved by focusing on how genes became similar in sequence (*i.e.*, by evolutionary processes) rather than on the sequence similarity itself (Eisen, *Genome Res.* 8: 163-167, 1998). Many specific examples exist in which gene function has been shown to correlate well with gene phylogeny (Eisen, *Genome Res.* 8: 163-167, 1998). By using a phylogenetic analysis, one skilled in the art would recognize that the ability to deduce similar functions conferred by closely-related polypeptides is predictable.

[0134] When a group of related sequences are analyzed using a phylogenetic program such as CLUSTAL, closely related sequences typically cluster together or in the same clade (a group of similar genes). Groups of similar genes can also be identified with pair-wise BLAST analysis (Feng and Doolittle, *J. Mol. Evol.* 25: 351-360, 1987). Analysis of groups of similar genes with similar function that fall within one clade can yield sub-sequences that are particular to the clade. These sub-sequences, known as consensus sequences, can be used not only to define the sequences within each clade, but to define the functions of these genes; genes within a clade may contain paralogous sequences, or orthologous sequences that share the same function (see also, for example, *Mount, Bioinformatics: Sequence and Genome Analysis*, p. 543. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2001).

[0135] To find sequences that are homologous to a reference sequence, BLAST nucleotide searches can be performed with the BLASTN program, score=100, wordlength=12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the disclosure. BLAST protein searches can be performed with the BLASTX program, score=50, wordlength=3, to obtain amino acid sequences homologous to a protein or polypeptide of the disclosure. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul et al. (*Nucleic Acids Res.* 25:3389, 1997). Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul et al. (1997) supra. When utilizing BLAST, Gapped BLAST, or PSI-BLAST, the default parameters of the respective programs (*e.g.*, BLASTN for nucleotide sequences, BLASTX for proteins) can be used.

Sequence Alignment / Sequence Similarity and Identity Analysis

[0136] Methods for the alignment of sequences and for the analysis of similarity and identity of polypeptide and polynucleotide sequences are well known in the art.

[0137] As used herein "sequence identity" refers to the percentage of residues that are identical in the same positions in the sequences being analyzed. As used herein "sequence similarity" refers to the percentage of residues that have similar biophysical / biochemical characteristics in the same positions (*e.g.*, charge, size, hydrophobicity) in the sequences being analyzed.

[0138] Methods of alignment of sequences for comparison are well-known in the art, including manual alignment and computer assisted sequence alignment and analysis. This latter approach is a preferred approach in the present disclosure, due to the increased throughput afforded by computer assisted methods. As noted below, a variety of computer programs for performing sequence alignment are available, or can be produced by one of skill.

[0139] The determination of percent sequence identity and/or similarity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms include the algorithm of Myers and Miller, *CABIOS* 4:11-17 (1988); the local homology algorithm of Smith et al., *Adv. Appl. Math.* 2:482 (1981); the homology alignment algorithm of Needleman and Wunsch, J. *Mol. Biol.* 48:443-453 (1970); the search-for-similarity-method of Pearson and Lipman, *Proc. Natl. Acad. Sci.* 85:2444-2448 (1988); the algorithm of Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 87:2264-2268 (1990), modified as in Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 90:5873-5877 (1993).

[0140] Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity and/or similarity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, Calif.); the AlignX program, version10.3.0 (Invitrogen, Carlsbad, CA); and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wis., USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins et al. *Gene* 73:237-244 (1988); Higgins et al. *CABIOS* 5:151-153 (1989); Corpet et al. *Nucleic Acids Res.* 16:10881-90 (1988); Huang et al. *CABIOS* 8:155-65 (1992); and Pearson et al. *Meth. Mol. Biol.* 24:307-331 (1994). The BLAST programs of Altschul et al. *J. Mol. Biol.* 215:403-410 (1990) are based on the algorithm of Karlin and Altschul (1990) supra.

Hybridization methods

[0141] Polynucleotides homologous to a reference sequence can be identified by hybridization to each other under stringent or under highly stringent conditions. Single stranded polynucleotides hybridize when they associate based on a variety of well

characterized physical-chemical forces, such as hydrogen bonding, solvent exclusion, base stacking and the like. The stringency of a hybridization reflects the degree of sequence identity of the nucleic acids involved, such that the higher the stringency, the more similar are the two polynucleotide strands. Stringency is influenced by a variety of factors, including temperature, salt concentration and composition, organic and non-organic additives, solvents, etc. present in both the hybridization and wash solutions and incubations (and number thereof), as described in more detail in references cited below (*e.g.*, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989 ("Sambrook"); Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology*, vol. 152 Academic Press, Inc., San Diego, Calif., 1987 ("Berger and Kimmel"); and Anderson and Young, "Quantitative Filter Hybridisation." In: Hames and Higgins, ed., *Nucleic Acid Hybridisation, A Practical Approach.* Oxford, TRL Press, 73-111, 1985).

[0142] Encompassed by the disclosure are nucleic acid sequences that are capable of hybridizing to the disclosed nucleic acid sequences, including any polynucleotide within the Sequence Listing, and fragments thereof under various conditions of stringency (see, for example, Wahl and Berger, *Methods Enzymol.* 152: 399-407,1987; and Kimmel, *Methods Enzymo.* 152: 507-511, 1987). In addition to the nucleotide sequences in the Sequence Listing, full length cDNA, homologs, orthologs, and paralogs of the present nucleotide sequences may be identified and isolated using well-known polynucleotide hybrization methods.

[0143] With regard to hybridization, conditions that are highly stringent, and means for achieving them, are well known in the art. See, for example, Sambrook et al. (1989) (supra); Berger and Kimmel (1987) pp. 467-469 (supra); and Anderson and Young (1985)(supra).

[0144] Hybridization experiments are generally conducted in a buffer of pH between 6.8 to 7.4, although the rate of hybridization is nearly independent of pH at ionic strengths likely to be used in the hybridization buffer (Anderson and Young (1985) (supra)). In addition, one or more of the following may be used to reduce non-specific hybridization: sonicated salmon sperm DNA or another non-complementary DNA, bovine serum albumin, sodium pyrophosphate, sodium dodecylsulfate (SDS), polyvinyl-pyrrolidone, ficoll and Denhardt's solution. Dextran sulfate and polyethylene glycol 6000 act to exclude DNA from solution,

thus raising the effective probe DNA concentration and the hybridization signal within a given unit of time. In some instances, conditions of even greater stringency may be desirable or required to reduce non-specific and/or background hybridization. These conditions may be created with the use of higher temperature, lower ionic strength and higher concentration of a denaturing agent such as formamide.

[0145] Stringency conditions can be adjusted to screen for moderately similar fragments such as homologous sequences from distantly related organisms, or to highly similar fragments such as genes that duplicate functional enzymes from closely related organisms. The stringency can be adjusted either during the hybridization step or in the post-hybridization washes. Salt concentration, formamide concentration, hybridization temperature and probe lengths are variables that can be used to alter stringency. As a general guideline, high stringency is typically performed at T_m-5°C to T_m-20°C, moderate stringency at T_m-20°C to T_m-35°C and low stringency at T_m-35°C to T_m-50° C for duplex >150 base pairs. Hybridization may be performed at low to moderate stringency (25-50°C below T_m), followed by post-hybridization washes at increasing stringencies. Maximum rates of hybridization in solution are determined empirically to occur at T_m-25°C for DNA-DNA duplex and T_m-15°C for RNA-DNA duplex. Optionally, the degree of dissociation may be assessed after each wash step to determine the need for subsequent, higher stringency wash steps.

[0146] High stringency conditions may be used to select for nucleic acid sequences with high degrees of identity to the disclosed sequences. An example of stringent hybridization conditions obtained in a filter-based method such as a Southern or Northern blot for hybridization of complementary nucleic acids that have more than 100 complementary residues is about 5°C to 20°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH.

[0147] Hybridization and wash conditions that may be used to bind and remove polynucleotides with less than the desired homology to the nucleic acid sequences or their complements that encode the present transcription factors include, for example: 6X SSC and 1% SDS at 65°C; 50% formamide, 4X SSC at 42°C; 0.5X SSC to 2.0 X SSC, 0.1% SDS at 50°C to 65°C; or 0.1X SSC to 2X SSC, 0.1% SDS at 50°C - 65°C; with a first wash step of, for example, 10 minutes at about 42°C with about 20% (v/v) formamide in 0.1X SSC, and

with, for example, a subsequent wash step with 0.2 X SSC and 0.1% SDS at 65°C for 10, 20 or 30 minutes.

[0148] For identification of less closely related homologs, wash steps may be performed at a lower temperature, *e.g.*, 50° C. An example of a low stringency wash step employs a solution and conditions of at least 25°C in 30 mM NaCl, 3 mM trisodium citrate, and 0.1% SDS over 30 min. Greater stringency may be obtained at 42°C in 15 mM NaCl, with 1.5 mM trisodium citrate, and 0.1% SDS over 30 min. Wash procedures will generally employ at least two final wash steps. Additional variations on these conditions will be readily apparent to those skilled in the art (see, for example, U.S. Patent Application Publication No. 2001/0010913).

[0149] If desired, one may employ wash steps of even greater stringency, including conditions of 65°C -68°C in a solution of 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS, or about 0.2X SSC, 0.1% SDS at 65° C and washing twice, each wash step of 10, 20 or 30 min in duration, or about 0.1 X SSC, 0.1% SDS at 65° C and washing twice for 10, 20 or 30 min. Hybridization stringency may be increased further by using the same conditions as in the hybridization steps, with the wash temperature raised about 3°C to about 5°C, and stringency may be increased even further by using the same conditions except the wash temperature is raised about 6°C to about 9°C.

[0150] Polynucleotide probes may be prepared with any suitable label, including a fluorescent label, a colorimetric label, a radioactive label, or the like. Labeled hybridization probes for detecting related nucleic acid sequences may be produced, for example, by oligolabeling, nick translation, end-labeling, or PCR amplification using a labeled nucleotide.

Methods of Producing Plants with Elevated Levels of Glucan

[0151] Methods for producing a plant with elevated levels of glucan are described herein. In some aspects, plants having elevated levels of glucan may be produced by inducing one or more mutations in one or more licheninase genes, or by reducing the gene expression of one or more licheninase genes. Additionally, plants having elevated levels of glucan may be produced by reducing the expression of one or more polypeptides encoded by one or more licheninase genes. In other embodiments, plants having elevated levels of glucan may are produced by reducing the gene expression in a plant of one or more licheninase genes that are

homologous, paralogous, or orthologous to the licheninase genes having the nucleic acid sequences of SEQ ID NOs: 3 and 7. Moreover, plants having elevated levels of glucan may be produced by reducing the expression in a plant of one or more polypeptides encoded by one or more licheninase genes that are homologous, paralogous, or orthologous to the licheninase genes having the nucleic acid sequences of SEQ ID NOs: 3 and 7. Methods for determining sequences homologous, paralogous, or orthologous to a sequence of interest are provided herein. Expression of a target gene or polypeptide may be reduced by any method provided herein for decreasing gene and/or polypeptide expression.

Methods of Evaluating Plants with Elevated Levels of Glucan

[0152] After a plant has been altered to potentially elevate the levels of glucan, one or more parts of the plants may be evaluated to determine the level of one or more target gene expression in a part of the plant that expresses the target gene(s), *e.g.*, by evaluating the level of mRNA or protein of the target gene(s), or determining the levels of glucan in the plants. These analyses can be performed using any number of methods known in the art.

[0153] Levels of glucan can be measured. For example, cell walls are prepared from plant material. Several methods are known, in the simplest method, the plant material is ground and extracted repeatedly with 96% and 70% ethanol. The resulting 'alcohol insoluble residue' is highly enriched in cell wall material. The sample is dried and resuspended in buffer at neutral pH. An aliquot of the sample is destarched by treatment with 50 µg/1mL alpha-amylase at 80°C for 20 min. Following destarching of cell wall material, the matrix polysaccharide composition is determined by acid hydrolysis with 2 M trifluoroacetic acid. The polysaccharide content of acid hydrolysis treated detstarched cell wall material can be determined in several different ways, *e.g.* by gas chromatography or HPLC on an appropriate column, or mass spectrometry.

[0154] Plants selected for elevated levels of glucan may further be evaluated to further confirm that the plants provide for improved yield during a saccharification or fermentation process using material from the plant. For example, plant material from a plant with elevated levels of glucan can be compared to plant material from plants that that do not have elevated levels of glucan in a saccharification and/or fermentation process as described below.

Methods of Saccharification Biomass / Making Fermentation Product

[0155] Plants that exhibit elevated levels of glucan can be used in a variety of methods. In some embodiments, biomass from plants having elevated levels of glucan is degraded into oligosaccharides and/or monosaccharides. In some embodiments, biomass from plants having elevated levels of glucan is degraded into oligosaccharides and/or monosaccharides, and the oligosaccharides and/or monosaccharides are fermented to produce a biofuel and/or commodity chemical.

[0156] Examples of biofuels and/or commodity chemicals include, without limitation, hydrocarbons, such as methane, ethane, ethane, ethyne, propane, propene, propyne, cyclopropane, allene, butane, isobutene, butane, butyne, cyclobutane, methylcyclopropane, butadiene, pentane, isopentane, neopentane, pentene, pentyne, cyclopentane, methylcyclobutane, ethylcyclopropane, pentadiene, isoprene, hexane, hexane, hexyne, cyclohexane, methylcyclopentane, ethylcyclobutane, propylcyclopropane, hexadiene, heptane, heptene, heptyne, cycloheptane, methylcyclohexane. heptadiene, octane, octane, octyne, cyclooctane, octadiene, nonane, nonene, nonyne, cyclononane, nonadiene, decane, decene, decyne, cyclodecane, and decadiene; hydrocarbon derivatives, such as alcohols (*e.g.*, arabinitol, butanol, ethanol, glycerol, methanol, 1,3-propanediol, sorbitol, and xylitol), organic acids (*e.g.*, acetic acid, adipic acid, ascorbic acid, citric acid, 2,5-diketo-D-gluconic acid, formic acid, fumaric acid, glucaric acid, gluconic acid, glucuronic acid, glutaric acid, 3-hydroxypropionic acid, itaconic acid, lactic acid, malic acid, malonic acid, oxalic acid, propionic acid, succinic acid, and xylonic acid), esters, ketones (*e.g.*, acetone), aldehydes (*e.g.*, furfural), amino acids (*e.g.*, aspartic acid, glutamic acid, glycine, lysine, serine, and threonine), and gases (*e.g.*, carbon dioxide and carbon monoxide); and lipids.

[0157] Plant material from a plant having elevated levels of glucan may be subjected to a saccharification procedure. A first step in a saccharification of biomass process is typically a "pretreatment" step. Many different pretreatment procedures may be used and are known in the art, including dilute acid or alkali treatment, steam explosion or ionic liquid treatments. As the beneficial effect of elevated levels of glucan will differ depending on the exact procedure used, several different pretreatment methods can be evaluated. For example, a dilute acid treatment method can be used. The pretreated plant material may then be subjected to enzymatic hydrolysis using a mixture of cell wall degrading enzymes.

[0158] Procedures for cell wall pretreatment and enzymatic digestion are well known to those skilled in the art. The yield or efficiency of the procedure can be readily determined by measuring the amount of reducing sugar released, using a standard method for sugar detection, *e.g.*, the dinitrosalicylic acid method well known in the art. Plants engineered in accordance with the disclosure to have elevated levels of glucan provide a higher sugar yield.

[0159] Plants having elevated levels of glucan may also be evaluated in comparison to non-modified plants to test for the effect of elevated levels of glucan on subsequent fermentation. For example, degraded biomass may be subjected to fermentation using an organism such as yeast or *E. coli* that can convert the biomass into compounds such as ethanol, butanol, hydrocarbons, lipids, etc. In the simplest test, the yield of ethanol obtained with a given amount of starting plant material and a standard yeast fermentation can be determined. Yield can be determined not only with organisms that can ferment glucose, but also with organisms that have the ability to ferment pentoses and or other sugars derived from the biomass. In addition to determining the yield of product, *e.g.*, ethanol, one can determine the growth rate of the organism. The plants of the disclosure that are engineered to have reduced expression in one or more licheninase genes will exhibit elevated levels of glucan in comparison to corresponding plants that have not been engineered to have reduced expression in one or more licheninase genes. The reduced expression in one or more licheninase genes may result in higher final yields of a fermentation reaction.

[0160] Plants having elevated levels of glucan can be used in a variety of reactions, including fermentation reactions. Such reactions are well known in the art. For example, fermentation reactions noted above, *e.g.*, a yeast or bacterial fermentation reaction, may employ plant material derived from a plant having elevated levels of glucan, to obtain ethanol, butanol, hydrocarbons, lipids, and the like. For example the plants with elevated levels of glucan may be used in industrial bioprocessing reactions that include fermentative bacteria, yeast, or filamentous fungi, such as *Corynebacterium spp., Brevibacterium spp., Rhodococcus spp., Azotobacter spp., Citrobacter spp., Enterobacter spp., Clostridium spp., Klebsiella spp., Salmonella spp., Lactobacillus spp., Aspergillus spp., Saccharomyces spp., Zygosaccharomyces spp., Pichia spp., Kluyveromyces spp., Candida spp., Hansenula spp., Dunaliella spp., Debaryomyces spp., Mucor spp., Torulopsis spp., Methylobacteria spp., Bacillus spp., Escherichia spp., Pseudomonas spp., Serratia spp., Rhizobium spp.*, and *Streptomyces spp., Zymomonas mobilis*, acetic acid bacteria (family Acetobacteraceae),

methylotrophic bacteria, *Propionibacterium, Acetobacter, Arthrobacter, Ralstonia, Gluconobacter, Propionibacterium*, and *Rhodococcus*.

EXAMPLES

[0161] The following Examples are merely illustrative and are not meant to limit any aspects of the present disclosure in any way.

Example 1: Generation and Characterization of "candy-leaf-1" (Cal-1) Maize Mutant

Introduction

[0162] Lignocellulosic plant materials are considered valuable feedstocks for the biorefinery industry, in particular for the production of biofuels such as ethanol. One way to unlock the energy in lignocellulosic feedstocks is to degrade the material to its monosaccharides, which can then be fermented by microbes to ethanol. The dominant sugar currently preferentially fermented by microbes is glucose. Accordingly, a mutational breeding screen was performed to identify a mutant plant that contains elevated levels of glucan in its lignocellulosic material, and to further identify the gene(s) responsible for the phenotype.

[0163] Candy-leaf-1 (Cal-1) is a non-transgenic maize mutant whose stover material contains elevated levels of glucan and which, under standard saccharification conditions, has an elevated glucose yield.

[0164] Cal-1 contains a point mutation in a licheninase gene ((1,3;1,4)-β-glucanase; Glycosylhydrolase family 17; genetic locus in maize: Chr. 6: GRMZM2G137535) that encodes a glutamic acid to a lysine substitution (Fig. 1). Since the glutamic acid is an active site residue, this particular licheninase is inactive. The loss of licheninase activity from this gene results in elevated levels of β-glucanan, which in turn results in higher saccharification yields.

[0165] Due to a higher glucan content in its lignocellulosic material (*e.g.*, corn stover), the Cal-1mutant gives a higher yield in biofuel output, and is thus suitable for use as a feedstock.

EMS Mutagenesis of Maize

[0166] Maize, A619 plants were grown in the field in Missouri and pollen was collected. The pollen was sifted to remove any anthers and added to a solution of 0.09% EMS well-dispersed in paraffin oil. The pollen mixed with the EMS for 45 minutes and then was applied to the silks of A619 plants using a paint brush. Resulting kernels were sent to the USDA in Albany and planted in Gill Tract. The plants were selfed. 20 kernels per ear were grown in trays in the greenhouse and tissue was collected from approximately 2 week old seedlings to send to for analysis. The Cal-1 mutant was identified as having high glucose and grown to maturity and crossed with the inbred Mo17 maize line. The resulting crosses were planted and self-pollinations were made. Kernels from those self-pollinations were grown in the greenhouse again, and 2 week seedlings were again harvested for analysis.

Analytical Method For Identifying Cal-1 Mutant From a Chemically Mutagenized Maize Seed Population

[0167] Selection of mutant lines, including the Cal-1 line, was based on analyzing alterations in matrix polysaccharide monosaccharide composition (Foster CE, et al., Comprehensive Compositional Analysis of Plant Cell Walls (Lignocellulosic biomass) Part II: Carbohydrates. *JoVE*, 37, 2010). The analysis of matrix polysaccharide composition was performed on whole leaf material from 14-day old seedlings, which were freeze-dried after harvest. Analysis of the freeze-dried samples consisted of preparing destarched cell wall material and identifying and quantifying the matrix polysaccharide composition of each mutant maize line.

Mapping of Mutation

[0168] The underlying mutation was mapped to a region of maize chromosome 6 that spans an interval of 140.31 to 144.16 Mbp (GRMZM2G137535). Classical mapping procedures were used to identify this region (Neuffer, MG, Mutation induction in maze. In WF Sheridan, ed, *Maize for Biological Research*. Plant Mol. Biol. Assoc., Charlottesville, VA, pp 61-64, 1982; and Neuffer, MG, Mutagenesis. In M Freeling, V Walbot, eds, *The Maize Handbook*. Springer-Verlag, New York, pp 212-219, 1993). Putative proteins encoded in this region were manually annotated by comparison to homologous proteins in rice. From among these putative proteins, the Cal-1 gene was identified as the mutation. The Cal-1 gene encodes a CH17 licheninase. The GRMZM2G137535 region contains two gene models, which were named Cal-1 T01 and Cal-1 T02. The genomic sequences of Cal-1 T01 and Cal-

1 T02 are set forth in SEQ ID NOs: 1 and 5, respectively. Additionally, Figure 1 shows protein models of the polypeptides encoded by Cal-1 T01 and Cal-1 T02.

[0169] The GRMZM2G137535 region containing the Cal-1 mutation was amplified and sequenced to identify the Cal-1 mutation. Briefly, the GRMZM2G137535 region containing the Cal-1 mutation was amplified by PCR in multiple segments. The segment carrying the mutation was produced using the forward primer 5'-ACG-TGC-TGT-CCA-ACA-TCG-3' (SEQ ID NO: 10) and the reverse primer 5'-AGG-TGA-TGA-GTC-AGC-CCT-AGC-3' (SEQ ID NO: 11). The PCR was performed using a primer concentration of 40 pmol for each primer in a total volume of 50 µl with Sigma REDTag Ready mix (Sigma-Aldrich). The reaction conditions consisted of an initial 2 min denaturing step at 94°C, followed by 35 cycles of denaturing, annealing and amplification at 94°C for 30 s, 58°C for 30 s, and 72°C for two min respectively. After completion of the last cycle, the reaction was left at 72°C for an additional 15 min before being cooled to 4°C. The PCR product was then purified using the QIAquick PCR purification kit (Qiagen). The mutation was identified by sequencing the purified PCR product using the primers 5'-ACG-TGC-TGT-CCA-ACA-TCG-3' (SEQ ID NO: 10) and 5'-ACC-AGA-ACC-TCT-TCG-ACA-CCA-3' (SEQ ID NO: 12) in independent sequencing reactions. The results of this analysis are described below.

[0170] The nucleotide and amino acid sequences of Cal-1 T01 from the Cal-1 mutant are shown in Figures 2A and 2B, respectively. The nucleotide and amino acid sequences of Cal-1 T01 from wild-type A619 maize is shown in Figures 2C and 2D, respectively. A comparison of the two sequences shows that the Cal-1 mutant contains a "g" to "a" point mutation in the nucleic acid sequence of Cal-1 T01, corresponding to a Glu to Lys substitution at position 262 of the amino acid sequence.

[0171] The nucleotide and amino acid sequences of Cal-1 T02 from the Cal-1 mutant are shown in Figures 3A and 3B, respectively. The nucleotide and amino acid sequences of Cal-1 T02 from wild-type A619 maize is shown in Figures 3C and 3D, respectively. A comparison of the two sequences shows that the that the Cal-1 mutant contains a "g" to "a" point mutation in the nucleic acid sequence of Cal-1 T02, corresponding to a Glu to Lys substitution at position 242 of the amino acid sequence.

Sequence Alignment

[0172] Figure 4 shows an amino acid sequence alignment of Cal-1 T01 with the GRMZM2G137535 P01 licheninase from the maize database, the Cal-1 T01 licheninase from A619 maize; and a barley licheninase, whose activity and active sites have been determined. The consensus sequence is also included. The sequence alignment showed that the maize Cal-1 T01 licheninase sequence has 78% identity and 99.7% similarity with the barley licheninase.

[0173] Figure 5 shows an amino acid sequence alignment of Cal-1 T02 with the GRMZM2G137535 P02 licheninase from the maize database, the Cal-1 T02 licheninase from A619 maize; and a barley licheninase, whose activity and active sites have been determined. The consensus sequence is also included. The sequence alignment showed that the maize Cal-1 T02 licheninase sequence has 82.3% identity and 99.7% similarity with the barley licheninase.

Example 2: Characterization of Cal-1 Maize Mutant

Materials and Methods

Destarched cell wall material preparation

[0174] The destarched cell wall material preparation was initiated by grinding approximately 60–70 mg of the freeze-dried maize leaf material (Foster CE, et al., Comprehensive Compositional Analysis of Plant Cell Walls (Lignocellulosic biomass) Part II: Carbohydrates. *JoVE*, 37, 2010). The freeze-dried maize leaf material was ground with 5.5 mm stainless steel balls in a 2 ml screw cap centrifugation tube using a retschmill for 1 min at 25 Hz. The steel balls were removed before continuing with the cell wall isolation procedure.

After grinding the plant material, 1.5 ml of 70% aqueous ethanol was added, and the mixture was vortexed thoroughly. Then, the mixture was centrifuged at 10,000 rpm for 10 min to pellet the alcohol-insoluble residue.

[0175] The supernatant was then either aspirated or decanted, and the pellet was washed with 1.5 ml of a chloroform/methanol (1:1 v/v) solution. The tube was shaken thoroughly to resuspend the pellet. The resuspended pellet was then centrifuged at 10,000 rpm for 10 min

and the supernatant was aspirated or decanted. The pellet was then resuspended in 500 µl of acetone. The acetone solvent was then evaporated with a stream of air at 35°C until dry. If needed, dried samples were stored at room-temperature until further processing.

[0176] Following the acetone wash, samples were treated with alpha-amylase to remove starch (Foster CE, et al., Comprehensive Compositional Analysis of Plant Cell Walls (Lignocellulosic biomass) Part II: Carbohydrates. *JoVE*, 37, 2010). To initiate the starch removal, the pellets were resuspended in 1.5 ml of a 0.1 M sodium acetate buffer at pH 5.0. The centrifugation tubes were then capped and heated for
20 min at 80°C in a heating block. After heat incubation, the tubes were cooled on ice.

[0177] After cooling, the following agents were added to digest the pellets: 35 µl of 0.01% sodium azide (NaN_3), 35 µl amylase (50 µg/1mL H_2O; from Bacillus species, SIGMA), and
17 µl pullulanase (18.7 units from *Bacillus acidopullulyticus*; SIGMA). The tubes were then capped and vortexed thoroughly. The pellet suspensions were then incubated overnight at 37°C in a shaker. The tubes were oriented horizontally to improve mixing.

[0178] After incubation, the pellet suspensions were heated at 100°C for 10 min in a heating block to terminate digestion. The suspensions were then centrifuged at 10,000 rpm for 10 min, and supernatants containing solubilized starch were discarded.

[0179] The remaining pellets were washed three times by adding 1.5 ml water, vortexing, centrifuging, and decanting the water.

[0180] After the washes, the pellets were resuspended in 500 µl of acetone. The acetone was evaporated with a stream of air at 35°C until dry. It was sometimes also necessary to break up the material in the tube with a spatula for better drying.

[0181] Dried samples were then stored at room-temperature until further processing.

Cell wall polysaccharide composition

[0182] Following preparation of the destarched cell wall material, the cell wall polysaccharide composition of each mutant maize line was determined (Foster CE, et al., Comprehensive Compositional Analysis of Plant Cell Walls (Lignocellulosic biomass) Part II: Carbohydrates. *JoVE*, 37, 2010).

[0183] First, 2 mg of cell wall material was weighed into 2 ml centrifugation tubes. Then,
20 µl of an inositol solution (5mg/ml) was added as an internal standard. Following addition of the inositol solution, the tube walls were rinsed with 250 µl of acetone to collect the cell wall material on the bottom of the tube, and then the acetone was evaporated under very gentle airflow.

[0184] Acid hydrolysis of the cell wall polysaccharides was then performed (Foster CE, et al., Comprehensive Compositional Analysis of Plant Cell Walls (Lignocellulosic biomass) Part II: Carbohydrates. *JoVE*, 37, 2010). For the acid hydrolysis, 250 µl of 2 M trifluoroacetic acid (TFA) was added to each sample. The TFA was added carefully to ensure that no material was splashed up onto the tube walls.

[0185] The tubes containing the samples with the TFA were capped and incubated for 90 min at 121°C in a heating block. After incubation, the heating blocks containing the sample tubes were cooled on ice. Then, the tubes were centrifuged at 10,000 rpm for 10 min.

[0186] After centrifugation, 100 µl of acidic supernatant containing the cell wall polysaccharide-derived monosaccharide from each tube was transferred to a glass screw cap vial, making sure that the pellet material was not disturbed. The TFA in the glass tube was then evaporated under a gentle stream of air in an evaporation device.

[0187] Then, 300 µl of 2-propanol was added to each sample, vortexed, and evaporated at 25°C. This procedure was repeated a total of three times.

[0188] Following acid hydrolysis, the released monosaccharides were derivatized into their alditol acetates (Foster CE, et al., Comprehensive Compositional Analysis of Plant Cell Walls (Lignocellulosic biomass) Part II: Carbohydrates. *JoVE*, 37, 2010). First, the monosaccharides were reduced to their corresponding alditols. To accomplish this, 200 µl of a freshly prepared sodium borohydride solution was added to each dried sample. The samples were then incubated in the glass vials at room temperature for 1.5 hours. After the incubation, the solution was neutralized by adding 150 µl of glacial acetic acid, vortexing the tubes, and evaporating the glacial acetic acid at 25°C.

[0189] Then, 250 µl of an acetic acid/ methanol (1:9, v/v) mixture was added to each sample, vortexed, and evaporated at 25°C and followed by adding 250 µl of methanol and evaporating it under a stream of air. The methanol wash was repeated a total of three times.

[0190] Next, the alditols in each sample were acetylated (Foster CE, et al., Comprehensive Compositional Analysis of Plant Cell Walls (Lignocellulosic biomass) Part II: Carbohydrates. *JoVE*, 37, 2010). The acetylation was performed by adding 50 µl of acetic anhydride and 50 µl of pyridine. Then the samples were vortexed and incubating for 20 min at 121°C in a heating block. The samples were then cooled in the block with ice until the temperature decreased to approximately room temperature. The reagents were then evaporated under a gentle stream of air at room temperature. The samples were then washed three times with toluene by adding 200 µl of toluene and evaporating under air.

[0191] The final part of the procedure was to extract the alditol acetates (Foster CE, et al., Comprehensive Compositional Analysis of Plant Cell Walls (Lignocellulosic biomass) Part II: Carbohydrates. *JoVE*, 37, 2010). To accomplish the extraction, 500 µl of ethyl acetate were first added to each sample, and the tubes were swirled lightly. Then, 2 ml of water were added. The tubes were then capped and vortexed. This was followed by centrifuging the tubes at 2,000 rpm for 5 min to obtain clear separate layers, which included ethyl acetate on top and water on bottom.

[0192] After centrifugation, 50 µl of the ethyl acetate layer were pipetted into GC/MS (gas chromatography/ mass spectrometry) vials with inserts (Foster CE, et al., Comprehensive Compositional Analysis of Plant Cell Walls (Lignocellulosic biomass) Part II: Carbohydrates. *JoVE*, 37, 2010). The samples were then diluted by adding 100 µl of acetone to the GC/MS vials. The vials were then capped and stored at 4°C when the GC/MS analysis was not immediately performed.

Gas chromatography/ mass spectrometry analysis

[0193] For gas chromatography analysis, the samples were injected into a gas chromatograph (GC) that was equipped with a quadrupole mass spectrometer (MS). A Supelco SP-2380 (30 mm X 0.25 mm x 0.25 µm film thickness) column was used with a 4 min solvent delay and a flow rate of 1.5 ml/min (Foster CE, et al., Comprehensive Compositional Analysis of Plant Cell Walls (Lignocellulosic biomass) Part II: Carbohydrates. *JoVE*, 37, 2010).

[0194] The injected samples were subjected to the following temperature program: initial hold at 160°C for 2 min; a 20°C/min ramp to 200°C and hold for 5 min; a 20°C/min ramp to 245°C and hold 12 min; spike to 270°C and hold for 5 min before cooling to the initial temperature of 160°C (Foster CE, et al., Comprehensive Compositional Analysis of Plant Cell Walls (Lignocellulosic biomass) Part II: Carbohydrates. *JoVE*, 37, 2010).

[0195] Peaks were then identified by mass profiles and/or retention times of standards. Monosaccharides were quantified based on standard curves (Foster CE, et al., Comprehensive Compositional Analysis of Plant Cell Walls (Lignocellulosic biomass) Part II: Carbohydrates. *JoVE*, 37, 2010).

Results

Cell wall polysaccharide composition

[0196] The Cal-1 maize mutant was characterized by comparing the hemicellulosic levels of glucan to wildtype maize ('Mo17'). The levels of glucan were determined by measuring the amount of glucose released by the glucan. Analysis was performed on two-week old (young seedling) and eleven-week old leaf material (adult leaves), as well as senesced leaf and stem material (Fig. 6). In all tissues, the Cal-1 mutant showed an increase of glucose released by weak acid hydrolysis (Fig. 6). Additionally, the Cal-1 mutant was shown to have approximately four-fold higher amounts of β-1,3-1,4-glucan content than the wildtype maize lines 'Mol7' and 'B73' (Fig. 7).

[0197] Further analysis of the elevated levels of glucan was also performed. An extraction of cell wall material from mature leaves of the Cal-1 mutant indicated that the elevated levels of glucan, as measured by the amount of released glucose, was found in a 4M potassium hydroxide fraction (Fig. 8A), indicating that the glucan is of hemicellulosic nature rather than cellulosic. Glycosidic linkage analysis of the 4M potassium hydroxide fraction indicated that the increased glucan content included a mixed-linked β-1,3-1,4-glucan, which is a grass-specific, transient hemicellulosic polymer (Fig. 8B).

[0198] The remaining residue, after 4M potassium hydroxide extraction of the leaf material, represented mainly crystalline cellulose. This was confirmed by monosaccharide composition analysis, which showed that glucose was the predominant component (Fig. 9A). Levels of cellulose in adult leaf material from the Cal-1 mutant were also compared with

levels in adult leaf material of wild-type maize. The comparison showed no statistically significant difference (Fig. 9A).

[0199] The amount of acetylbromide-soluble lignin was also determined for the Cal-1 maize mutant (Foster CE, et al., Comprehensive Compositional Analysis of Plant Cell Walls (Lignocellulosic biomass) Part I: Lignin. *JoVE*, 37, 2010). Levels of acetylbromide soluble lignin in adult leaf material from the Cal-1 maize mutant were compared with levels in adult leaf material of wild-type maize. This comparison showed no statistically significant difference in acetylbromide soluble lignin content (Fig. 9B).

[0200] The Cal-1 maize mutant was then evaluated to determine the amount of glucose released by saccharification of cell wall material. Destarched alcohol-insoluble residue from adult leaf material from the Cal-1 maize mutant and from wild-type maize was subjected to a saccharification assay. The assay was performed using a commercial enzyme mix containing multiple enzyme activities, mainly exoglucanase, endoglucanase, hemi-cellulase, and beta-glucosidase. After incubation for 17 hours, the released glucose amount was assayed using a Megazyme GOPOD kit (K-GLUC, Megazyme, Ireland). The Cal-1 maize mutant showed a 40% increase in glucose saccharification yield compared to the wild-type (Fig. 10A). Significant increases in glucose saccharification levels were also observed in senesced leaves (Fig. 10B) and senesced stems (Fig. 10C).

Grain and biomass yield

[0201] Moreover, field trial with the Cal-1maize mutant showed that there was no significant difference in kernel (i.e., grain) yield and biomass yield as compared to wildtype maize (Fig. 11).

Cal-1 protein

[0202] Additionally, the Cal-1 protein was isolated, and its licheninase activity was confirmed. The Cal-1 protein was cloned into an expression vector, and the vector was transformed into tobacco plants. The Cal-1 protein was then extracted and purified from the transformed tobacco. The Cal-1 protein was then shown to have licheninase (i.e., mixed-linked glucan endoglucanase) activity (Fig. 12A). Moreover, cellulase activity (endo-β-1,4-glucanase) and activity against laminarin (β-1,3-β-1,6 linked glucan) was not detected with the heterologously expressed protein.

Increased saccharification yield

[0203] The Cal-1 maize mutant was also crossed to lignin biosynthesis maize mutants *bm1* and *bm3*. The *BM1* gene is believed to encode cinnamyl alcohol dehydrogenase (CAD), while the *BM3* gene is believed to encode caffeic acid O-methyltransferase (COMT).

[0204] As shown in Figure 13, the crosses of the Cal-1 maize mutant to either the *bm1* maize mutant or the *bm3* maize mutant surprisingly resulted in a synergistic effect on saccharification yield. In particular, the Cal-1/*bm1* cross resulted in a 57% percent increase in saccharification yield as compared to wildtype maize, and the Cal-1/*bm3* cross resulted in a 43% increase in saccharification yield as compared to wildtype maize. These results were much higher than the approximate 25% increase over wildtype seen with the Cal-1 mutant, the approximate 10% increase over wildtype seen with the *bm1* mutant, or the negligible increase over wildtype seen with the *bm3* mutant (Fig. 13B).

Conclusion

[0205] These characterizations show that the Ca-1 maize mutant exhibits an increase in cell wall glucan level compared to wild-type maize. In particular, the Cal-1 maize mutant showed increased levels of cell wall-derived mixed-linked hemicellulosic glucan (Figs. 7 and 8). However, the Cal-1 maize mutant showed no change in crystalline cellulose content or acetylbromide-soluble lignin (Fig. 9). Moreover, when subjected to a saccharification assay, the Cal-1 maize mutant showed a 40% increase in cell wall released glucose compared to wild-type maize. Thus, the Cal-1 maize mutant has improved characteristics for use as a bioenergy crop.

Example 3: Cal-1 Homology Identification

Phylogenetic tree of *Zea mays* GH17 domains with at least 40 % identity to Cal-1 T01

[0206] The amino acid sequence of the GH17 domain of Cal-1 T01 was subjected to a BLASTP search and the GH17 domains of 86 proteins were identified that had at least 40% sequence identity with Cal-1 T01. Fifteen proteins with incomplete GH17 domains were eliminated. Five further proteins, the predicted active site was not preserved and were therefore eliminated. The remaining proteins were selected for sequence alignment (Fig. 14).

[0207] A consensus sequence of the 66 *Zea mays* GH17 domains was determined and is set forth below (SEQ ID NO: 9):

X-X-X-X-X-X-X-X-X-X-[I/L/V/H/A/F]-G-[V/I/A]-[N/T/S/C]-[Y/N/I/W/H]-G-X-[V/Q/M/S/I/L/T/N/R/A]-[A/S/G/M/V]-X-[N/H/D/T/S]-[L/P/Q/R/I]-[P/L/I/A]-X-[P/L//H/A/K/S/T]-X-X-[V/A/M/S/P/L/K/I]-[V/A/I/M/S/P/L/T]-X-[L/Q/R/K/E/D/M/I/F]-[L/V/M/G/Y/C/A/I]-[R/L/K/Q/A/E/S/V/L/T]-X-[S/D/G/A/R/K/L/Q]-X-X-[I/V/F/A]-X-[K/R/A/L/V/Y/D/S/G/N/M/H]-[V/A/M/L]-[R/K/T]-[L/S/M/I/T]-[Y/F/I/L]-[D/E/N/L/A/W/H/F/S/G]-[A/T/P/S/V]-[D/M/E/V/N/Q]-X-X-[V/A/P/T/I/L/F/M]-[L/M/P/V/I]-X-[A/S]-[L/F/V/A]-[A/V/G/R/S]-[G/D/H/K/N/R/A]-X-X-[T/S/A/P]-[G/S/D/R/N]-[I/V/L/W]-X-[V/A/L/F]-[V/M/T/A/I/D]-[V/L/A/P/I]-[G/A/D/S/M/T]-[V/I/A/L/T/E/F]-[P/T/L/G/A]-[N/D]-X-X-X-[L/R/A/D/E/G/S/K/I]-X-X-X-[A/P/DS/R/T/G/I/L/M]-[A/D/Y/S/G/R/Q/T/V/N]-[S/A/G/D/Y/P/V/M/Q/R/T/N]-X-X-X-X-X-X-[A/V/S]-X-X-[W/C/L]-[V/A/L]-X-X-[N/L/A/Y/T/S/H/R]-[V/I/L]-X-[P/A/R/K/T/S]-[Y/V/N/H/A/F/T/S]-X-[P/L/F/N/S/G/Q/D]-[A/D/K/R/S/Q/V]-X-X-X-X-X-X-X-[I/C/L/F/V/S/T]-X-X-[V/I/L/M]-[A/C/N/V/T/S]-[V/L/A/G]-[G/D/N]-[N/P/A/E/S/D]-[E/S/V]-[V/A/F/I/L/T]-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-[L/T/I/V]-[L/F/V/I/A/M]-[P/Q/G/D]-A-[M/L/V/I]-[R/Q/K/T/E/A/S/L]-[N/S/T/A/C/R/Y]-[L/I/V/M/A]-[H/Q/R/E/D/N/S/A/Y/L]-X-[A/S/G]-[L/I/V/A]-X-X-[A/L/H/R/V/S/G/N/E]-[G/N/S/R/H/A]-[L/I/F/H/V/F/M/D]-X-X-X-X-X-[V/I/A/T]-[K/H/P/R/T/E/N/A]-[V/A/L/C/I/F]-[S/T/V/G/F]-[T/V/C/S]-X-[V/L/C/H/I/N]-[S/N/A/K/R/Q/T/Y/P]-X-X-[V/I/A/D]-[L/Y/I/V/T/F/M]-[A/M/N/D/S/E/Q/G/T/R/L/V]-X-[S/P/Q/T/A]-X-X-X-X-[P/V/Q/I]-[P/S]-[S/A/Q]-[A/Q/G/R/D/S/T/N]-[G/Q/E/A/S/C]-X-[F/W/T/S]-[R/C/D/V/G/E/A/S/N/T/H]-X-X-[L/P/I/V/L/S/Y/A/D/E]-X-X-X-X-[M/L/V/I]-X-[P/D/E/S/T/Q/Y/R]-[L/M/I/V]-[L/V/A/I]-X-[F/Y/L/H]-[L/F/H]-[A/N/H/L/S/Q/E/D/V/R]-X-[T/N/S/H/K/R/I/V/A]-[G/D/R/N/Q/S]-[A/G/S/T/R]-[P/V/A/C/Y/F]-[L/F/Y/V]-[L/T/V/M/F/Y/P/W/L]-[V/I/A/C/L/I]-[N/S/D]-[I/H/A/V/L/C/P]-[Y/L]-[P/T]-[Y/R/F/C/W]-[F/S/L/Y]-[A/S/T/V/D]-[Y/P/H/W/Q/L]-X-X-X-X-X-X-X-X-X-X-[I/S/F/V/M/L/E/S/A]-X-[L/V/F/Q/M/I]-[D/E/N/A/S/G/P]-[Y/F/N]-[A/S/V/C]-[L/F/Y/I/T/V]-[F/L/G/S]-X-[P/G/S/A/M/V]-X-X-X-X-X-X-X-X-X-X-X-X-X-X-[V/G/A/S/N/K/H/T/Y/R/M]-[V/W/P/I/S/T/R/L/A/Q/M/Y]-[D/V/Q/I/L/T]-X-X-[T/S/H/N/G/A]-[G/R/N/S/P/A/E/K]-[L/V/I/A/M/F/N/Y]-X-Y-[T/S/Y/Q/N/G/D/H/A/P]-[N/D/S]-[M/V/A/L]-[F/L]-[D/Y/H/A/V]-[A/G/T/Q/E]-[Q/N/T/I/M/L/V]-[V/F/Y/H/A/M/L]-D-[A/T/S/C]-[V/L/F/T/I/A]-[Y/V/I/H/R/F/T/K]-[A/S/H/W/L/V/I/F/Y/T]-[A/S]-[L/M/V/I/A]-X-X-[L/V/A/H/N/I/E/M/K]-[G/N]-X-X-X-X-X-X-X-X-X-X-X-X-X-X-[V/M/L/I/P]-X-[V/I/L/A]-[V/M/I/A/H/R/T/K/L]-[V/I/L]-[S/G/T/A]-E-[T/V/A/I/S]-G-[W/H/C]-[P/A]-[S/T/N/Y/H]-X-[G/D/C/A]-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-[E/D/N/A/H/Q/Y]-X-[G/H/Y/N/S/A/Q/V/E/D]-[A/E/G/V]-[T/K/N/S/G]-X-[E/A/S/K/Q/T/G/D/R/H]-[N/Y/F/L/M/A/E]-[A/S]-X-X-[Y/F]-[N/Y/V/S/D/I]-X-[N/G/K/Y]-[L/F/I/V/A/M]-[I/L/F/M/V/A/R]-[R/Q/D/T/N/E/L/A/K/M/S]-X-[V/L/M/I/A/Q/C]-X-X-[G/N/S/R/D/Q/L/E]-X-X-X-G-T-P-X-[R/H/K/A/T/M]-[P/K/T/S]-[G/N/Q/R/D/K/H/A/S]-X-X-X-X-X-X-X-[Y/F/I/M/S]-[I/L/V/M]-[F/Y]-[A/G/S/D/E]-[L/M/T]-[F/L/V/I/Y]-[N/D]-E-[D/E/N]-X-[K/R]-X-X-X-[G/P/D/E/A]-X-X-[S/F/Q/E/T/V/I/A]-[E/N/H/K/R]-[R/Q/N/K/A]-X-[W/F/Y]-G-

[L/I/V/M]-[F/L/M/Y]-X-[P/Y/F/A/G/K/T/M]-X-[D/N/S]-[G/M/K/R/Q/E/L]-[T/Q/R/K/S/L/H/E/A/V]-[P/A/K/H/R/E/L/M/S/I]-[V/K/A/I/S/N/T]-[Y/F]-X-[L/M/I/V/F]-X-X

[0208] Figure 15 shows a phylogenetic tree of the aligned proteins that was generated using the Neighbor-Joining method (Saitou N. and Nei M. *Molecular Biology and Evolution* 4:406-425.2, 1987). The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein J. *Evolution* 39:783-791, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein J. *Evolution* 39:783-791, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site (Zuckerkandl E. and Pauling L. Evolutionary divergence and convergence in proteins. Edited in *Evolving Genes and Proteins* by V. Bryson and H.J. Vogel, pp. 97-166, 1965. Academic Press, New York). The analysis involved the 66 aligned amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 444 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura K. et al., *Molecular Biology and Evolution* 24:1596-1599, 2007).

Evolutionary relationships of *Zea mays, Oryza sativa, Sorghum bicolor, Brachypodium distachyon* and *Setaria italica* to Cal-1 T01

[0209] The amino acid sequence of the GH17 domain of Cal-1 T01 was subjected to a BLASTP search and the GH17 domains of 77 *Zea mays, Oryza sativa, Sorghum bicolor, Brachypodium distachyon* and *Setaria italica* proteins were identified that had at least 40% sequence identity with Cal-1 T01. These 77 proteins were selected for sequence alignment (Fig. 16).

[0210] Figure 17 shows a phylogenetic tree of the aligned proteins that was generated using the Neighbor-Joining method (Saitou N. and Nei M. *Molecular Biology and Evolution* 4:406-425, 1987). The optimal tree with the sum of branch length = 8.75872220 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein J. *Evolution*

39:783-791, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site (Zuckerkandl E. and Pauling L. Evolutionary divergence and convergence in proteins. Edited in *Evolving Genes and Proteins* by V. Bryson and H.J. Vogel, pp. 97-166. Academic Press, New York, 1965). The analysis involved the 77 aligned amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 374 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura K. et al., *Molecular Biology and Evolution* 24:1596-1599, 2007).

CLAIMS

What is claimed:

1. A non-naturally occurring mutant plant, comprising a mutation in at least one licheninase gene, wherein the mutant plant has elevated levels of glucan, compared to the levels of glucan in a corresponding plant lacking the mutation in the at least one licheninase gene.

2. The plant of claim 1, wherein the at least one licheninase gene encodes a polypeptide comprising consensus sequence SEQ ID NO: 9.

3. The plant of claim 1 or claim 2, wherein the at least one licheninase gene comprises a nucleic acid sequence selected from the group consisting of:

(a) SEQ ID NO: 3 or 7;

(b) a homolog of SEQ ID NO: 3 or 7;

(c) a paralog of SEQ ID NO: 3 or 7; and

(d) an ortholog of SEQ ID NO: 3 or 7.

4. The plant of any one of claims 1-3, wherein the at least one mutant licheninase gene encodes a polypeptide sequence having a Glu to Ly substitution at position 262 of SEQ ID NO: 4, a Glu to Ly substitution at a position analogous to position 262 of SEQ ID NO: 4, a Glu to Lys substitution at position 242 of SEQ ID NO: 8, or a Glu to Ly substitution at a position analogous to position 242 of SEQ ID NO: 8.

5. The plant of any one of claims 1-4, wherein the plant has at least a 20% increase in levels of glucan as compared to a corresponding plant lacking the mutation.

6. The plant of any one of claims 1-5, further comprising a mutation in at least one additional licheninase gene.

7. The plant of claim 6, wherein the at least one additional licheninase gene encodes a polypeptide comprising consensus sequence SEQ ID NO: 9

8. The plant of any one of claims 1-7, wherein the at least one licheninase gene or the at least one additional licheninase gene comprises a partial deletion or a complete deletion of the gene.

9. The plant of any one of claims 1-8, further comprising a mutation in at least one *bm1* gene, a homolog thereof, a paralog thereof, or an ortholog thereof, wherein the plant exhibits an increased saccharification yield compared to the saccharification yield in a corresponding plant lacking the mutation in the at least one licheninase gene and the mutation in at least one *bm1* gene.

10. The plant of any one of claims 1-8, further comprising a mutation in at least one *bm3* gene, a homolog thereof, a paralog thereof, or an ortholog thereof, wherein the plant exhibits an increased saccharification yield compared to the saccharification yield in a corresponding plant lacking the mutation in the at least one licheninase gene and the mutation in at least one *bm3* gene.

11. A seed of the mutant plant of any one of claims 1-10.

12. A plant comprising an RNAi-inducing vector, wherein the vector generates RNAi against a licheninase gene.

13. The plant of claim 12, wherein the licheninase gene encodes a polypeptide comprising consensus sequence SEQ ID NO: 9.

14. The plant of claim 12 or claim 13, wherein the licheninase gene comprises a nucleic acid sequence selected from the group consisting of:

(a) SEQ ID NO: 3 or 7;

(b) a homolog of SEQ ID NO: 3 or 7;

(c) a paralog of SEQ ID NO: 3 or 7; and

(d) an ortholog of SEQ ID NO: 3 or 7.

15. The plant of any one of claims 12-14, further comprising one or more additional RNAi-inducing vectors, wherein the vectors generate RNAi against one or more additional licheninase genes.

16. The plant of claim 15, wherein the one or more additional licheninase genes encode a polypeptide comprising consensus sequence SEQ ID NO: 9.

17. The plant of any one of claims 12-16, further comprising an additional RNAi-inducing vector, wherein the additional vector generates RNAi against a *bm1* gene, a homolog thereof, a paralog thereof, or an ortholog thereof, wherein the plant exhibits an increased saccharification yield compared to the saccharification yield in a corresponding plant lacking the vectors generating RNAi against a licheninase genes and a *bm1* gene.

18. The plant of any one of claims 12-16, further comprising an additional RNAi-inducing vector, wherein the additional vector generates RNAi against a *bm3* gene, a homolog thereof, a paralog thereof, or an ortholog thereof, wherein the plant exhibits an increased saccharification yield compared to the saccharification yield in a corresponding plant lacking the vectors generating RNAi against a licheninase genes and a *bm3* gene.

19. The plant of any one of claims 12-18, wherein the RNAi-inducing vector or one or more additional RNAi-inducing vectors are stably transformed in the plant.

20. A seed of the plant of any one of claims 12-19.

21. A plant having reduced expression of at least one licheninase gene encoding a polypeptide comprising consensus sequence SEQ ID NO: 9, wherein the plant has elevated levels of glucan compared to the levels of glucan in a corresponding plant lacking the reduced expression of the at least one licheninase gene.

22. The plant of claim 21, wherein the polypeptide comprises an amino acid sequence selected from the group consisting of:

(a) SEQ ID NO 4 or 8;

(b) a homolog of SEQ ID NO: 4 or 8;

(c) a paralog of SEQ ID NO: 4 or 8; and

(d) an ortholog of SEQ ID NO: 4 or 8.

23. The plant of claim 21 or claim 22, further comprising reduced expression of at least one additional licheninase gene encoding a polypeptide comprising consensus sequence SEQ ID NO: 9.

24. The plant of any one of claims 21-23, further comprising reduced expression of at least one *bm1* gene, a homolog thereof, a paralog thereof, or an ortholog thereof, wherein the plant exhibits an increased saccharification yield compared to the saccharification yield in a corresponding plant having reduced expression of the at least one licheninase gene and reduced expression of the at least one *bm1* gene.

25. The plant of any one of claims 21-23, further comprising reduced expression of at least one *bm3* gene, a homolog thereof, a paralog thereof, or an ortholog thereof, wherein the plant exhibits an increased saccharification yield compared to the saccharification yield in a corresponding plant having reduced expression of the at least one licheninase gene and reduced expression of the at least one *bm3* gene.

26. The plant of any one of claims 21-25, wherein the reduced expression of the at least one licheninase gene, the at least one additional licheninase gene, the at least one *bm1* gene, and/or the at least one *bm3* gene is a result of RNAi, antisense RNA, T-DNA insertion, transposon insertion, or TILLING.

27. A seed of the plant of any one of claims 21-26.

28. The plant of any one of claims 1-27, wherein the plant is selected from the group consisting of corn (*Zea mays)*, barley (*Hordeum vulgare*), rice (*Oryza sativa*), sorghum (*Sorghum bicolor*), foxtail millet (*Setaria italica*), sugar cane (*Saccharum spp.*), wheat (*Triticum spp.*), soy (*Glysine sp.*), cotton (*Gossypium sp.*), sugar beet (*Beta vulgaris*), sunflower (*Helianthus sp.*), miscanthus (*Miscanthus sp.*), giant miscanthus (*Miscanthus giganteus*), rape (*Brassica napus*), grass (*Poaceae sp.*), switchgrass (*Panicum virgatum*), giant reed (*Arundo donax*), reed canary grass (*Phalaris arundinacea*), sericea lespedeza (*Lespedeza cuneata*), millet *(Panicum miliaceum*), ryegrass (*Lolium sp.*), timothy-grass (*Phleum sp.*), kochia (*Kochia sp.*), kenaf (*Hibiscus cannabinus*), bahiagrass (*Paspalum sp.*), bermudagrass (*Cynodon dactylon*), pangolagrass (*Digitaria decumbens*), bluestem grass (*Andropogon sp.*), indiangrass (*Sorghastrum sp.*), bromegrass (*Bromus sp.*), elephant grass (*Pennisetum purpureum*), jatropha (*Jatropha sp.*), alfalfa (*Medicago sp.*), clover (*Trifolium*), sunn hemp (*Crotalaria juncea*), fescue (*Festuca sp.*), orchard grass (*Dactylis sp.*), purple

false brome (*Brachypodium distachyon*), sesame (*Sesamum indicum*), poplar (*Populus trichocarpa*), spruce (*Picea sp.*), pine (*Pinaceae spp.*), willow (*Salix sp.*), eucalyptus (*Eucalyptus sp.*), castor oil plant (*Ricinus communis*), and palm tree (*Arecaceae sp.*).

29. A method of increasing levels of glucan in a plant, the method comprising reducing the expression in a plant of at least one licheninase gene.

30. The method of claim 29, further comprises reducing the expression of at least one additional licheninase gene.

31. The method of claim 29 of claim 30, wherein the plant has at least a 20% increase in levels of glucan as compared to a corresponding plant lacking the reduced expression.

32. A method of increasing the amount of glucose generated from biomass in a saccharification procedure, the method comprising:

a) obtaining biomass from a plant having reduced expression of at least one licheninase gene; and

b) subjecting the biomass to an enzymatic or chemical saccharification procedure,

wherein an increased amount of glucose is generated from the plant having reduced expression of a licheninase gene, as compared to the amount of glucose generated from a corresponding plant lacking the reduced expression.

33. The method of claim 32, wherein the plant further comprises reduced expression of at least one additional licheninase gene.

34. The method of claim 29, wherein the amount of glucose generated is increased by at least 20%, as compared to the amount of glucose generated from a corresponding plant lacking the reduced expression.

35. The method of claim 32 or claim 33, wherein the plant further comprises reduced expression of at least one *bm1* gene, a homolog thereof, a paralog thereof, or an ortholog thereof.

36. The method of claim 32 or claim 33, wherein the plant further comprises reduced expression of at least one *bm3* gene, a homolog thereof, a paralog thereof, or an ortholog thereof.

37. The method of claim 35 or claim 36, wherein the amount of glucose generated is increased by at least 40%, as compared to the amount of glucose generated from a corresponding plant lacking the reduced expression of at least one licheninase gene and the at least one *bm1* gene or the at least one *bm3* gene.

38. A method of increasing the yield of fermentation product from a fermentation reaction, the method comprising:

a) obtaining biomass from a plant having reduced expression of at least one licheninase gene;

b) subjecting the biomass to an enzymatic or chemical saccharification procedure; and

c) incubating the degraded biomass with a fermentative organism under conditions suitable to yield a fermentation product, wherein an increased yield of fermentation product from the fermentation reaction is obtained, as compared to the yield of fermentation product obtained from a fermentation reaction using degraded biomass from a corresponding plant lacking the reduced expression.

39. The method of claim 38, wherein the plant further comprises reduced expression of at least one additional licheninase gene.

40. The method of claim 38 or claim 39, wherein the plant further comprises reduced expression of at least one *bm1* gene, a homolog thereof, a paralog thereof, or an ortholog thereof.

41. The method of claim 38 or claim 39, wherein the plant further comprises reduced expression of at least one *bm3* gene, a homolog thereof, a paralog thereof, or an ortholog thereof.

42. The method of any one of claims 29-41, wherein the at least one licheninase gene or the at least one additional licheninase gene encodes a polypeptide comprising consensus sequence SEQ ID NO: 9.

43. The method of any one of claims 29-42, wherein the at least one licheninase gene or the at least one additional licheninase gene comprises a nucleic acid sequence is selected from the group consisting of:

(a) SEQ ID NO: 3 or 7;

(b) a homolog of SEQ ID NO: 3 or 7;

(c) a paralog of SEQ ID NO: 3 or 7; and

(d) an ortholog of SEQ ID NO: 3 or 7.

44. The method of any one of claims 29-43, wherein the reduced expression of the at least one licheninase gene or the at least one additional licheninase gene is a result of mutagenesis of the gene.

45. The method of any one of claims 29-44, wherein the reduced expression of the at least one *bm1* gene or the at least one *bm3* gene is a result of mutagenesis of the gene.

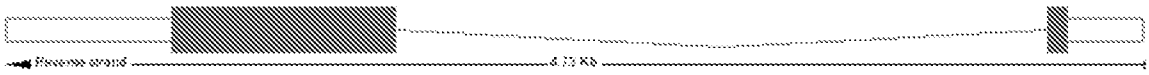
46. The method of claim 44 or claim 45, wherein the mutagenesis of the gene is by TILLING, T-DNA insertion, or transposon insertion.

47. The method of any one of claims 44-46, wherein the mutagenesis of the gene results in a partial deletion or a complete deletion of the gene.

48. The method of any one of claims 29-43, wherein the reduced expression of the at least one licheninase gene or the at least one additional licheninase gene is a result of RNAi or antisense RNA.

49. The method of any one of claims 29-44, wherein the reduced expression of the at least one *bm1* gene or the at least one *bm3* gene is a result of RNAi or antisense RNA.

A



B

Figure 1

A

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B

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C

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gcaggccatcctgggcgtgtacagcccgccgtccgccgcggagttcaccggcgaggcgcgcggatacatgggccccgtg
ctgcagttcctggcgcgcaccgggtcgccgctcatggccaacatctaccgtacctggcctgggcatacaaccccagcgc
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D

MASRQGVAASMFATALLLGVFASIPQSAEAIGVCYGMSANNLPAASTVVSMYKANGISAM
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GNEVAGGAAQDLAPAMENVHAALAAAGLGHIKVTTSVSQAILGVYSPPSAAEFTGEARGY
MGPVLQFLARTGSPLMANIYPYLAWAYNPSAMDMSYALFTSSGTVVQDGAYGYQNLFDTT
VDAFYVAMGKNGGSGVPLVVSESGWPSGGGVQATPANARVYNQYLINHVGRGTPRHPGAI
ETYLFSMFNENQKESGVEQNWGLFYPNMQHVYPISF

Figure 2

A

atgtgcgtttcgatcgcaggtgctgaggccatcggggtgtgctacggcatgagcgccaacaacctgccggcggcgagca
cggtggtgagcatgtacaaggcgaacggcatctcggcgatgcggctgtacgcgccggaccagggcgcgctgcaggcgg
tgggcggcacgggcatcagcgtggccgtgggcgcccccaacgacgtgctgtccaacatcgcggctagccccgcggcggc
cgcgtcgtgggtgcgcaacaacatccaggcgtacccgtccgtgtcgttccgctacgtgtgcgtgggcaacgaggtggccg
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gcggatacatgggccccgtgctgcagttcctggcgcgcaccgggtcgccgctcatggccaacatctacccgtacctggcct
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cgtggtgtcgaagagcgggtggccgtccggcggcggcgtccaggccacgccggccaacgcgagggtgtacaaccagta
cctcatcaaccacgtcgggcgcgggacgccgcgccacccgggcgccatcgagacctacctcttctccatgttcaacgaga
accagaaggagagcggcgtggagcagaactgggggctcttctaccccaacatgcagcacgtctaccccatcagcttctg
a

B

mcvsiagaeaigvcygmsannlpaastvvsmykangisamrlyapdqgalqavggtgiavavgapndvlsntaa
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aaeftgeargymgpvlqflartgsplmaniypylawaynpsamdmsyalftsngtvvqdgayggyqnlfdttvdaf
yvamgnnggsgvplvvskgwpsgggvqatpanarvynqylinhvgrgtprhpgaietylfsmfnenqkesgv
eqnwglfypnmqhvypisf*

C

Atgtgcgtttcgatcgcaggtgctgaggccatcggggtgtgctacggcatgagcgccaacaacctgccggcggcgagca
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a

D

MCVSIAGAEAIGVCYGMSANNLPAASTVVSMYKANGISAMRLYAPDQGALQAVGGTGIAV
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AALAAAGLGHIKVTTSVSQAILGVYSPPSAAEFTGEARGYMGPVLQFLARTGSPLMANIY
PYLAWAYNPSAMDMSYALFTSNGTVVQDGAYGYQNLFDTTVDAFYVAMGNNGGSGVPLVV
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Figure 3

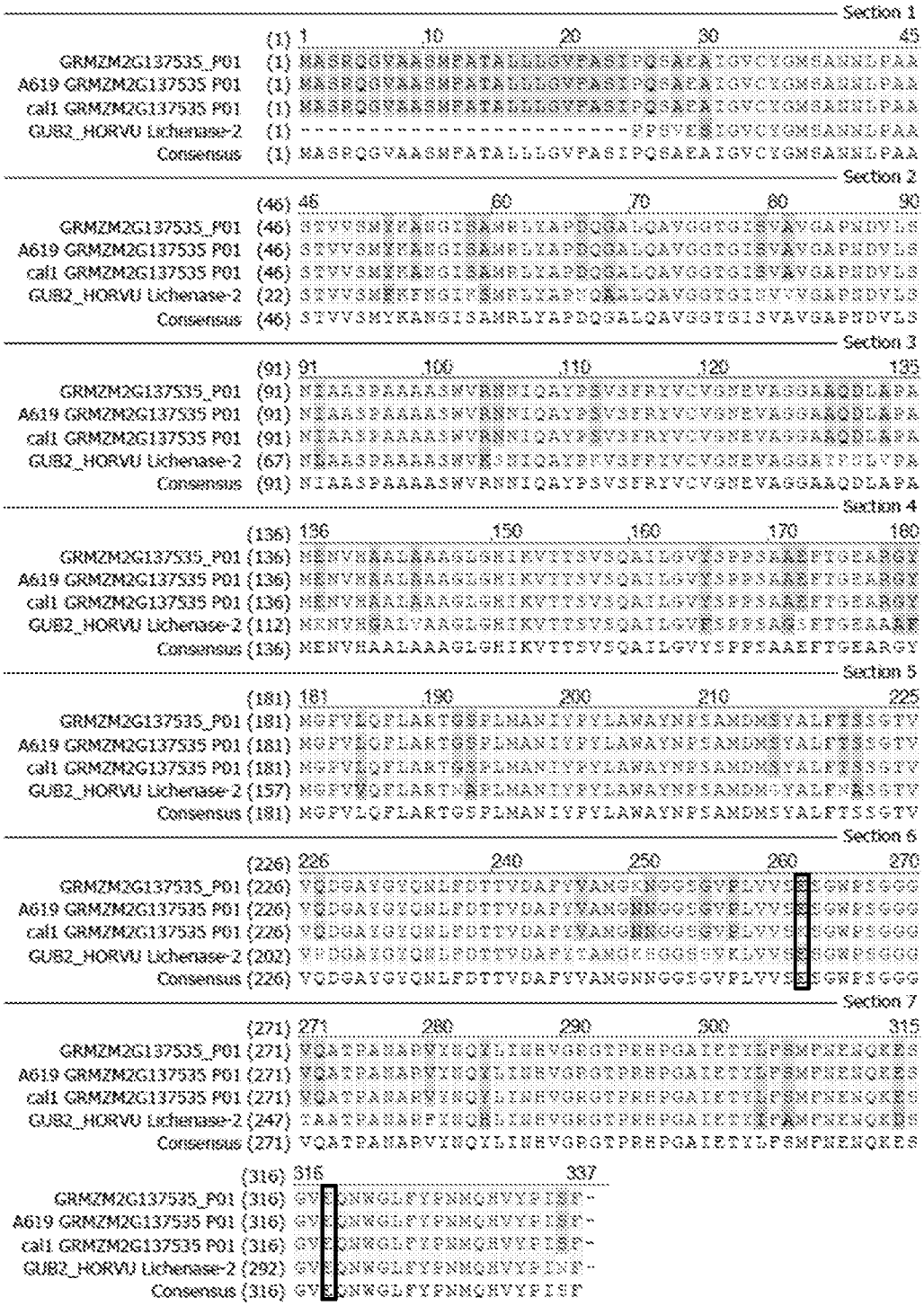

Section 1
Section 2
Section 3
Section 4
Section 5
Section 6
Section 7
GRMZM2G137535_P01
A619 GRMZM2G137535 P01
cal1 GRMZM2G137535 P01
GUB2_HORVU Lichenase-2
Consensus

Figure 4

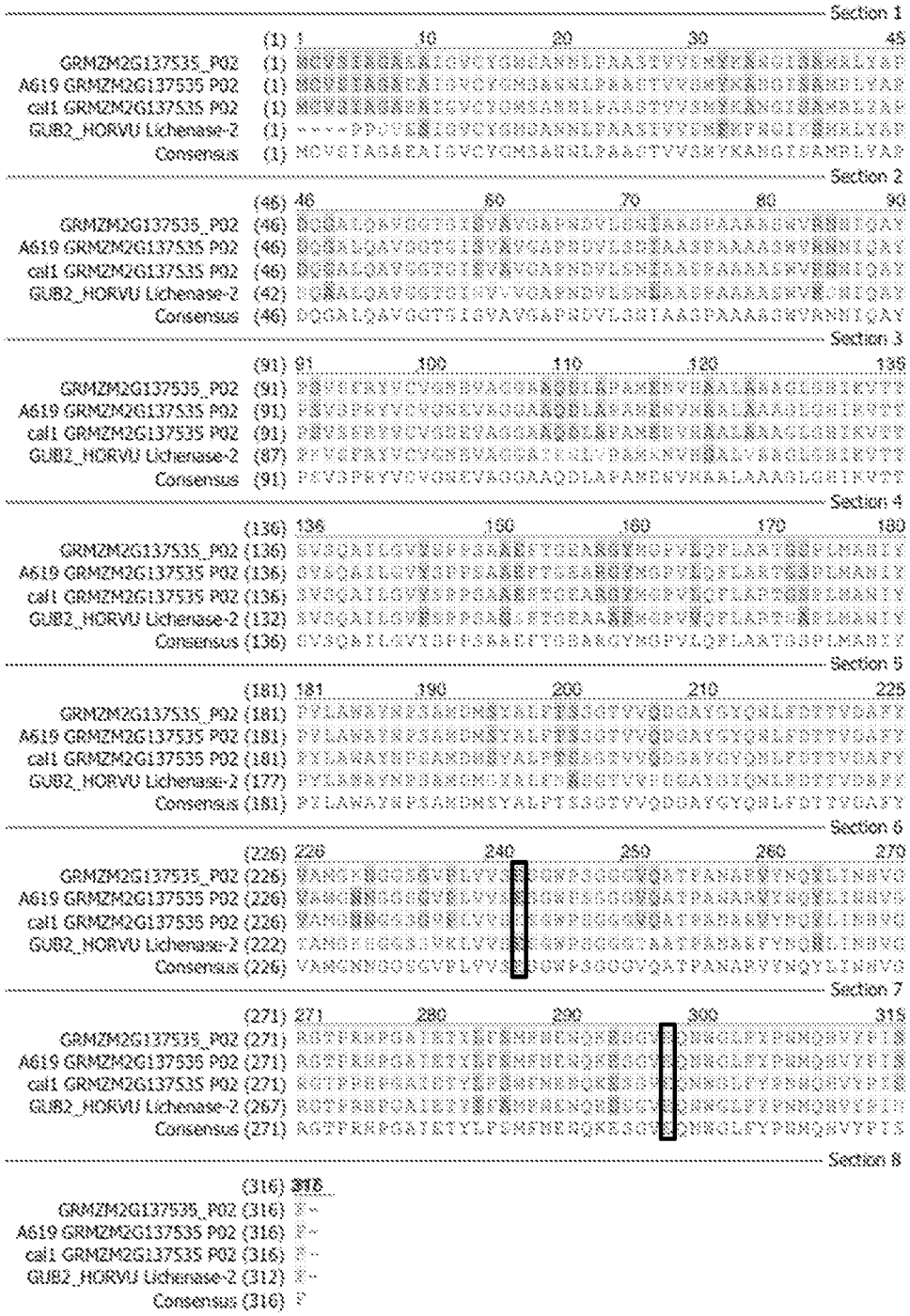

Section 1
Section 2
Section 3
Section 4
Section 5
Section 6
Section 7
Section 8
GRMZM2G137535_P02
A619 GRMZM2G137535 P02
cal1 GRMZM2G137535 P02
GUB2_HORVU Lichenase-2
Consensus

Figure 5

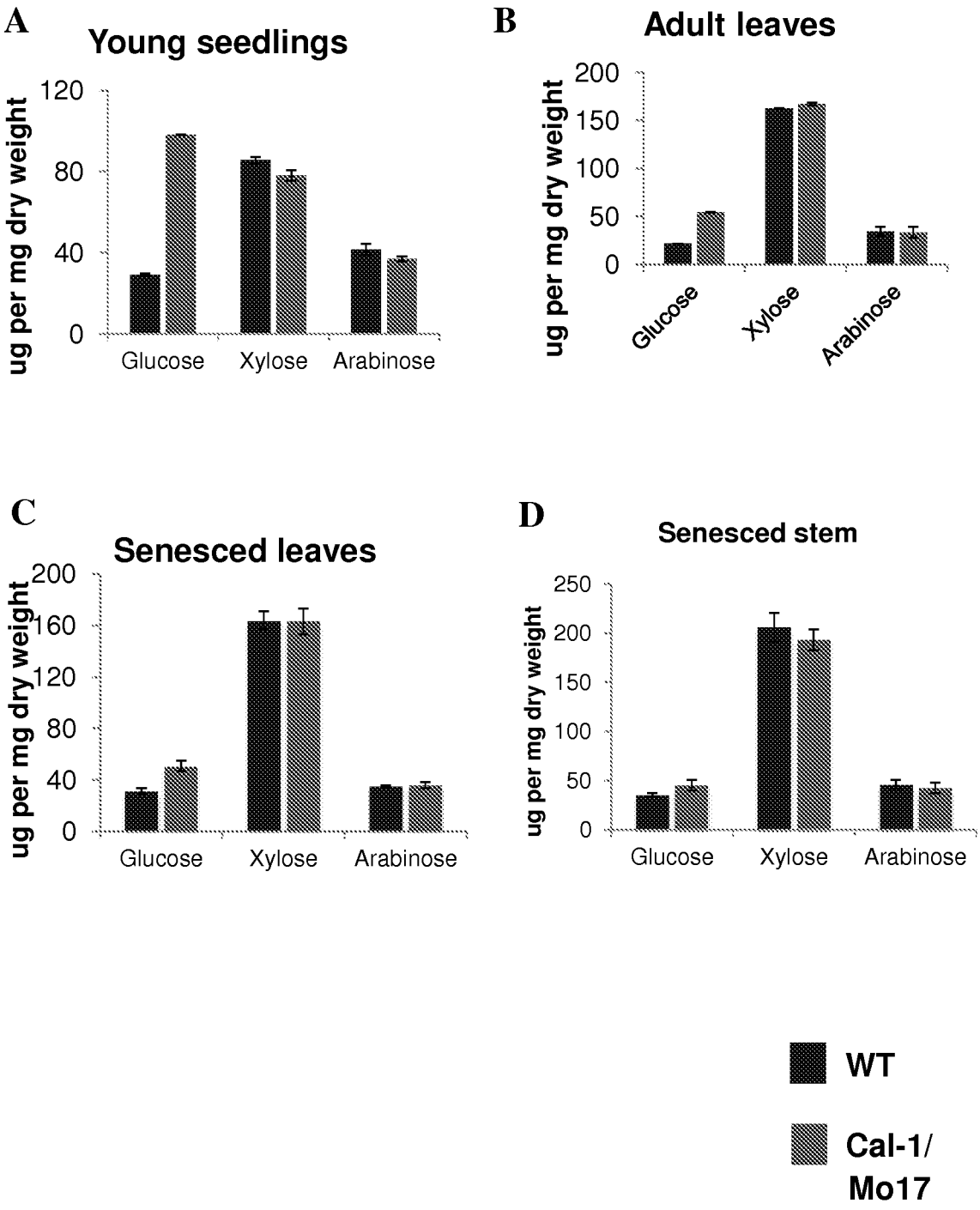

A
Young seedlings
ug per mg dry weight
120
80
40
0
Glucose
Xylose
Arabinose
B
Adult leaves
ug per mg dry weight
200
150
100
50
0
Glucose
Xylose
Arabinose
C
Senesced leaves
ug per mg dry weight
200
160
120
80
40
0
Glucose
Xylose
Arabinose
D
Senesced stem
ug per mg dry weight
250
200
150
100
50
0
Glucose
Xylose
Arabinose
WT
Cal-1/
Mo17

Figure 6

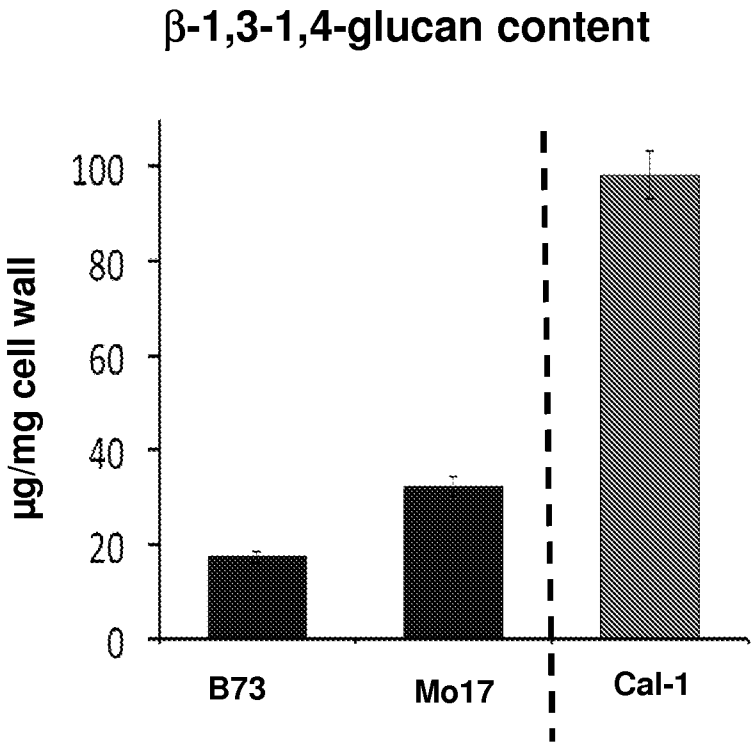
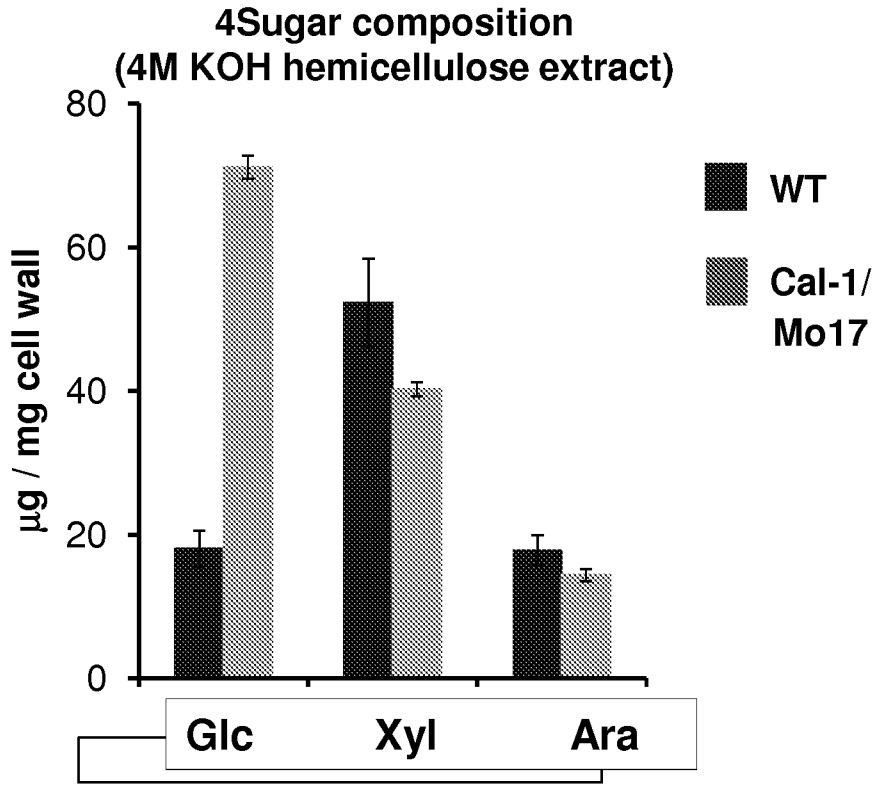

β-1,3-1,4-glucan content
µg/mg cell wall
100
80
60
40
20
0
B73
Mo17
Cal-1

Figure 7

A


4Sugar composition
(4M KOH hemicellulose extract)
µg / mg cell wall
80
60
40
20
0
WT
Cal-1/
Mo17
Glc
Xyl
Ara

B

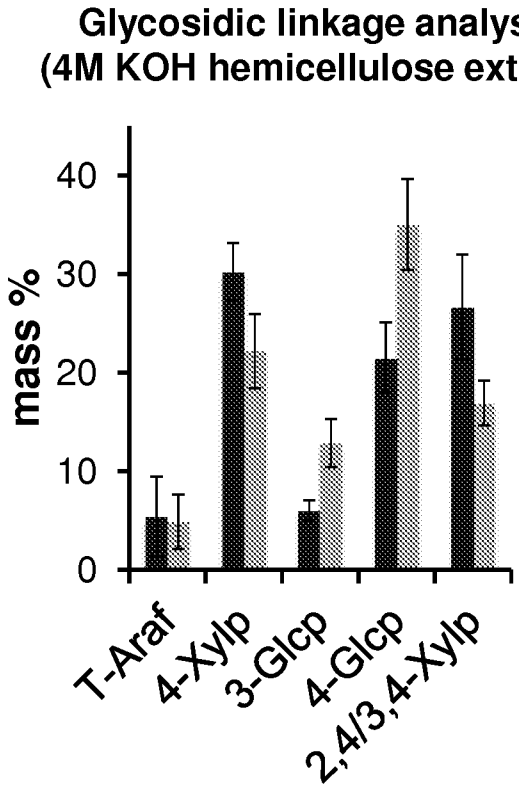
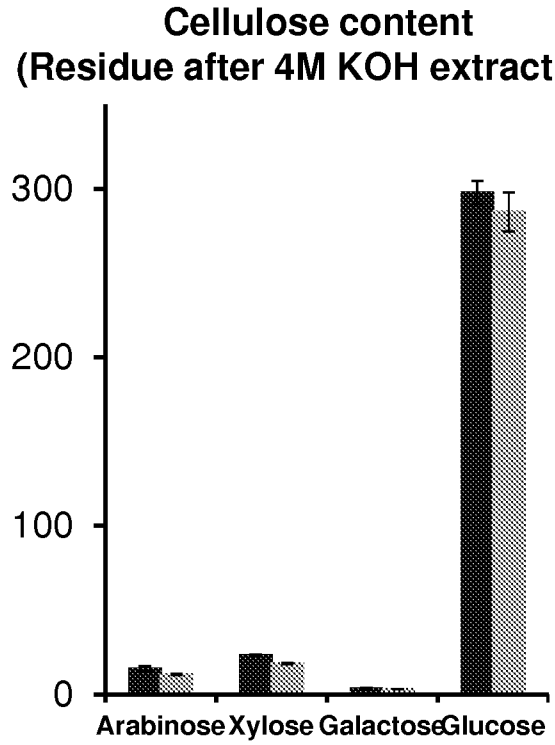

Glycosidic linkage analysis
(4M KOH hemicellulose extract)
mass %
40
30
20
10
0
T-Araf
4-Xylp
3-Glcp
4-Glcp
2,4/3,4-Xylp

Figure 8

A


Cellulose content
(Residue after 4M KOH extraction)
300
200
100
0
Arabinose
Xylose
Galactose
Glucose

B

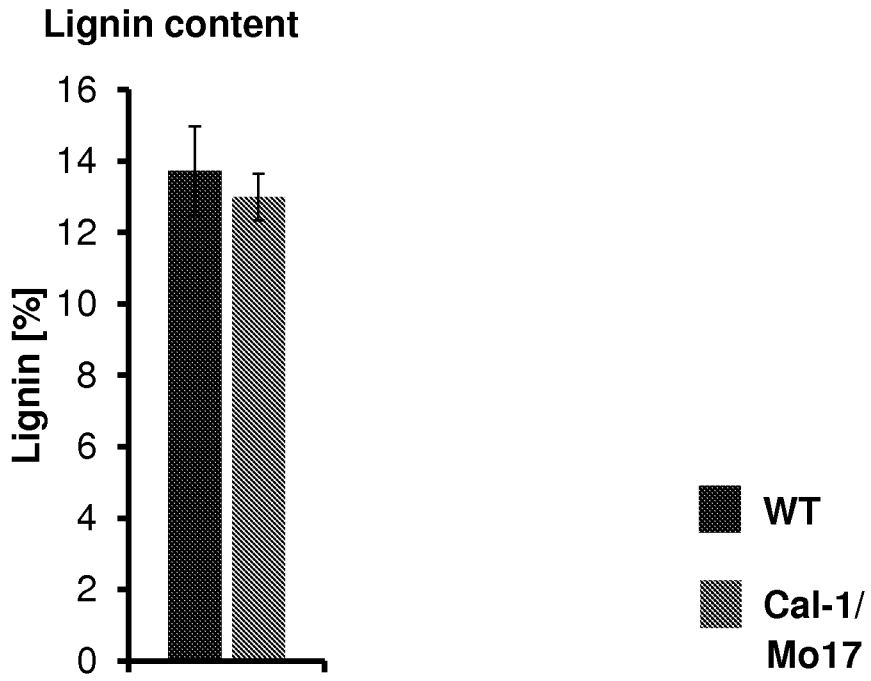

Lignin content
Lignin [%]
16
14
12
10
8
6
4
2
0
WT
Cal-1/
Mo17

Figure 9

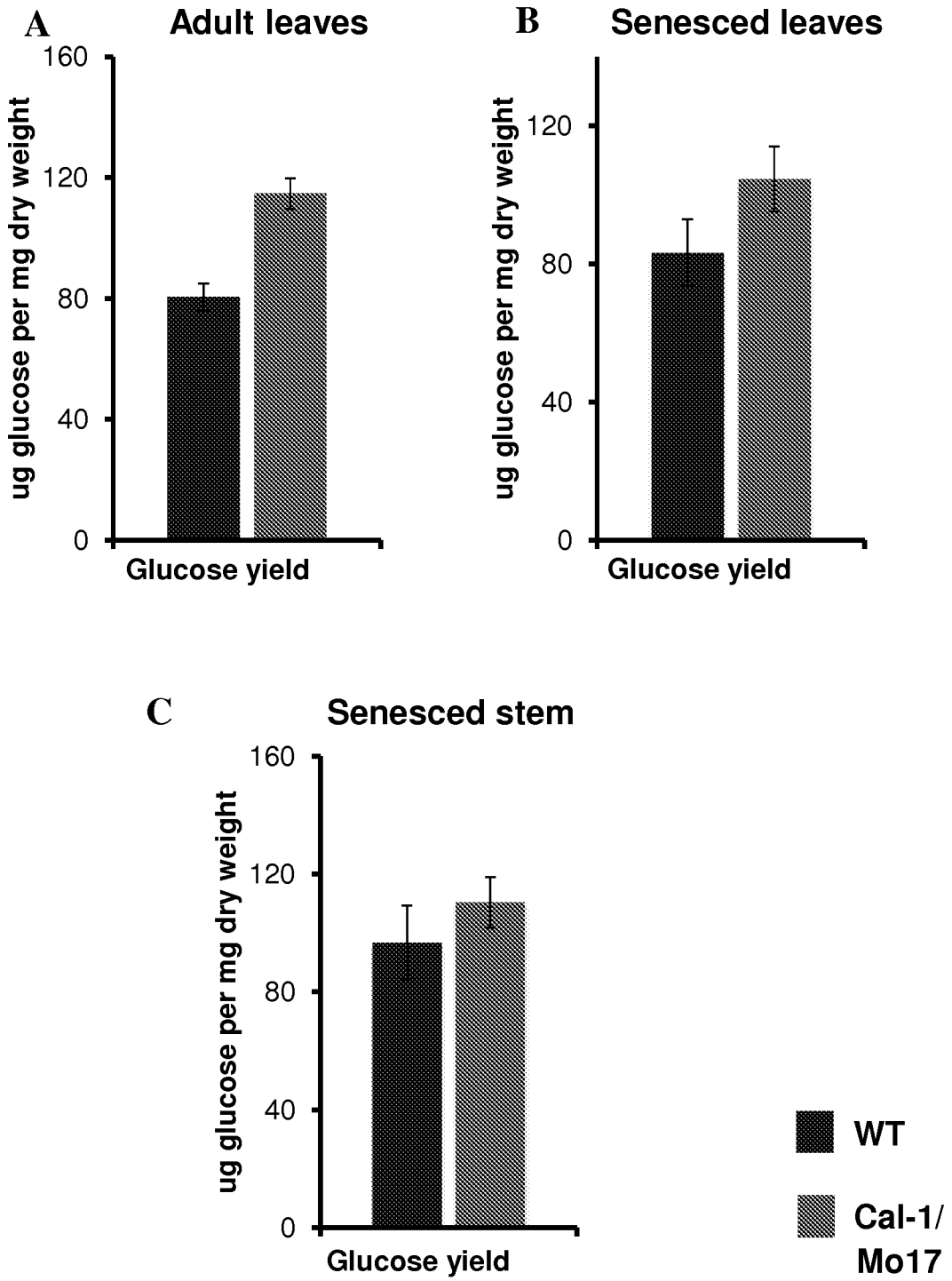

A
Adult leaves
160
120
80
40
0
ug glucose per mg dry weight
Glucose yield
B
Senesced leaves
120
80
40
0
ug glucose per mg dry weight
Glucose yield
C
Senesced stem
160
120
80
40
0
ug glucose per mg dry weight
Glucose yield
WT
Cal-1/
Mo17

Figure 10

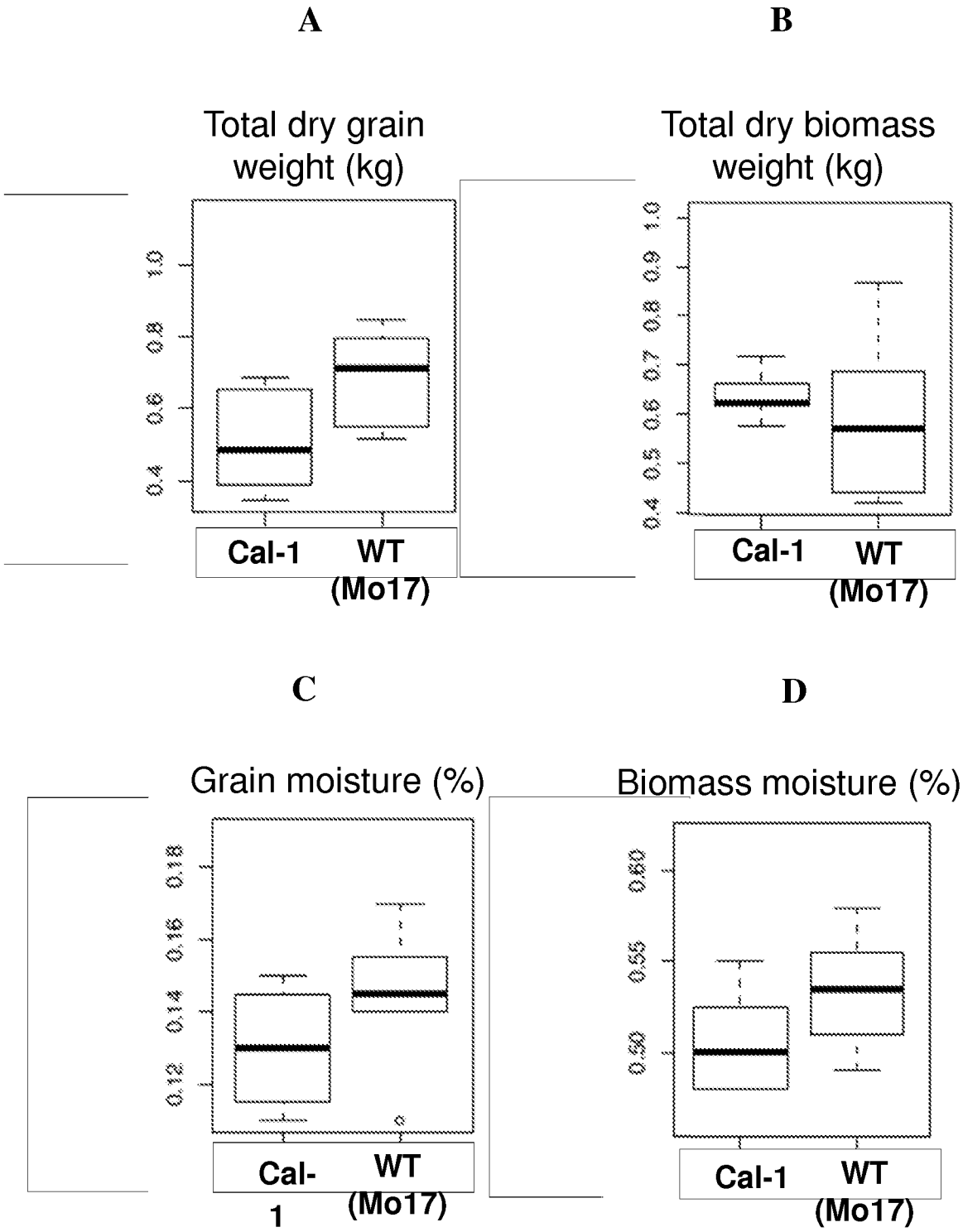

A
Total dry grain weight (kg)
0.4
0.6
0.8
1.0
Cal-1
WT (Mo17)
B
Total dry biomass weight (kg)
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Cal-1
WT (Mo17)
C
Grain moisture (%)
0.12
0.14
0.16
0.18
Cal-1
WT (Mo17)
D
Biomass moisture (%)
0.50
0.55
0.60
Cal-1
WT (Mo17)

Figure 11

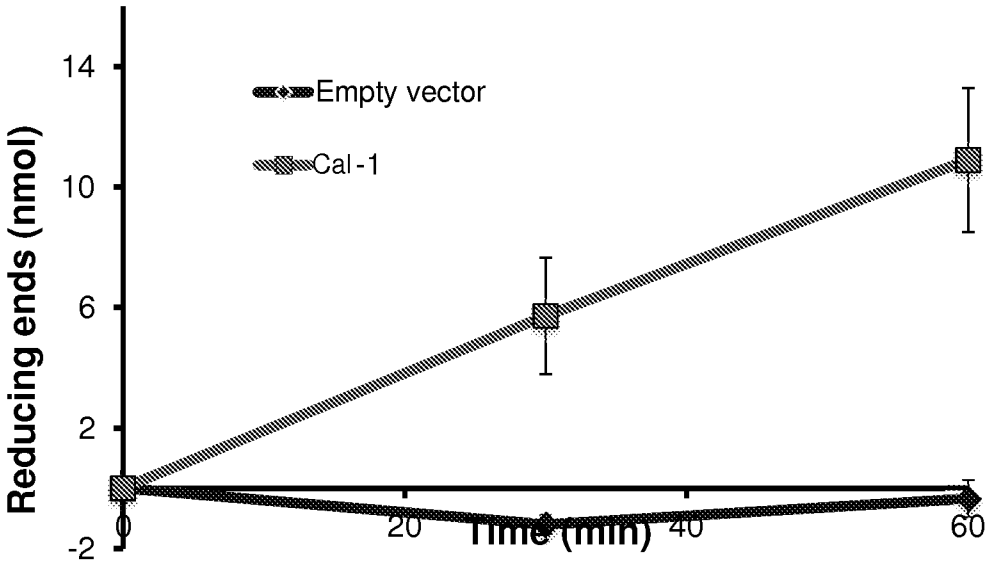
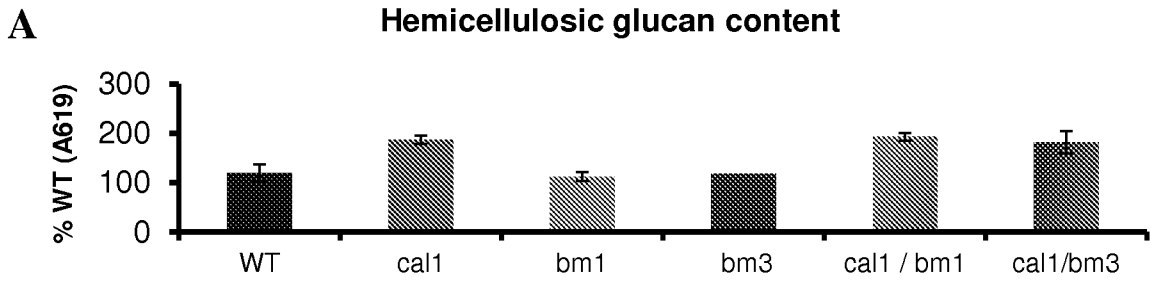

14
10
6
2
-2
Reducing ends (nmol)
Empty vector
Cal-1
0
20
40
60
Time (min)

Figure 12


A
Hemicellulosic glucan content
% WT (A619)
300
200
100
0
WT
cal1
bm1
bm3
cal1 / bm1
cal1/bm3

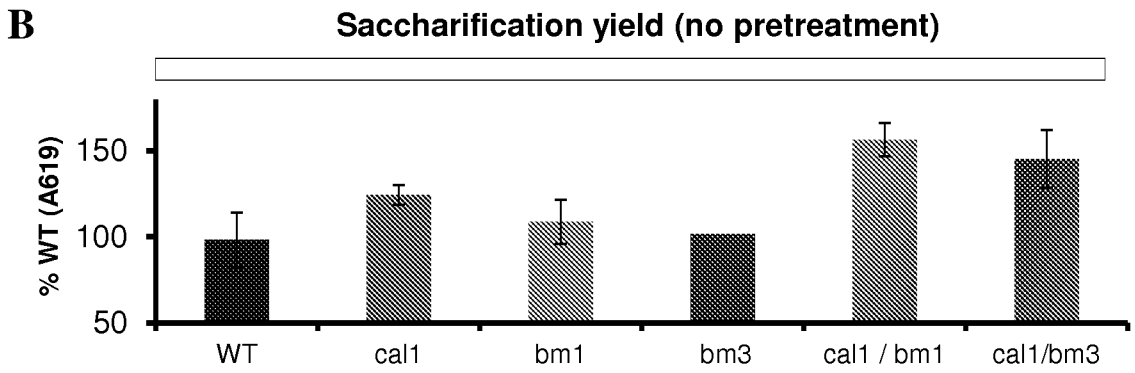

B
Saccharification yield (no pretreatment)
% WT (A619)
150
100
50
WT
cal1
bm1
bm3
cal1 / bm1
cal1/bm3

Figure 13

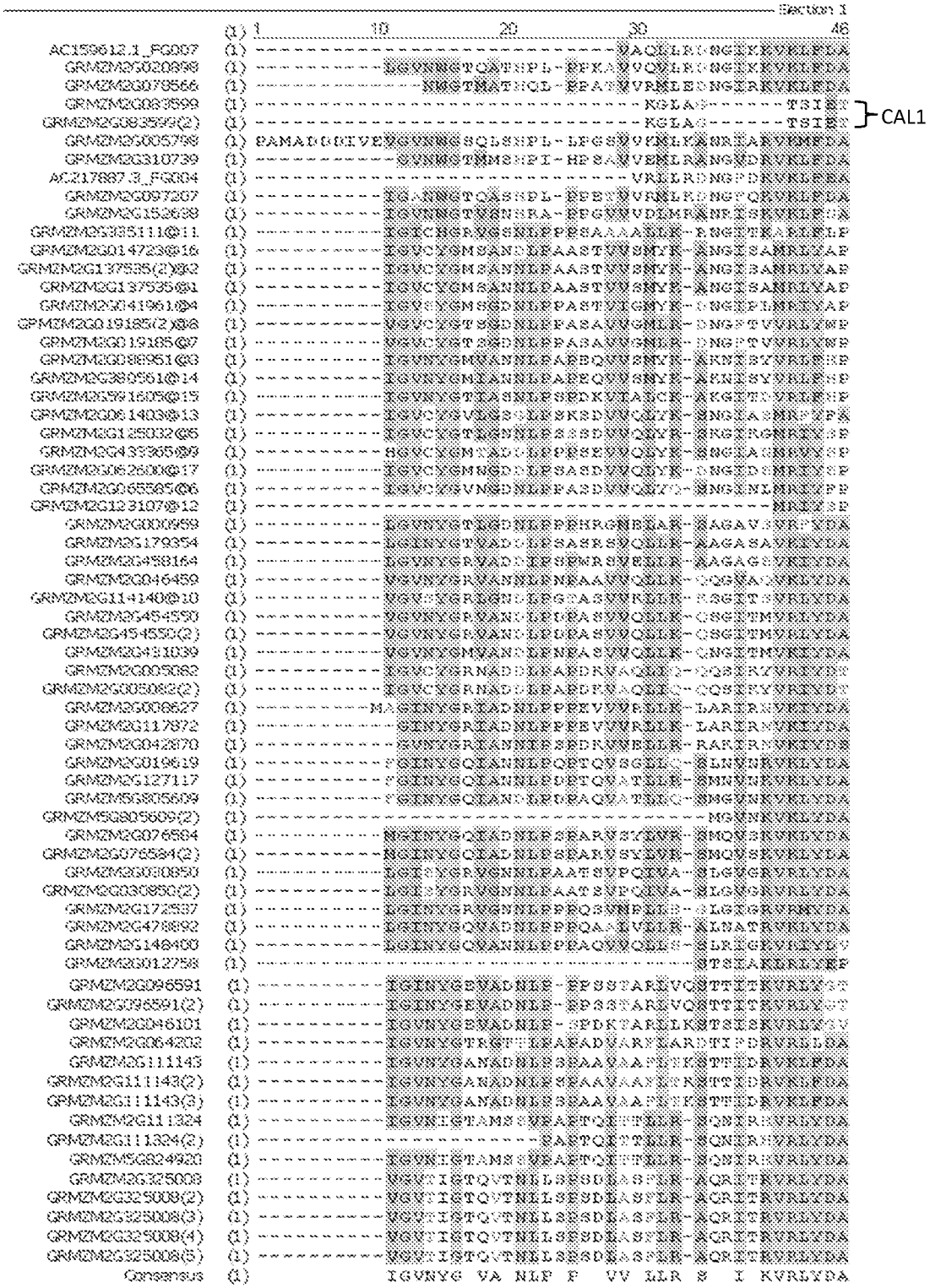


Figure 14

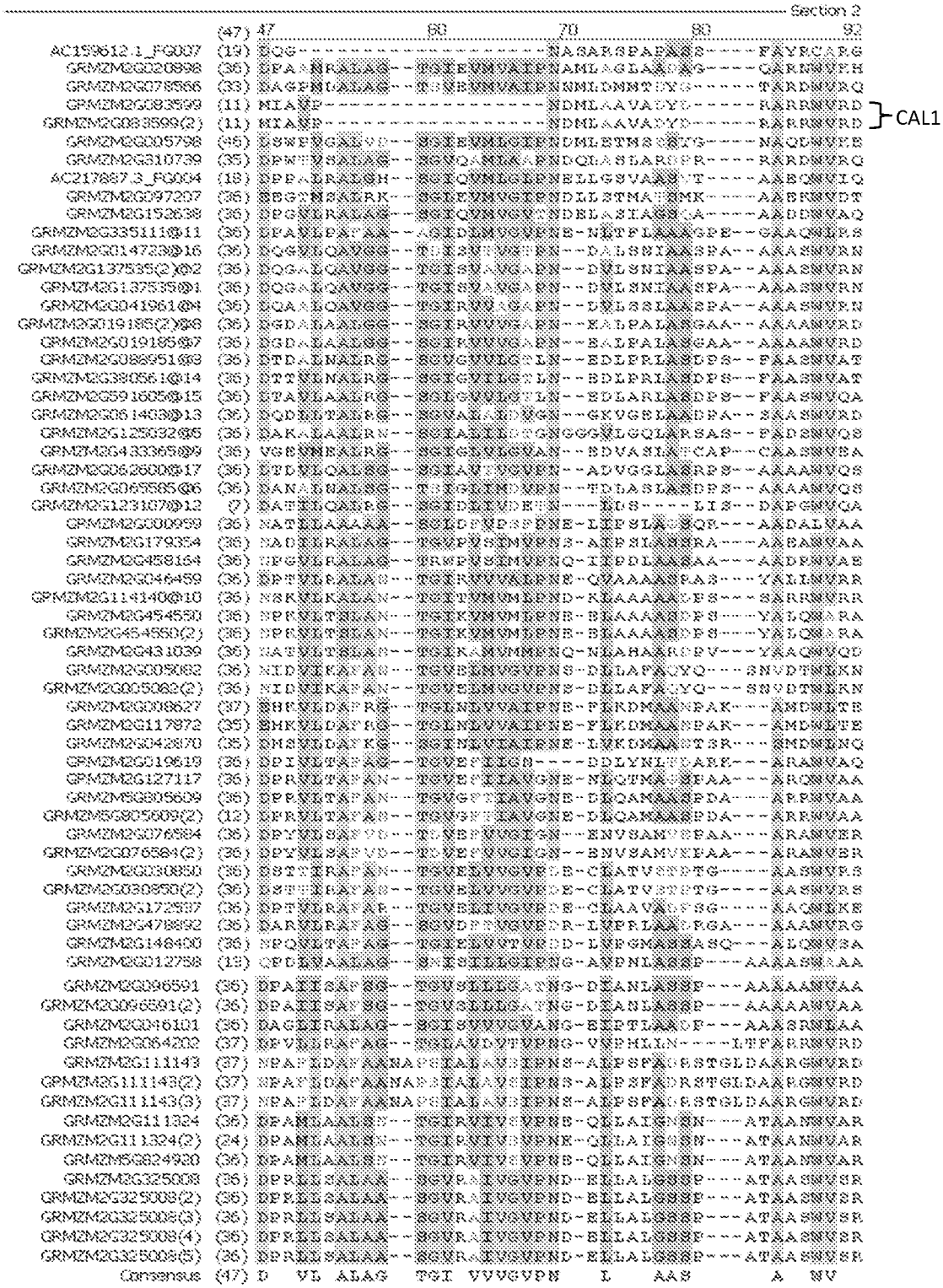


Figure 14 Continued

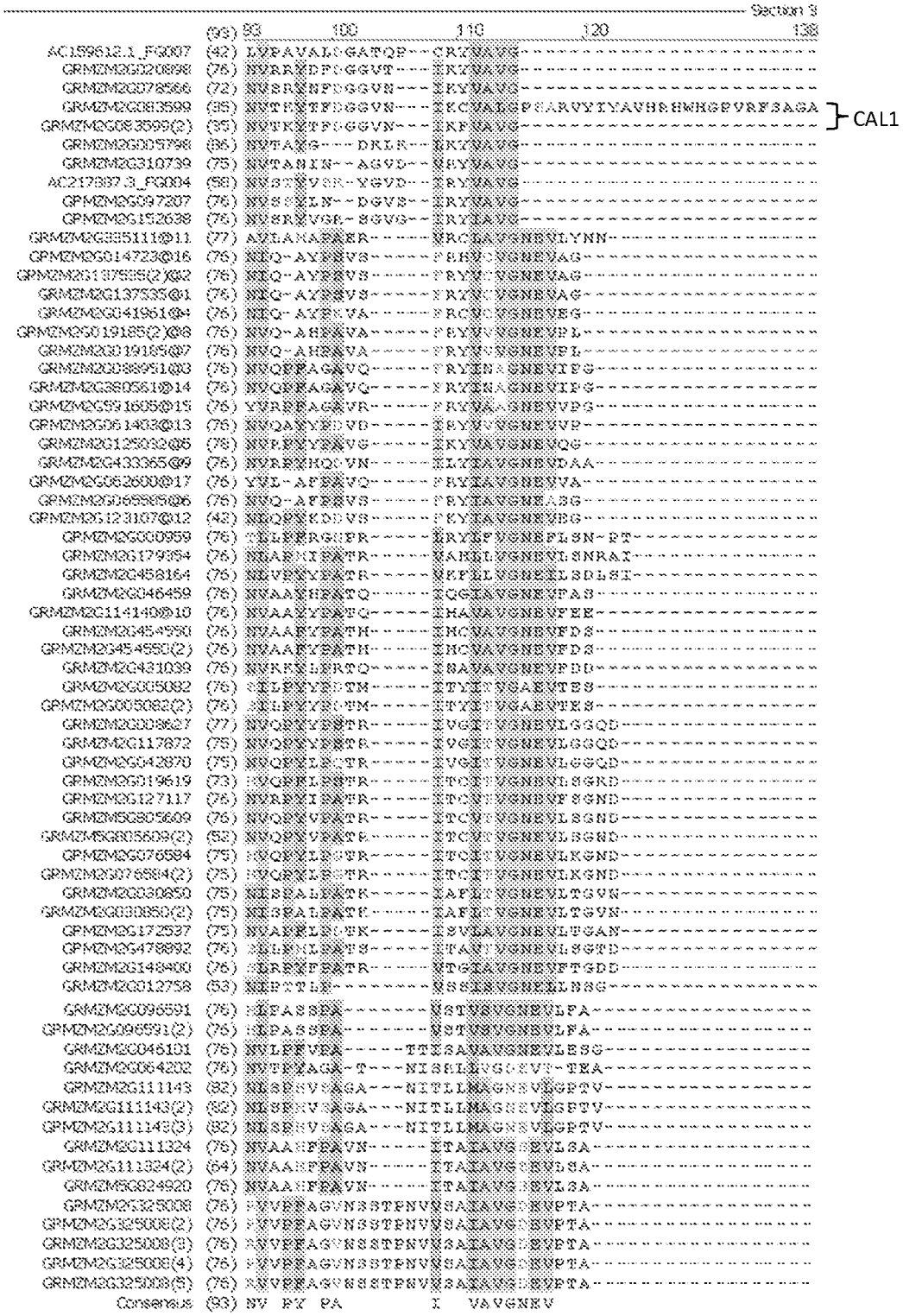


Figure 14 Continued

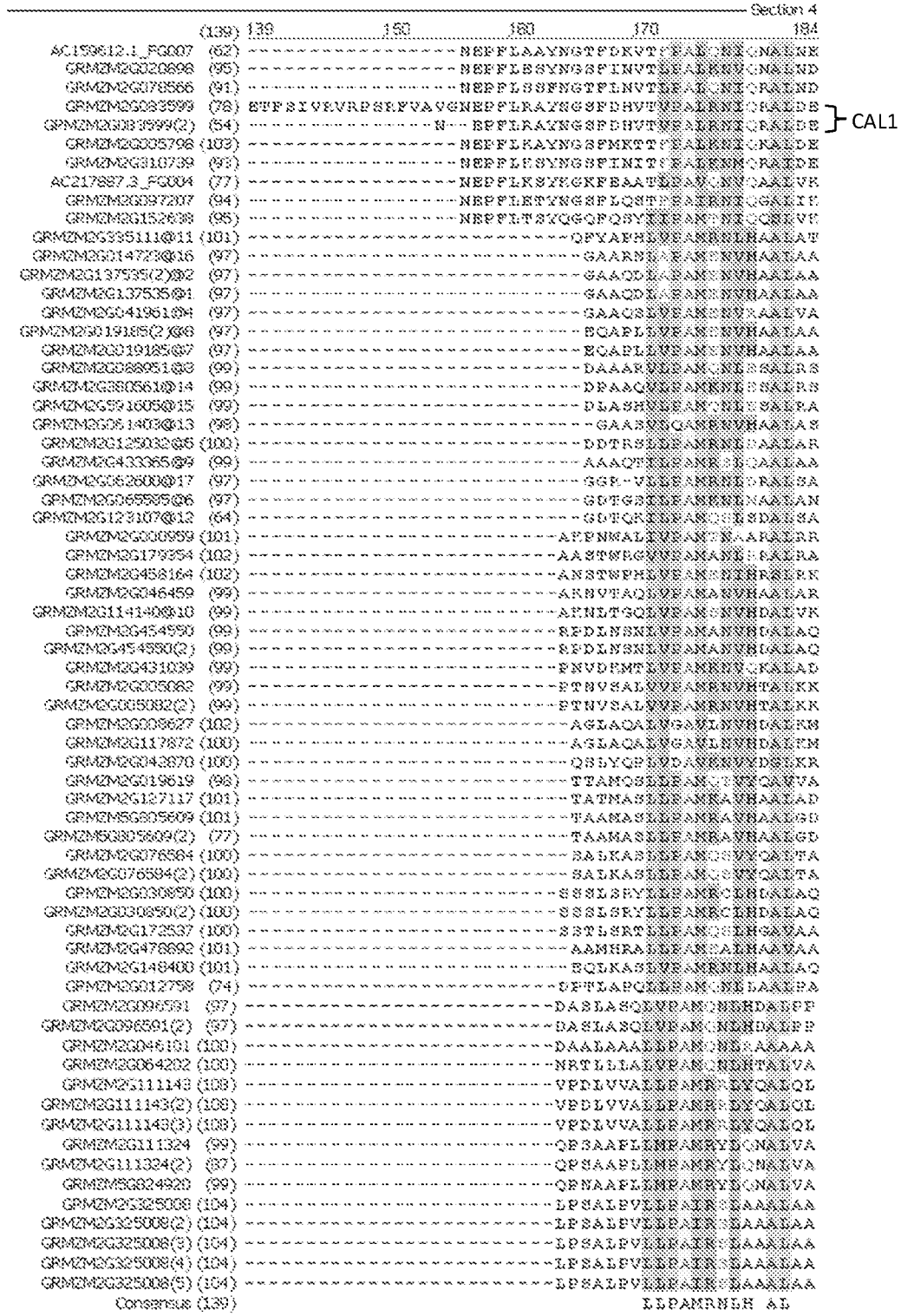


Figure 14 Continued

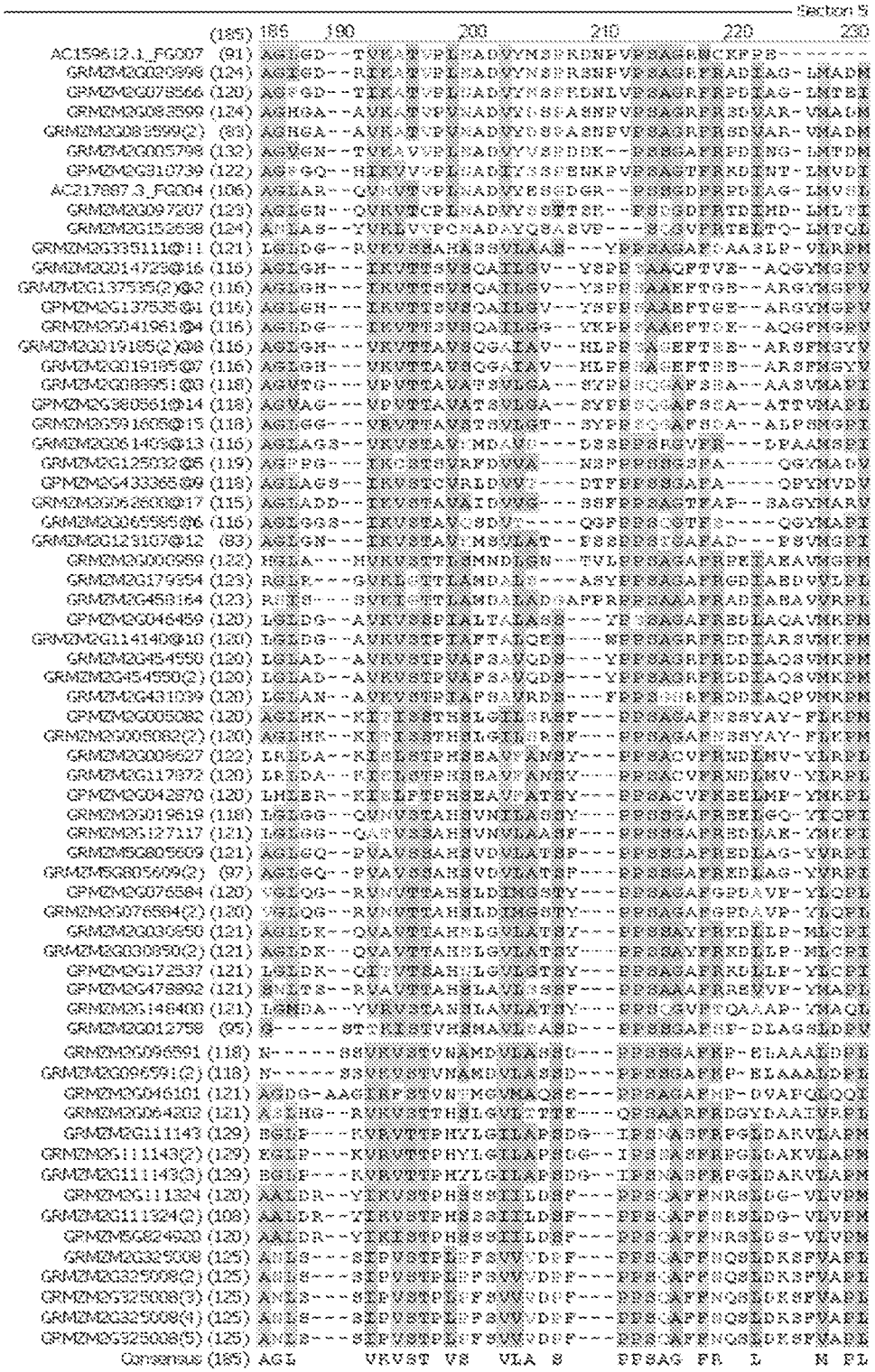


Figure 14 Continued

Figure 14 Continued

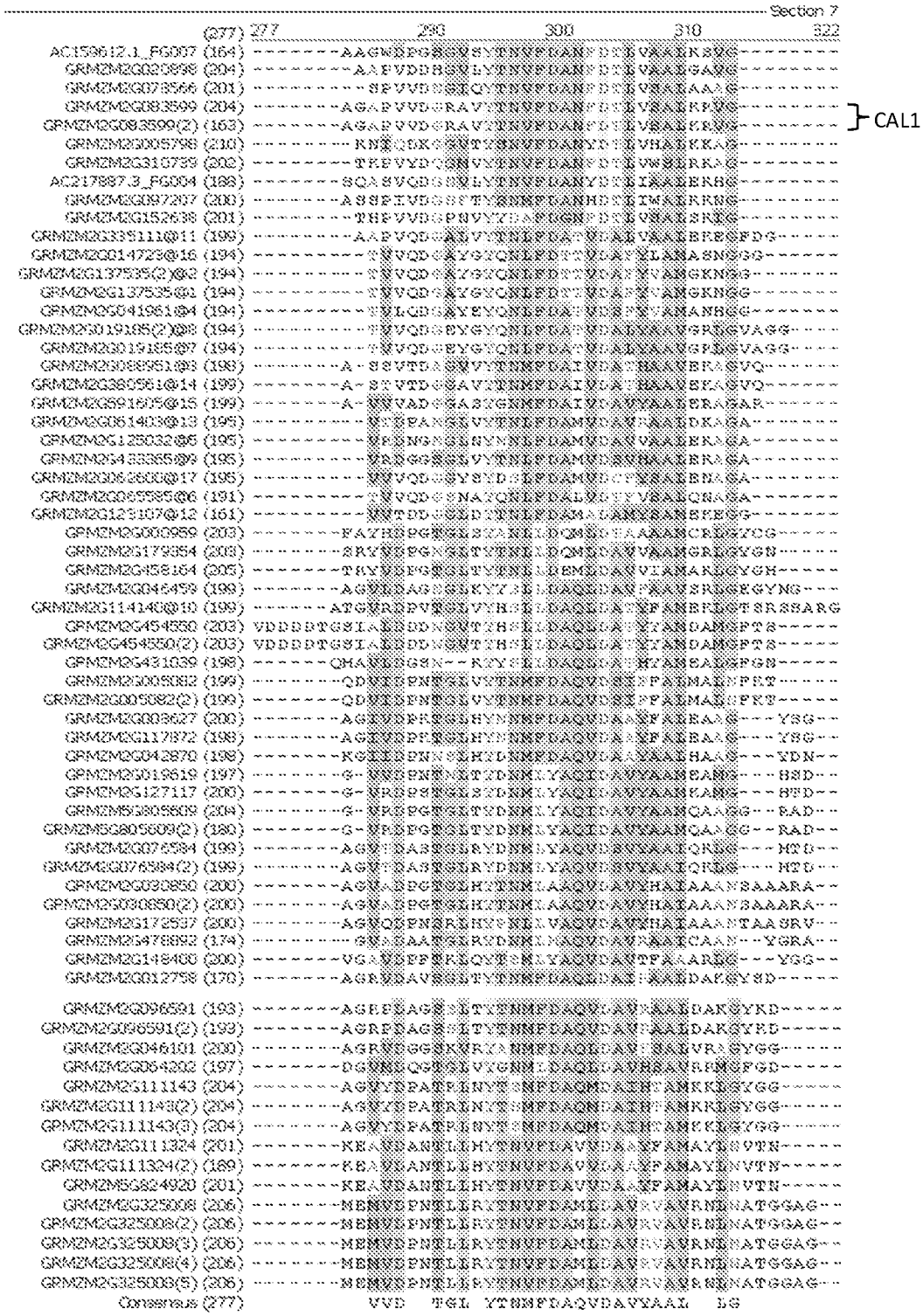


Figure 14 Continued

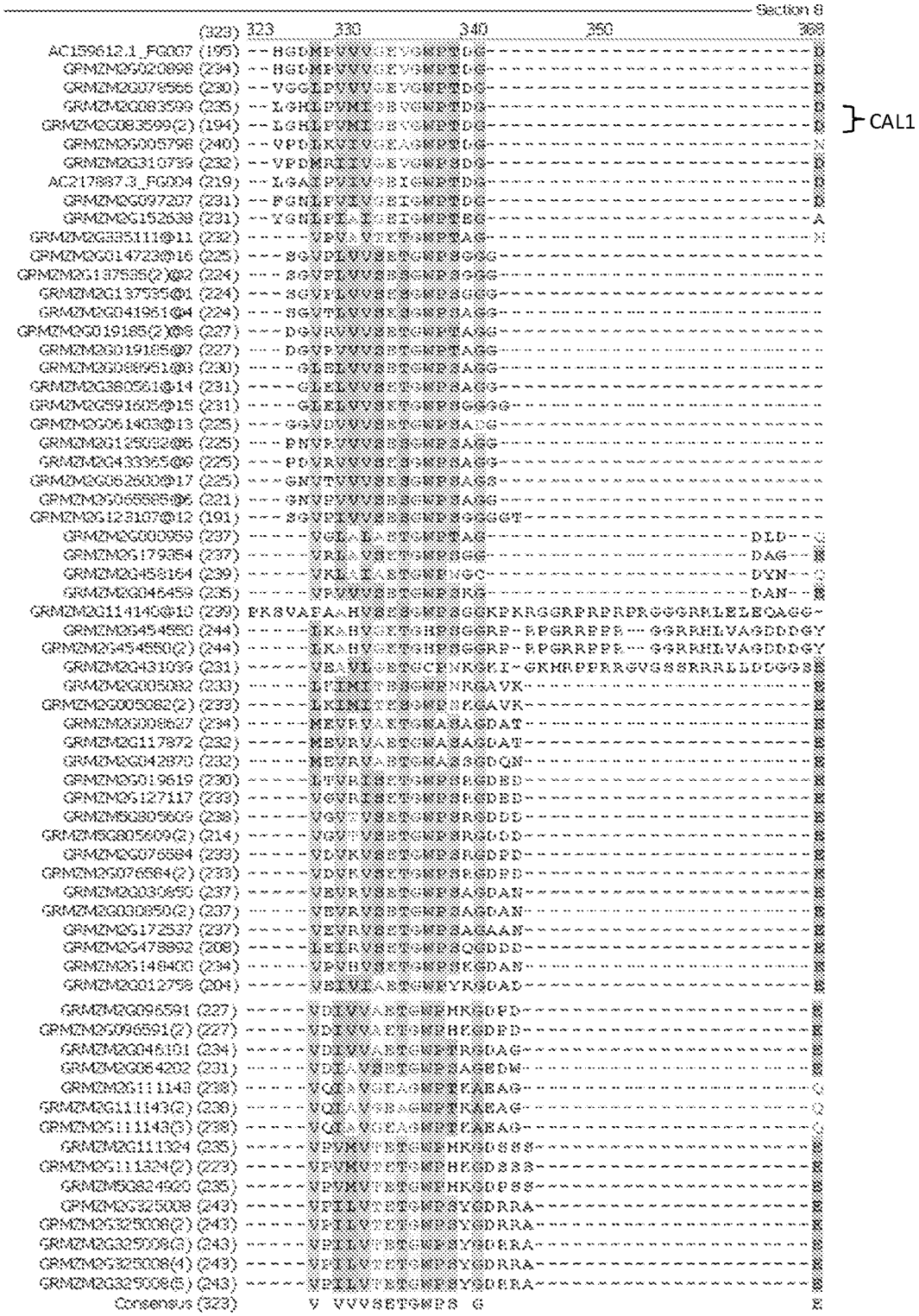


Figure 14 Continued

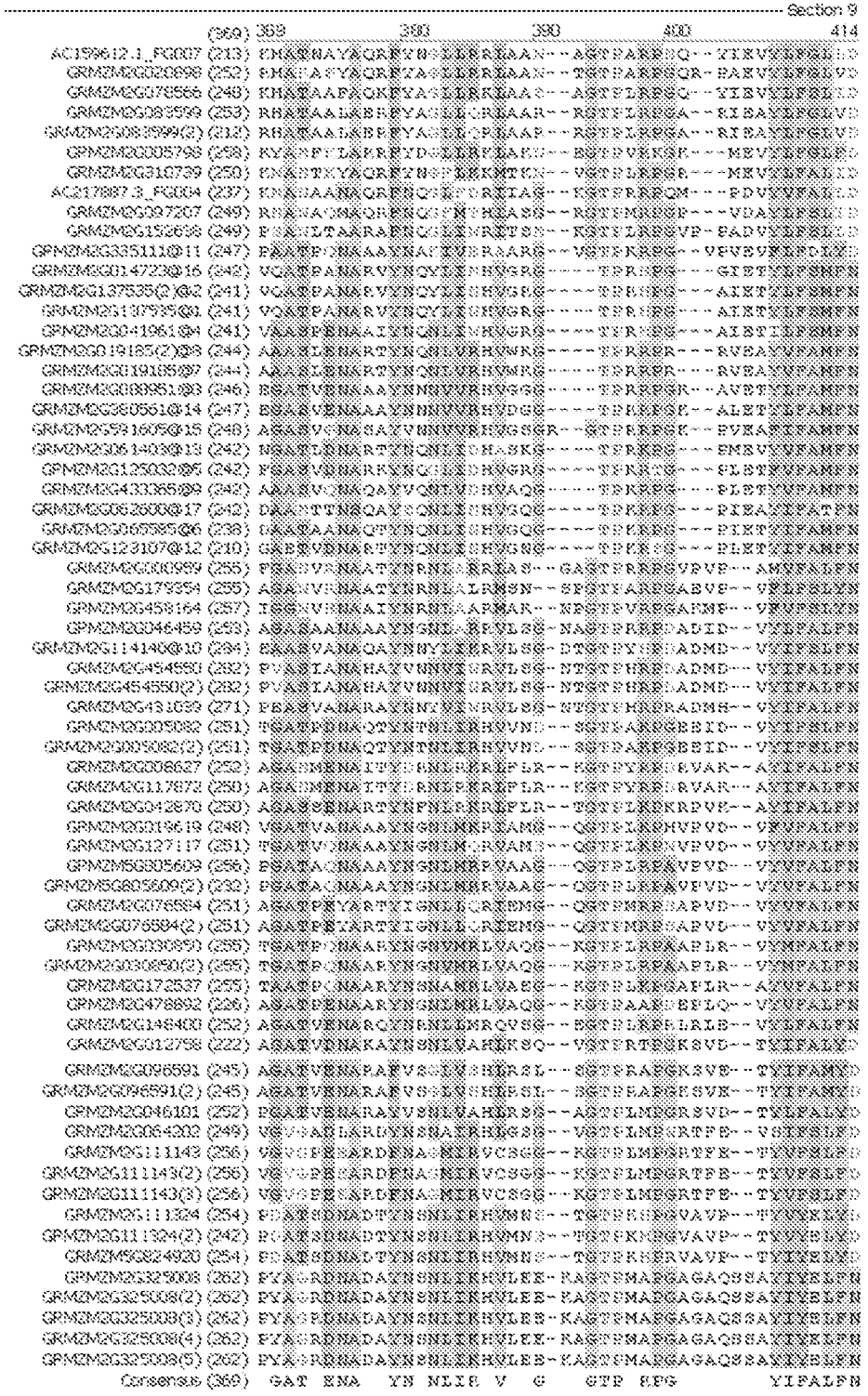


Figure 14 Continued

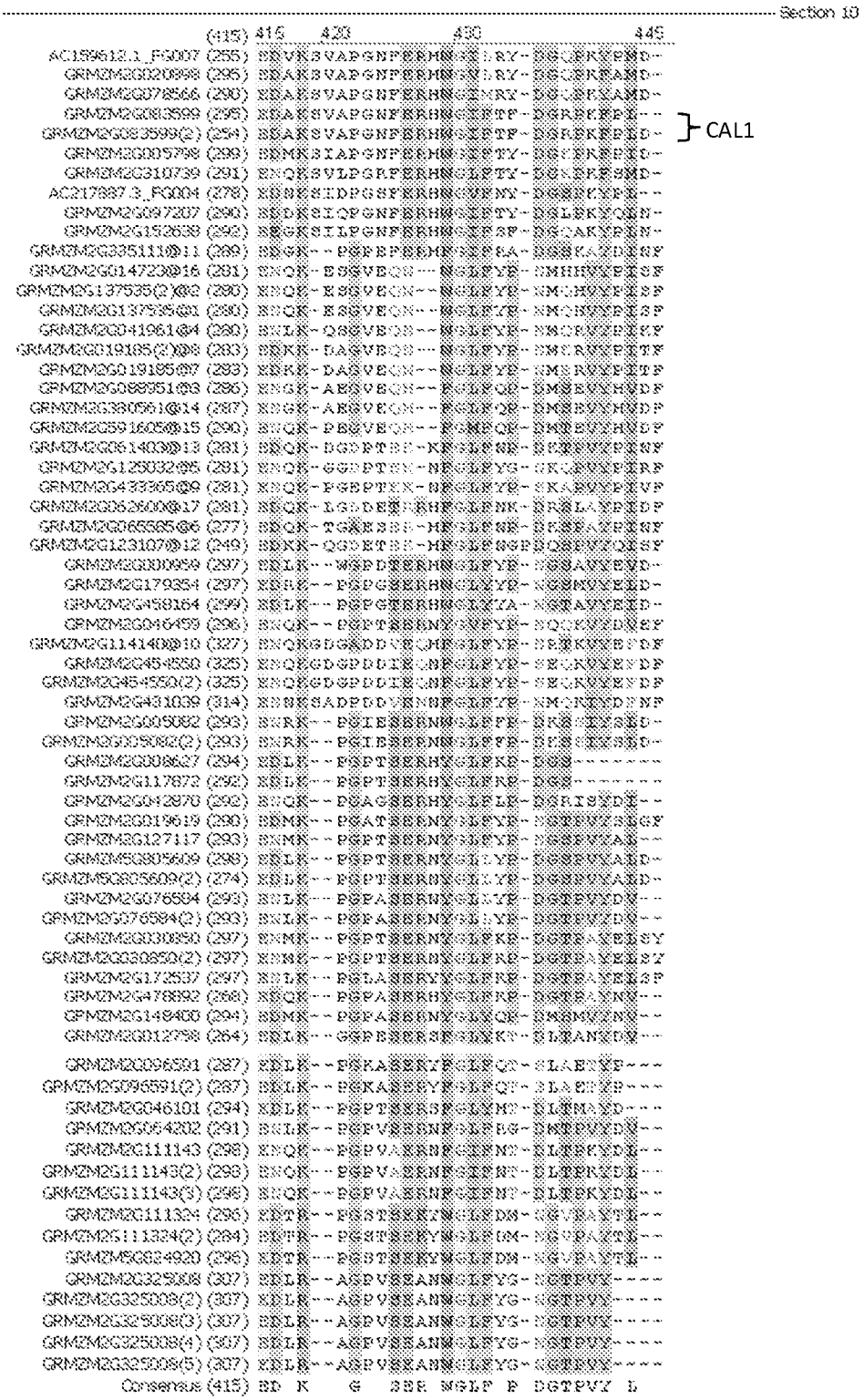


Figure 14 Continued

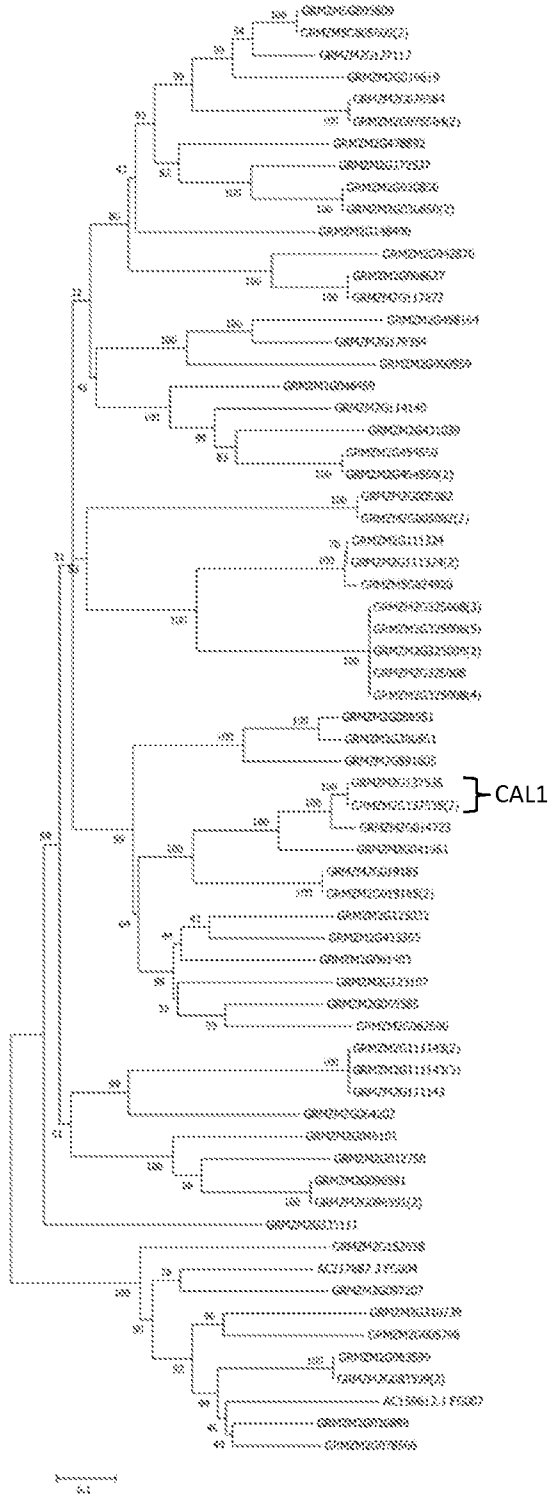


Figure 15

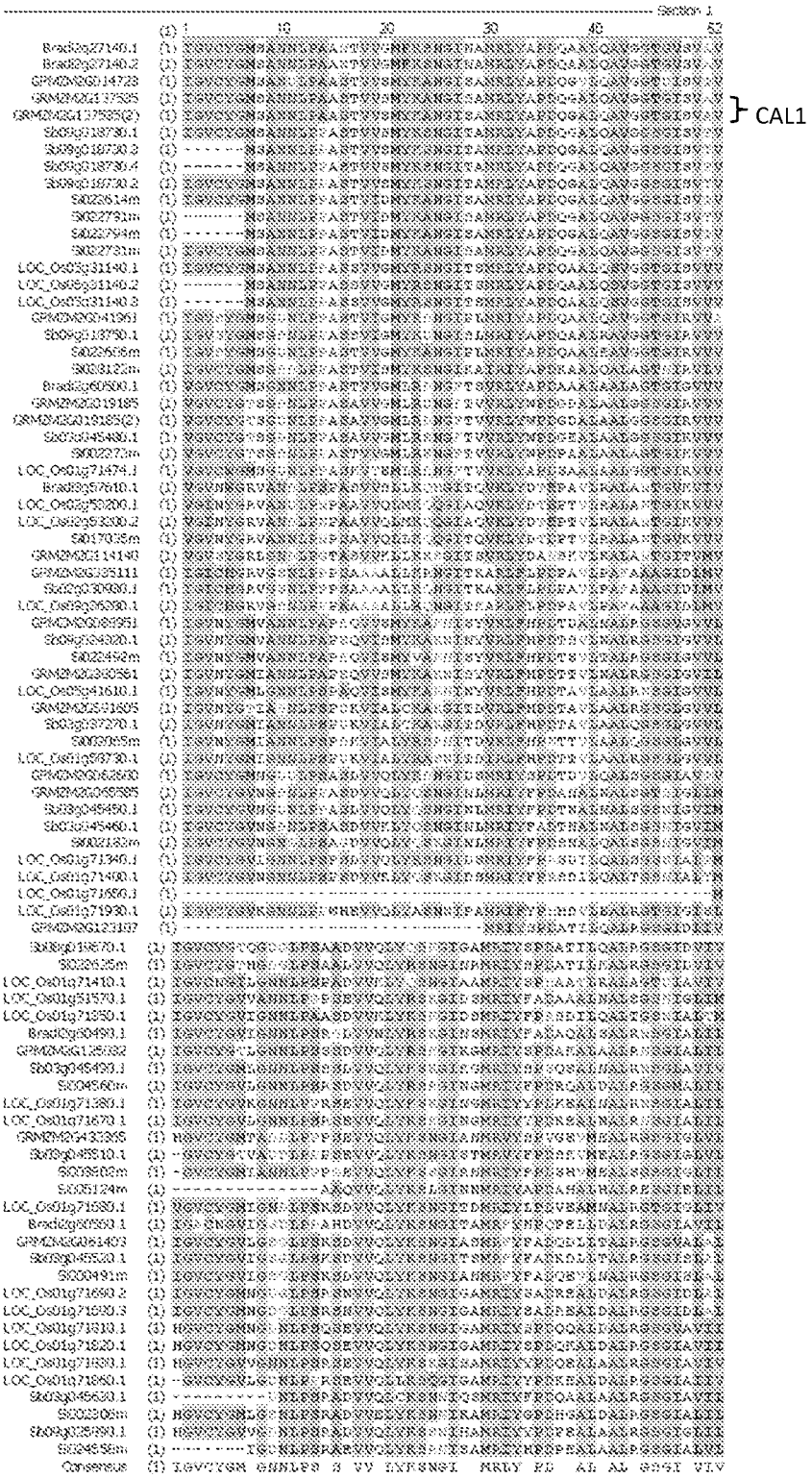


Figure 16

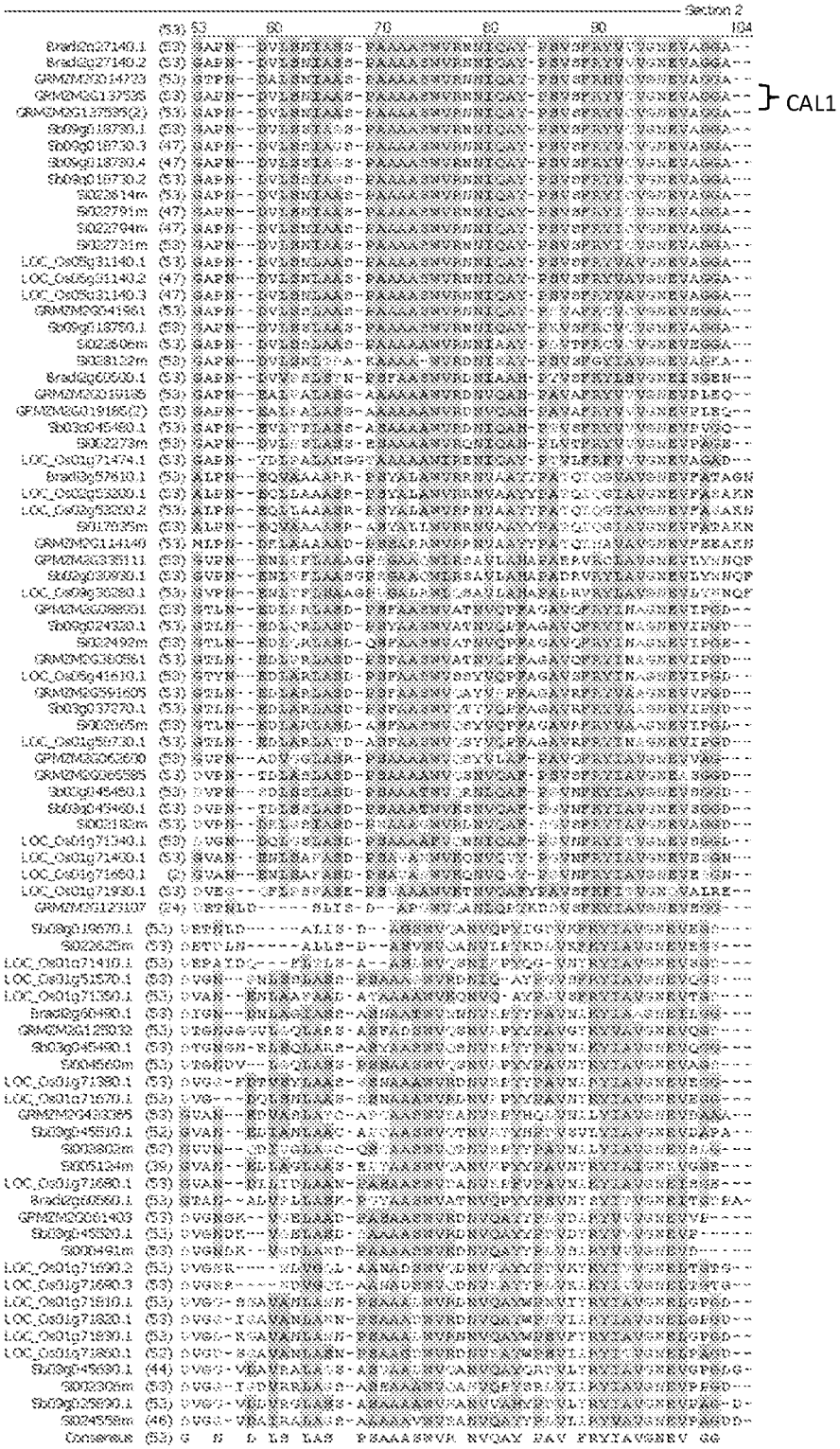


Figure 16 Continued

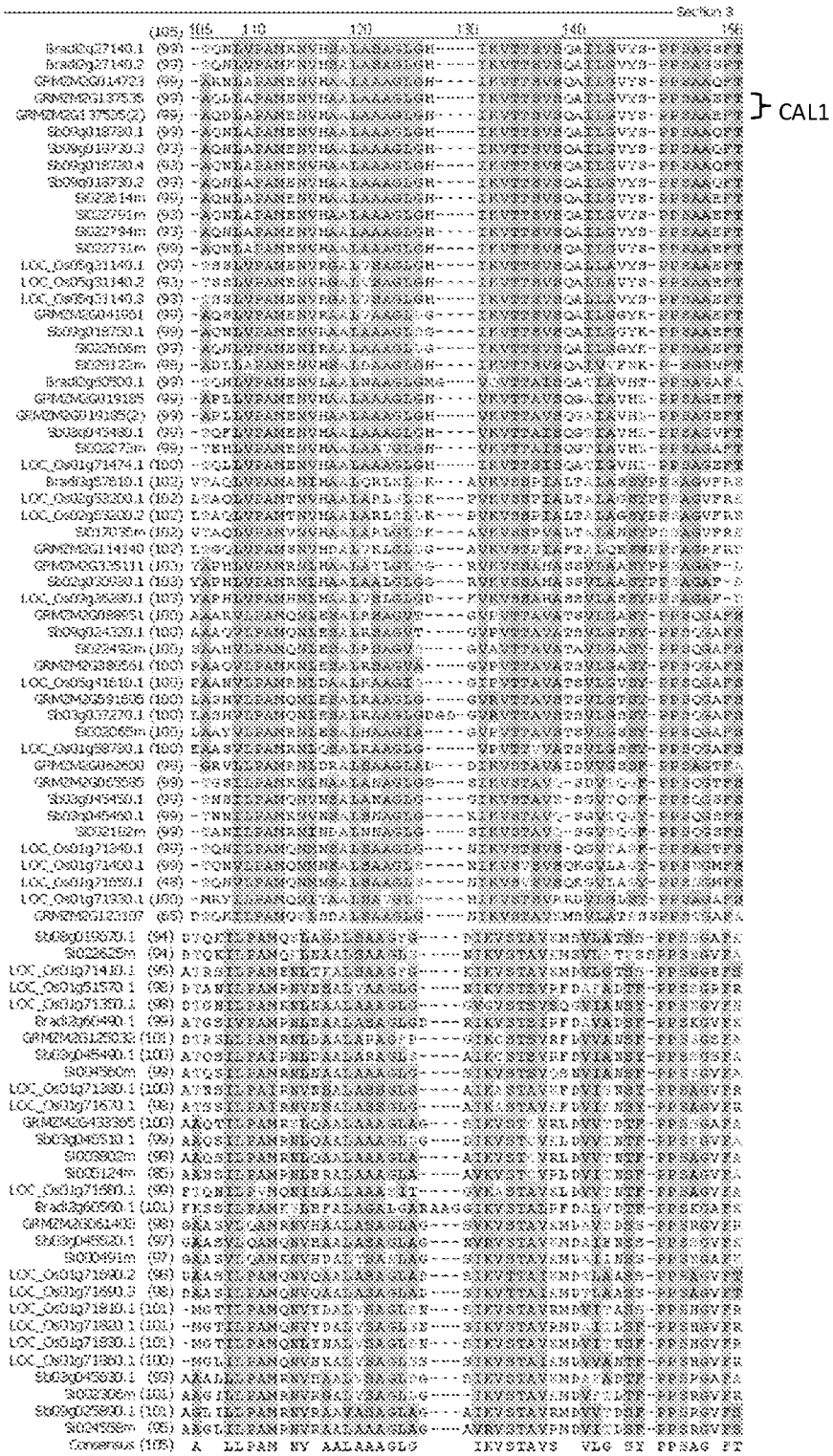


Figure 16 Continued

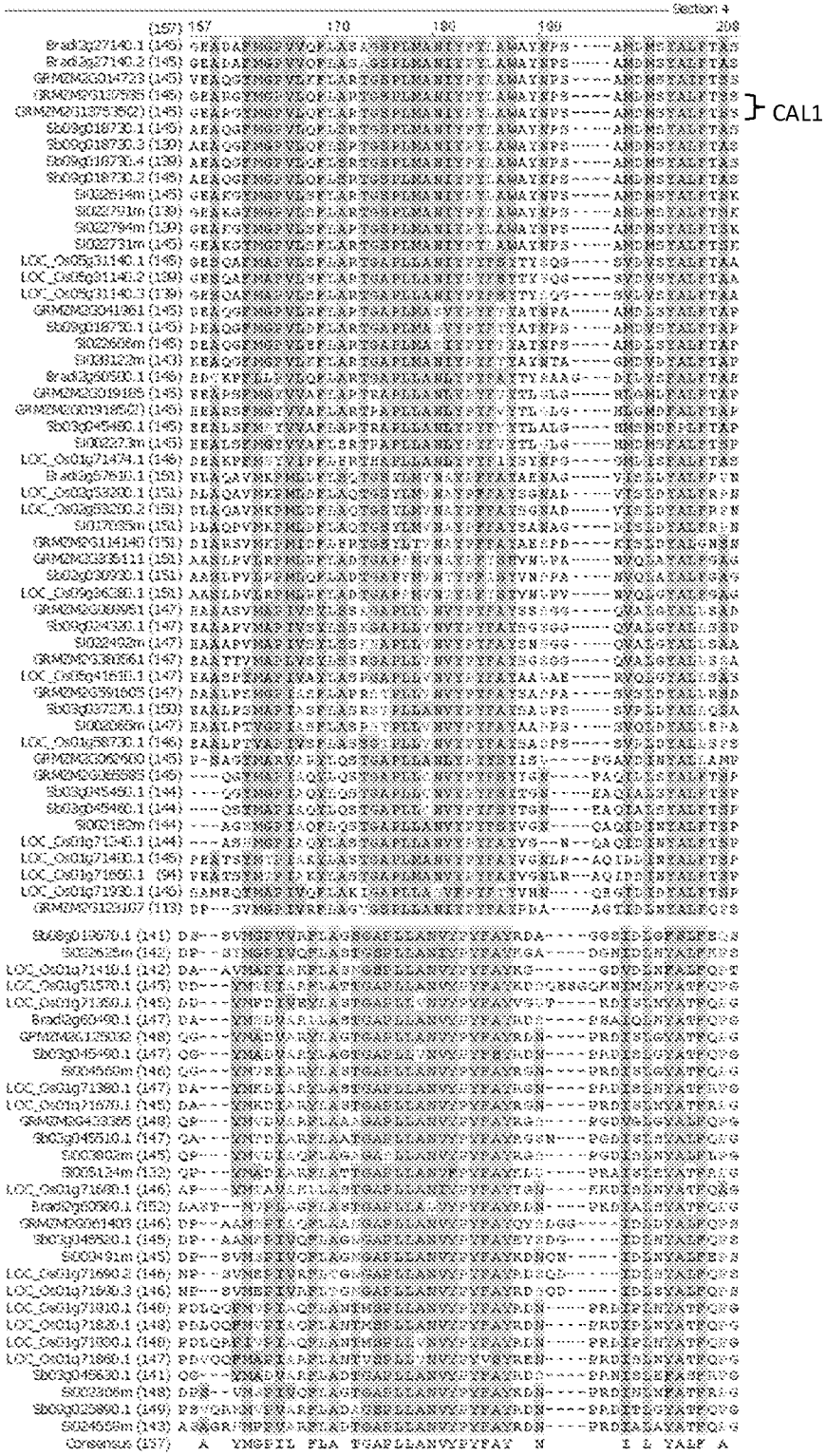


Figure 16 Continued

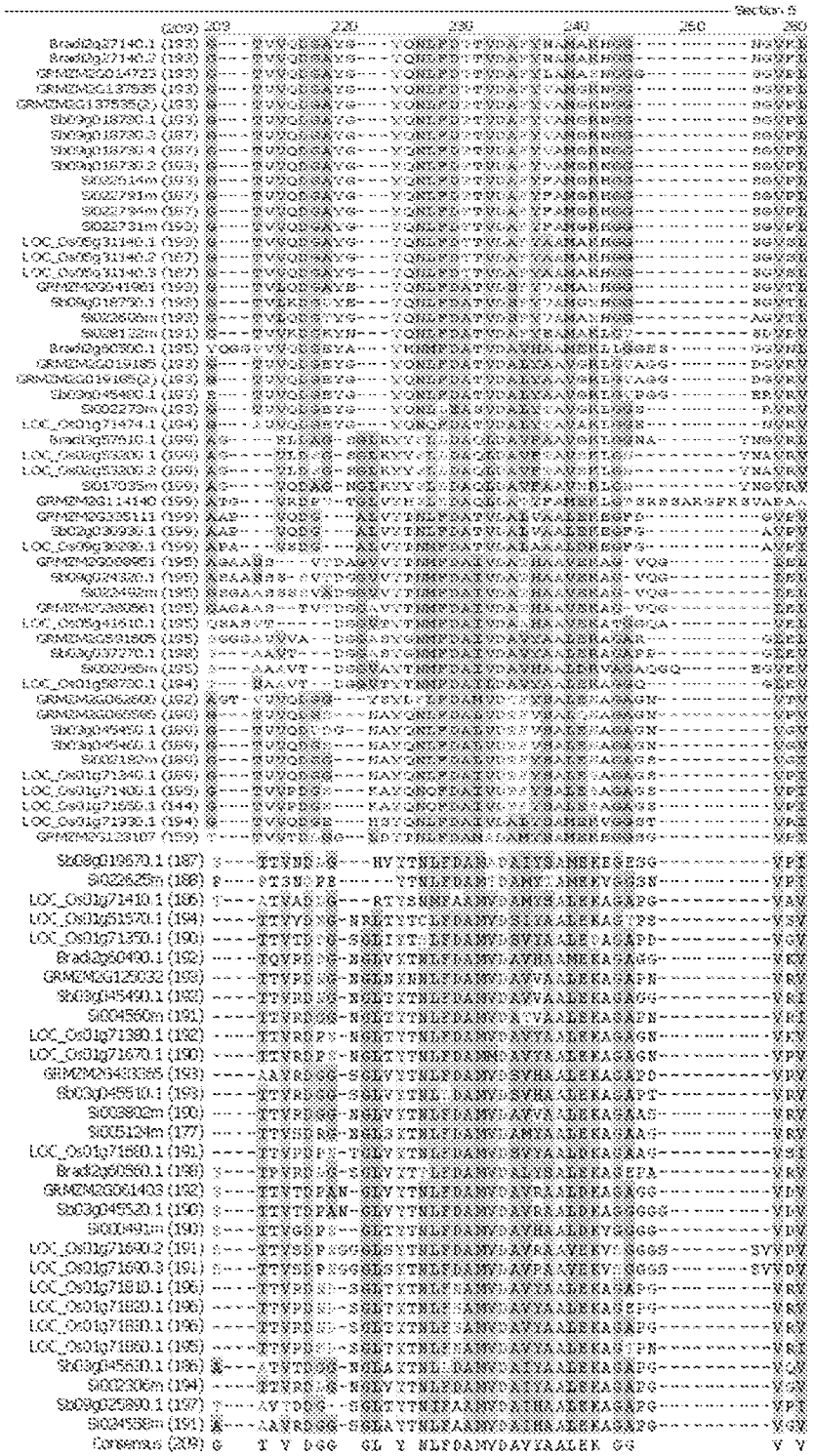


Figure 16 Continued

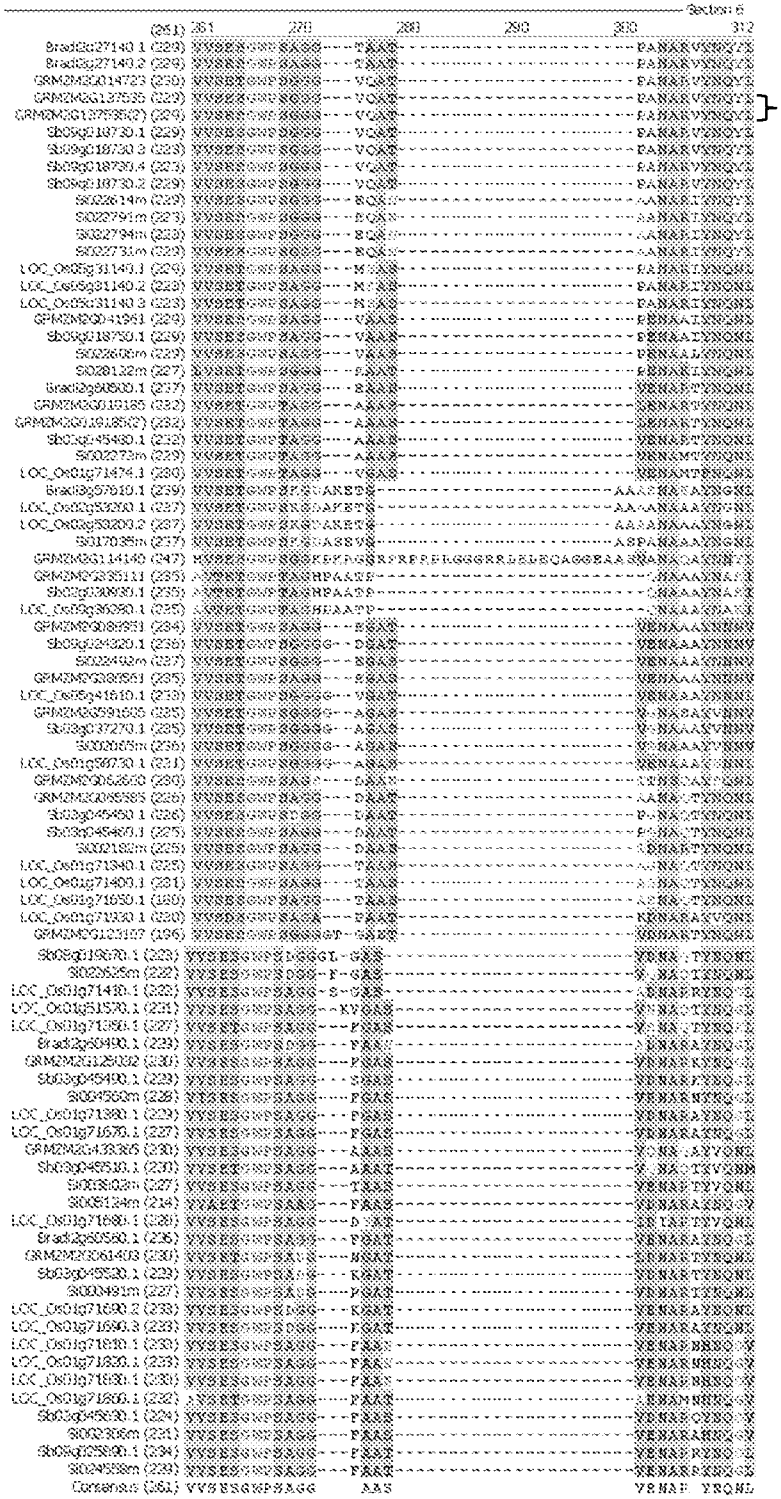


Figure 16 Continued

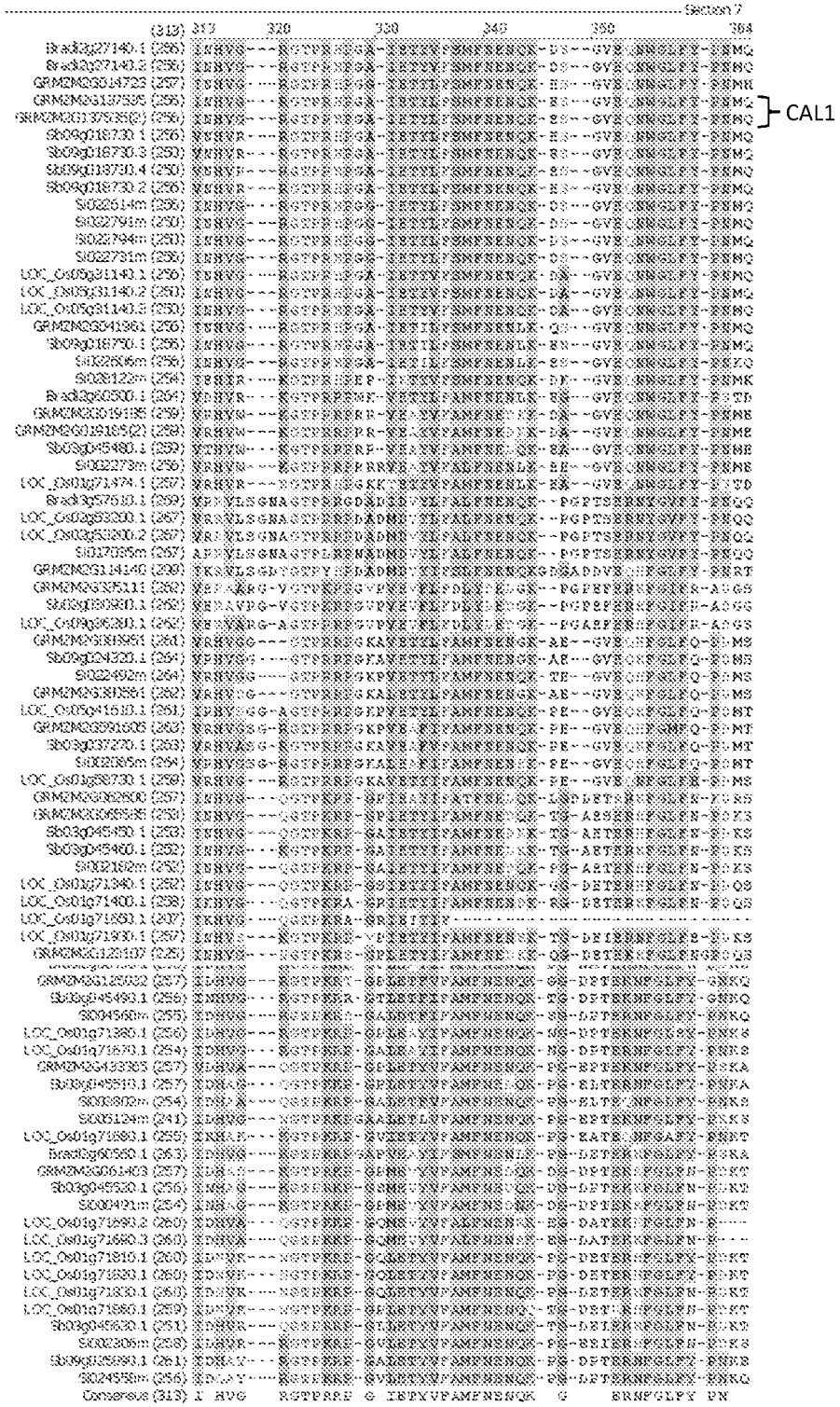


Figure 16 Continued

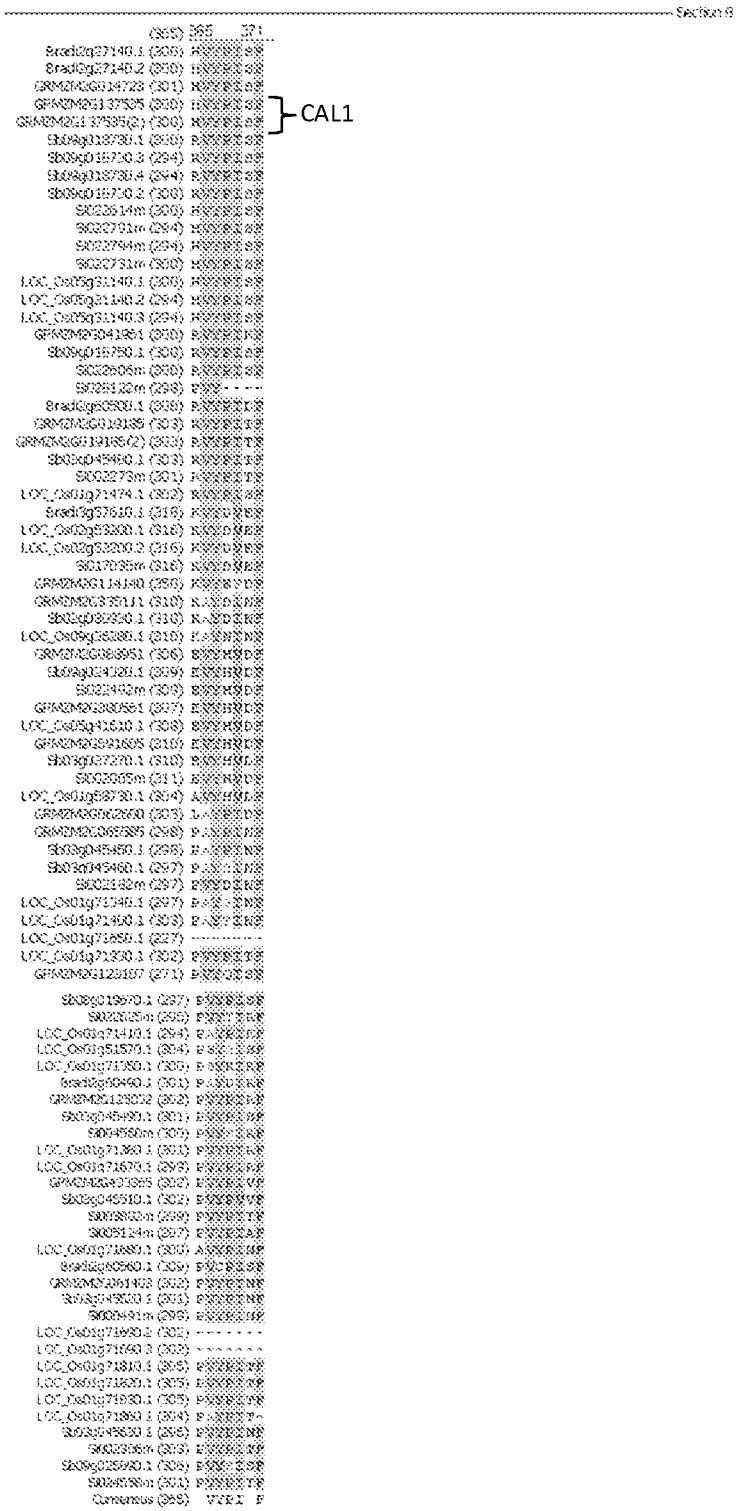


Figure 16 Continued

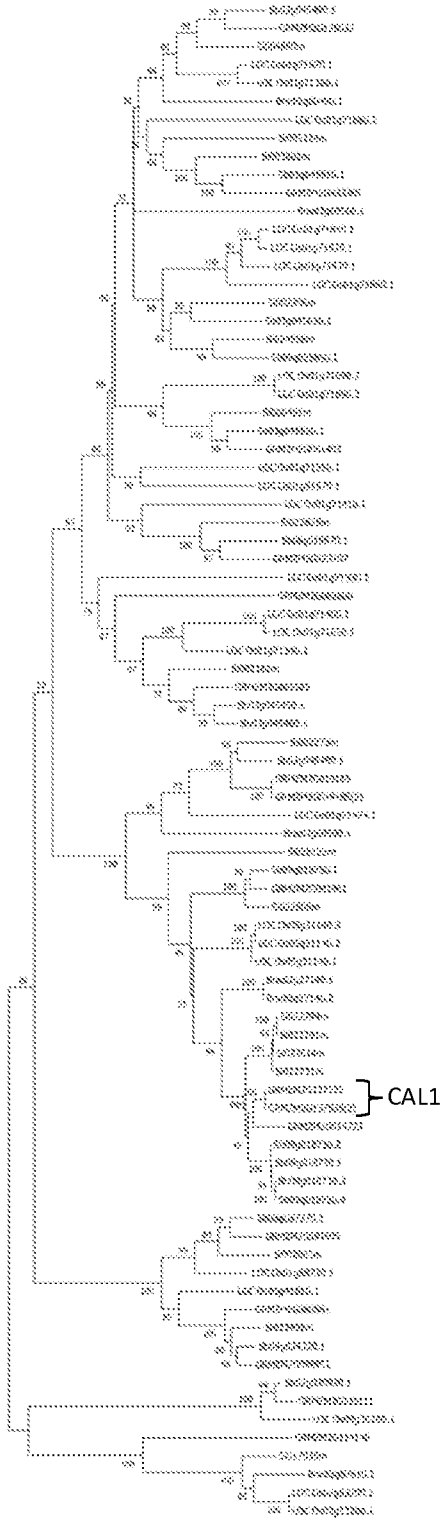


Figure 17