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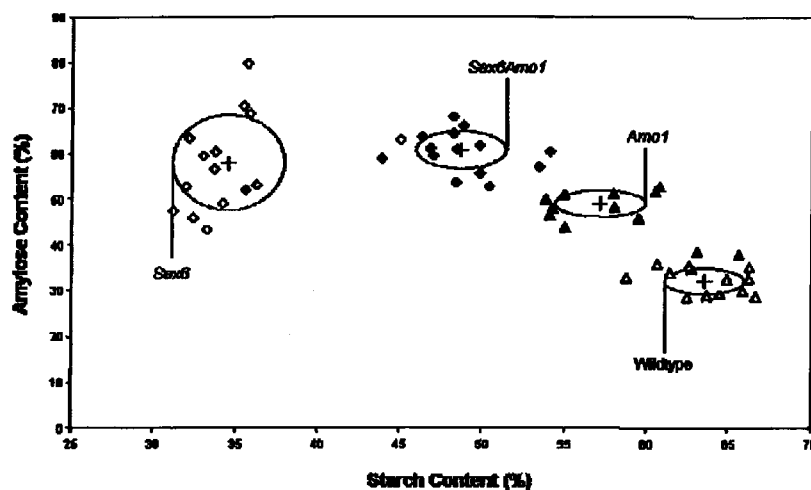
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(54) Title: BARLEY WITH MODIFIED SSIII

**FIGURE 1**

(57) Abstract: Barley grain, plants and parts thereof producing same and methods of producing and using same are described, the grain comprising (a) starch (b) a first genetic variation which is (i) an induced mutation in an endogenous gene encoding a starch synthase HI (SSIII), or (ii) a transgene which encodes an RNA molecule which reduces expression of an endogenous gene encoding an SSIII, and (c) a reduced level or activity of SSIII protein relative to the level or activity in barley grain lacking the first genetic variation. The grain may further comprise a second genetic variation in a gene encoding a protein involved in starch synthesis, starch catabolism, starch phosphorylation or non-starch carbohydrate synthesis. The second genetic variation is illustratively (i) a mutation in a gene encoding a starch synthase II (SSII), in some embodiments a starch synthase IIa (SSIIa), or (ii) a transgene which encodes an RNA molecule which inhibits expression of a gene encoding an SSII, preferably an SSIIa. The grain illustratively comprises (i) an allele of SSIIIa other than an allele comprising the nucleic acid sequence of SEQ ID NO: 33.

sequence of SEQ ID NO: 3 and (ii) an allele of SSIIIa other than an allele comprising the nucleic acid sequence of SEQ ID NO: 33. The SSIII is illustratively SSIIIa. The SSII is illustratively SSIIa.



— with sequence listing part of description (Rule 5.2(a))

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TITLE**"BARLEY WITH MODIFIED SSIII"****FIELD**

[0001] The present specification describes variant barley grain comprising a reduced level or activity of SSIII and exhibiting desirable starch and non-starch components in relatively high yield.

BACKGROUND

[0002] Barley is widely used for food production. Wildtype barley seed contains approximately 50 to 60% of starch, contained in its endosperm, that has approximately 25% amylose and 75% amylopectin. Amylose is a mostly linear α -(1-4) linked glucosyl chain with a few α -(1-6) linked glucan chains and has a molecular weight of 10^4 to 10^5 daltons. Amylopectin is a highly branched glucan in which α -(1-4) linked glucosyl chains with mostly 3 to 60 glucosyl units are connected by α -(1,6)-linkages, so that approximately 5-6% of the glucosyl linkages are α -(1,6)-linkages, and has a molecular weight of 10^5 to 10^6 daltons.

[0003] A suite of enzymes are involved in cereal starch biosynthesis including ADP-glucose pyrophosphorylases (EC 2.7.7.27), starch synthases (EC 2.4.1.21), starch branching enzymes (EC 2.4.1.18) and starch debranching enzymes (EC 3.2.1.41 and 3.2.1.68). The first committed step of starch synthesis is synthesis of ADP-glucose from Glucose-1-P and ATP, catalyzed by the enzyme ADP-glucose pyrophosphorylase. The ADP-glucose is then used as substrate for the synthesis of starch by starch synthases which transfer glucose to the non-reducing end of pre-existing α -(1-4) linked glucosyl chain of starch. The branched glucan chains of starch, linked with α -(1-6) linkages, are formed by starch branching enzymes through the cleavage of a region of the α -(1-4) linkage glucan and subsequent transfer of the short glucan to a position on the α -(1-4) linkage glucan of starch. Excess α -(1-6) linked glucan chains are removed by debranching enzymes to maintain starch in a defined structure.

[0004] Ten starch synthase genes have been identified in the rice genome and are grouped into five distinct classes: granule-bound starch synthase (GBSS),

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starch synthase I (SSI), starch synthase II (SSII), starch synthase III (SSIII) and starch synthase IV (SSIV). There are two GBSS isoforms (GBSSI and GBSSII), one SSI isoform, three SSII isoforms (SSIIa [SSII-3], SSIIb [SSII-2], and SSIIc [SSII-1]), two SSIII isoforms (SSIIIa [SSIII-2] and SSIIIb [SSIII-1]), and two SSIV isoforms (SSIVa [SSIV-1] and SSIVb [SSIV-2]) in rice. Proteins corresponding to SSI, SSIIa and GBSSI have been detected within starch granules, whereas SSIIIa protein has been only detected in the soluble phase of amyloplastids. The precise role of these starch synthases individually and cooperatively in determining the final structure of the starch granule largely remains undefined although the potential roles of some starch synthases have been characterized in different organs and different species.

[0005] Mutants in starch synthases have been useful in determining the roles in some cereal species. GBSSI plays a crucial role in the biosynthesis of amylose, (Nelson *et al.*, *Biochem. Biophys. Res. Comm.*, 9: 297-300, 1962) but it may also contribute to the synthesis of the long chains of amylopectin (Maddelein *et al.*, *J. Bio. Chem.*, 269: 25150-25157, 1994).

[0006] A barley *SSIIa* mutant has been shown to have a high amylose phenotype with reduced starch content and reduced seed weight due to the reduction of starch biosynthesis. The mutant barley lines M292 and M342 which were homozygous for a null mutation in the gene encoding *SSIIa* were obtained following mutagenesis of grains of the barley variety 'Himalaya' with sodium azide. Mutant seeds were initially selected from progeny grain of the mutagenized population on the basis of a shrunken grain phenotype. The mutant lines were further characterized by their altered starch properties, reduced *SSIIa* protein level and activity, and genetically by the presence of a premature stop codon in the protein coding region of the gene encoding *SSIIa* which caused loss of the *SSIIa* enzyme in the endosperm (Morell *et al.*, *Plant Journal* 34: 173-185, 2003) incorporated herein in its entirety by reference). The *SSIIa* mutant grain also had substantially reduced starch content and this was associated with a moderate reduction in yield when the barley plants were grown in the field. It was not known if the yield could be improved, or how, while still maintaining the high amylose phenotype.

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[0007] There is a need for barley grain with improved agronomic performance and methods of producing same.

SUMMARY

[0008] In one embodiment barley grain is described comprising: (a) starch, (b) a first genetic variation which is: (i) an induced mutation in an endogenous gene encoding a starch synthase III (SSIII), or (ii) a transgene which encodes an RNA molecule which reduces expression of an endogenous gene encoding an SSIII, and (c) a reduced level or activity of SSIII protein relative to the level or activity in barley grain lacking the first genetic variation.

[0009] In some embodiments, the barley grain comprises an allele of *SSIIIa* other than a allele comprising the nucleic acid sequence of SEQ ID NO: 3.

[0010] In an illustrative embodiment, the grain further comprises a second genetic variation in a gene encoding a protein involved in starch synthesis, starch catabolism, starch phosphorylation or non-starch carbohydrate synthesis. In some embodiments, the second genetic variation is (i) a mutation in a gene encoding a starch synthase II (SSII), preferably a starch synthase IIa (SSIIa), or (ii) a transgene which encodes an RNA molecule which inhibits expression of a gene encoding an SSII, preferably an SSIIa.

[0011] In some embodiments, the grain comprises (i) an allele of *SSIIIa* other than an allele comprising the nucleic acid sequence of SEQ ID NO: 3 and (ii) an allele of *SSIIa* other than an allele comprising the nucleic acid sequence of SEQ ID NO: 33. In another version of this embodiment, the grain lacks the allele of *SSIIIa* comprising the nucleic acid sequence of SEQ ID NO: 3 and lacks the allele of *SSIIa* comprising the nucleic acid sequence of SEQ ID NO: 33.

[0012] In another embodiments, the description enables barley grain comprising: (a) starch, (b) a first genetic variation which is (i) a mutation in a gene encoding an SSIII, or (ii) a transgene which encodes an RNA molecule which inhibits expression of a gene encoding an SSIII, and (c) a reduced level or activity of SSIII protein relative to the level or activity in barley grain lacking the first genetic variation, (d) a second genetic variation which is (i) a mutation in a gene encoding an

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SSII, or (ii) a transgene which encodes a nucleic acid molecule which inhibits expression of a gene encoding an SSII, (e) a reduced level or activity of SSII protein relative to the level or activity in barley grain lacking the second genetic variation, and wherein the grain comprises an allele of SSIIa other than an allele comprising the nucleic acid sequence of SEQ ID NO: 3.

[0013] In exemplary embodiments, the SSIII is SSIIa. In further illustrative embodiments, the SSII is SSIIa.

[0014] In further illustrative embodiments the starch content of the grain is at least 43% (w/w), at least 45% (w/w), at least 47% (w/w), at least 50% (w/w), or 41-65% (w/w).

[0015] In still further illustrative embodiments, the amylose content of the grain is at least 40%, at least 50% or at least 60% as a proportion of the total starch in the grain.

[0016] In further embodiments, the β -glucan content of the grain is 5-9% (w/w), or greater than 9% (w/w).

[0017] In still further embodiments, the fructan content of the grain as described herein is 2-11%, 3-11%, or 4-11% (w/w). Illustratively, the fructan comprises a degree of polymerization from about 3 to about 12.

[0018] In yet further embodiments, the grain comprises an elevated protein content relative to wild-type barley, or relative to HAG.

[0019] In some embodiments, the grain is homozygous for the *sex6-292* allele of *SSIIa*.

[0020] As described herein, in some embodiments the *SSIII* gene encodes an SSIII protein comprising an amino acid sequence having at least 98% sequence identity to SEQ ID NO: 4.

[0021] The grain of the present invention is, in some embodiments, wholegrain or cracked, ground, polished, milled, kibbled, rolled or pearled grain. In particular embodiments, the grain is processed and is unable to germinate.

[0022] The present invention extends of course to barley plants that produce or are capable of producing the grain are hereinabove described and as herein described. In some embodiments, the barley plants produce or are capable of

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producing barley grain comprising: (a) starch, (b) a first genetic variation which is (i) an induced mutation in an endogenous gene encoding a starch synthase III (SSIII), or (ii) a transgene which encodes an RNA molecule which reduces expression of an endogenous gene encoding an SSIII, and (c) a reduced level or activity of SSIII protein relative to the level or activity in barley grain lacking the first genetic variation.

[0023] In some embodiments, the plant or part therefrom comprises an allele of *SSIIIa* other than a allele comprising the nucleic acid sequence of SEQ ID NO: 3.

[0024] In some embodiments, the barley plants are a population of at least 1000 plants growing in a field.

[0025] The description encompasses barley wholemeal or flour produced from the grain as herein above described.

[0026] In another aspect the description provides a process for producing a food ingredient or beverage ingredient, wherein the process comprises: (i) obtaining or producing barley grain as described hereinabove or as described herein; and (ii) processing the grain to produce the ingredient.

[0027] In some embodiments, the ingredient is isolated wholemeal, flour, starch, bran, β -glucan, fructan, non-starch polysaccharides, a baking ingredient, a baking mix, a thickening agent, or cracked, ground, polished, milled, kibbled, rolled or pearled grain, or any combination thereof.

[0028] In further embodiments, the description provides for a process for producing a food or animal feed or beverage product, wherein the process comprises: (i) obtaining or producing an ingredient produced according to a process as described herein above or herein and (ii) Mixing the ingredient with another food ingredient or beverage ingredient to produce the product.

[0029] In some embodiments of the process, the ingredient is used as a bulking agent, a dietary fibre, a texturizing agent, a preservative, a sweetener, a thickening agent, a probiotic agent or any combination of these, in the food or beverage product. In other embodiments, the process further comprises assessing the level or type of starch or starch synthase activity, starch content, amylose,

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amylopectin, β -glucan, fructan, non-starch polysaccharides, dietary fibre, resistant starch in the barley grain or the ingredient or the product produced therefrom.

[0030] In some embodiments of the process the food product is a breakfast cereal, biscuit, muffin, muesli bar, noodle, bread, cake, chip, pancake, tortilla, buns, pastry, cracker, pizza, croissants, bagels, pretzels, pasta, soup, sauce, confectionary, and other farinaceous goods.

[0031] In yet another aspect the description enables a barley plant capable of producing grain as herein described, wherein the process comprises: (i) introducing into a barley plant a nucleic acid which down-regulates the level or activity of an SSIII in the plant, or introducing into a barley plant a mutation in an endogenous gene encoding SSIII in the plant, and (ii) selecting a barley plant from step (i) which is capable of producing said grain. In some embodiments of the process the SSIII gene of the barley plant encodes a protein having at least 98% sequence identity to SEQ ID NO: 4.

[0032] In an illustrative embodiment, the process further comprises assessing the level, activity and/or type of starch, starch content, the level or activity of SSIIIa and/or a non-SSIII starch synthase such as SSI and/or GBSSI, amylose, amylopectin, β -glucan, fructan, non-starch polysaccharide, dietary fibre or resistant starch in the barley grain or a product therefrom, or analysing the plant with one or more genetic markers.

[0033] In some embodiments of the process, the level or activity of SSIII protein is less than 25%, less than 10%, less than 5%, or is essentially lacking relative to that of a control plant or the plant prior to the introduction of the nucleic acid or mutation.

[0034] In another aspect, the description enables a process for producing the herein described barley grain, the process comprising the steps of growing a barley plant and harvesting the grain. In some useful embodiments, the process comprises processing the grain to produce grain that is cracked, ground, polished, milled, kibbled, rolled or pearled.

[0035] Food production is an aspect of the present invention. The present invention provides barley grain as described herein or an ingredient produced

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according to the processes described herein, wholemeal or flour therefrom, when used or for use in the production of a product to increase the level of starch, amylose, amylopectin, resistant starch, dietary fibre, protein, lipid, water soluble carbohydrate, β -glucan, fructan or non-starch carbohydrate in said product or to decrease the glycemic index (GI) of said product.

[0036] Accordingly, the present description provides for the use of a grain as described herein or an ingredient produced according to a process described herein such as flour, wholemeal, starch, β -glucan or fructan isolated from a plant, grain, wholemeal or flour in the production of a food product.

[0037] In one embodiment, the present invention provides a food product comprising a food ingredient at a level of at least 10% on a dry weight basis, wherein the food ingredient is a barley grain according to any one of the herein described embodiments or an ingredient produced according to the process embodiments described herein such as wholemeal or flour.

[0038] Illustratively, the product is selected from the group consisting of bread, buns, breakfast cereal, cake, biscuit, pastry, crackers, muffins, pizza, croissants, bagels, pretzels, pasta, noodles, soup, sauce, confectionary and other farinaceous goods. Illustratively, the barley grain used to produce the above products comprises the *sex6-292* allele. Accordingly, the present invention provides a process for preparing a food or beverage, comprising mixing the barley grain or ingredient such as barley wholemeal or flour with another food or beverage ingredient.

[0039] The invention also encompasses methods of improving the health of a subject. In some embodiments, the invention provides a process for providing starch, amylose, amylopectin, β -glucan, fructan, non-starch polysaccharide, dietary fibre or resistant starch to improve one or more indicators of health in a mammal, wherein the process comprises administering, to the mammal, a composition comprising barley grain as herein described, or wholemeal or flour therefrom or the food product or ingredient as described herein. In some embodiments, the grain, flour, starch, amylose, amylopectin, β -glucan, fructan, non-starch polysaccharide, dietary fibre or resistant starch is in the form of a food product, a beverage or a pharmaceutical composition.

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[0040] Illustratively, the one or more indicators of health is an increased number of beneficial intestinal bacteria, reduced number of aberrant crypt foci, increased mineral absorption, reduced level of insulin, reduced glycaemic index, reduced glycaemic load, reduced blood glucose, reduced blood pressure, reduced body weight, reduced blood cholesterol level, increased HDL cholesterol level, increased bone density, increased calcium levels, more frequent bowel movement, or improved blood serum cardiovascular profile.

[0041] Thus, in some embodiments the invention provides a process/method for ameliorating one or more symptoms of a condition associated with low levels of dietary starch, amylose, amylopectin, β -glucan, fructan, non-starch polysaccharide, dietary fibre or resistant starch in a subject, said process comprising administering orally to the subject grain as herein described or a processed product or ingredient comprising starch, starch content, amylose, amylopectin, β -glucan, fructan, non-starch polysaccharide, dietary fibre or resistant starch obtained therefrom for a time and under conditions sufficient to ameliorate one or more symptoms. In some embodiments, the condition is selected from the group consisting of diabetes, obesity, heart disease, hypertension, constipation, osteoporosis and cancer.

[0042] In another aspect, the present invention provides a process for analyzing a barley plant or a part thereof, comprising the steps of: (i) analysing DNA, RNA, protein, starch granules, starch or grain obtained from the barley plant or part thereof, and (ii) determining from step (i) whether the barley plant or part thereof comprises an allele of *SSIIIa* which comprises the nucleic acid sequence of SEQ ID NO: 3 or which comprises a nucleic acid sequence other than SEQ ID NO: 3.

[0043] In some embodiments of the process, a plant or part therefrom is selected which has SEQ ID NO: 3. In some other embodiments of the process, a plant or part therefrom is selected which an allele other than SEQ ID NO: 3.

[0044] In some embodiments of the process, the barley plant or part thereof is a member of a population of barley plants or parts thereof. Illustratively, the process will find broad application in breeding programs.

[0045] The above summary is not and should not be seen in any way as an exhaustive recitation of all embodiments of the present invention.

BRIEF DESCRIPTION OF THE FIGURES

[0046] Figure 1 is a graphical representation illustrating the relationship between amylose content and starch content is shown for 53 homozygous BC3F6 lines. Lines containing the wildtype *ssIIa* gene (triangular symbols) contained the three markers from the mutant *amo1* locus (solid triangles) or were wildtype for all three *amo1* markers (open triangles). The lines with the *sex6* mutant genotype (diamond symbols) contained the wild type *amo1* markers (open diamonds), or mutant *amo1* markers (solid diamonds).

[0047] Figure 2 is a schematic representation of the gene constructs encoding hairpin RNA for suppression of gene expression targeting (A) barley *SSIIa* or (B) barley *SSIIb*. See Example 8 for details of the constructs.

[0048] Figure 3 is a comparison of amino acid sequences of barley *ssIIa* gene from HAG, Glacier, Himalaya292 and Himalaya showing the *amo* mutation at L1480R in HAG.

BRIEF DESCRIPTION OF THE TABLES

[0049] Table 1 provides a statistical analysis of genotypes and phenotypes of BC3F6 lines of barley.

[0050] Table 2 provides a summary of grain constituents of barley genotypes on a per seed basis.

[0051] Table 3 describes the intron and exon structure of barley *ssIIa* gene and SNPs among barley *ssIIa* genes from HAG, Glacier, Himalaya292 and Himalaya.

[0052] Table 4 provides the results of an analysis of RS content and GI level of barley wholemeal.

[0053] Table 5 provides the results of an analysis of RS content and GI level of bread produced using 100% barley wholemeal.

[0054] Table 6 provides the results of an analysis of the effects of genotype on RS contents of bread produced with 30% or 100% barley flour.

[0055] Table 7 provides the results of an analysis of RS content and GI level of breads produced with 30% barley flour.

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[0056] Table 8 provides the results of an analysis of the effects of genotype on RS content (mg RS per g starch) of bread produced with 100% barley flour.

[0057] Table 9 provides the results of an analysis of the effects of genotype on RS content (mg RS per g starch) of breads produced with 30% barley flour.

[0058] Table 10 provides the results of an analysis of the effects of genotype on GI level of the 10g breads produced with 30% or 100% barley flour.

[0059] Table 11 provides a description of the SEQ ID NOs provided herein.

[0060] Table 12 provides an amino acid sub-classification.

[0061] Table 13 provides exemplary amino acid substitutions.

DETAILED DESCRIPTION

[0062] The description includes a mutational approach to produce barley grain having, or that has been subject during development to, a reduced level or activity of an endogenous SSIII protein or an SSIII gene.

[0063] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers. Thus, for example, reference to grain comprising 45% amylose includes grains having 45% amylose or any greater percentage value of amylose.

[0064] As used herein the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a mutation" includes a single mutation, as well as two or more mutations; reference to "an agent" includes one agent, as well as two or more agents (i.e. to at least one); and so forth.

[0065] Accordingly, in one aspect the present invention is directed to barley grain comprising: (a) starch; (b) a reduced level or activity of SSIIIa protein; and (c) a genetic variation which is (i) an induced mutation in a gene encoding SSIIIa, or (ii) a transgene which encodes an RNA molecule which reduces expression of a gene encoding SSIIIa, wherein the genetic variation causes said reduced level or activity of SSIIIa protein. SSIIIa activity is typically in the developing endosperm.

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[0066] In another embodiment, the present invention is directed to barley grain comprising (a) starch; (b) a reduced level or activity of SSIII protein; and (c) a genetic variation which is (i) an induced mutation in a gene encoding SSIII, or (ii) a transgene which encodes an RNA molecule which reduces expression of a gene encoding SSIII, wherein the genetic variation causes said reduced level or activity of SSIII protein, and further comprising a mutation in a gene encoding a protein involved in starch synthesis, starch catabolism, starch phosphorylation or non-starch carbohydrate synthesis. In an illustrative non-limiting embodiment, SSIII is SSIIIa.

[0067] In an illustrative embodiment, the further mutation or second genetic variation encodes a nucleic acid encoding a protein which is an ADP-glucose pyrophosphorylase, a starch synthase, a starch branching enzyme, a starch debranching enzyme, or an amylase or a combination of two or more of these. Debranching enzymes include an isoamylase and a pullulanase. Starch phosphorylation enzymes include a glucose-water dikinase (GWD) and a phosphoglucan water dikinase (PWD). Starch branching enzymes include SBEI, SBEII, SBEII, and starch synthase enzymes including an array of isoforms including GBSSI, GBSSII, SSI, SSIIa, SSIIb, SSIIc, SSIIIa, SSIIIb, SSIVa, SSIVb and CSL.

[0068] In some embodiments, the barley grain does not comprise an allele of SSIIIa comprising the nucleic acid sequence of SEQ ID NO: 3. This allele encompasses the SSIIIa-38 mutation, also referred to as the *amol*-38 mutation and comprises a leucine to arginine substitution at a position corresponding to amino acid 1480 of SEQ ID NO:4. In some embodiments, the barley grain does not comprise the combination of an allele of SSIIIa comprising the nucleic acid sequence of SEQ ID NO: 3 (SSIIIa-38, *amol*-38) and an allele of SSIIa comprising the nucleic acid sequence of SEQ ID NO: 33 (SSIIa-292, *sex6*-292).

[0069] In another embodiment, the present invention provides barley grain comprising starch and: (a) a genetic variation which is (i) an induced mutation in a gene encoding SSIII; or (ii) a transgene which encodes an RNA molecule which inhibits expression of a gene encoding SSIII, wherein the genetic variation leads to said reduced level or activity of SSIII protein; and (b) a genetic variation which is (i) a mutation in a gene encoding SSII, or (ii) a transgene which encodes an RNA molecule

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which inhibits expression of a gene encoding SSII, wherein the genetic variation leads to said reduced level or activity of SSII protein. As above, the grain does not comprise a combination of an allele of SSIII comprising the nucleic acid sequence of SEQ ID NO: 3 (SSIIIA-38, *amoI*-38) and an allele of SSIIa comprising the nucleic acid sequence of SEQ ID NO: 33 (SSIIa-292, *sex6*-292). In some embodiments, the SSIII and the SSII mutations are homozygous null mutations. In some of these embodiments, the SSII mutation is an SSIIa mutation. In other embodiments, the SSIIa mutation is the *sex6*-292 mutation.

[0070] In another embodiment, the invention provides barley grain comprising: (a) starch, (b) a reduced level or activity of SSIIIA protein, (c) a genetic variation which is (i) a mutation in a gene encoding SSIIIA, or (ii) a transgene which encodes an RNA molecule which inhibits expression of a gene encoding SSIIIA, wherein the genetic variation leads to said reduced level or activity of SSIIIA protein, and (d) a genetic variation which is (i) a mutation in a gene encoding SSIIa, or (ii) a transgene which encodes a nucleic acid molecule which inhibits expression of a gene encoding SSIIa, wherein the genetic variation leads to said reduced level or activity of SSIIa protein, and wherein the grain does not comprise a combination of an allele of SSIIIA comprising the nucleic acid sequence of SEQ ID NO: 3 (SSIIIA-38). In some embodiments, the SSIIIA and the SSIIa mutations are homozygous null mutation. In some of these embodiments, the SSIIa mutation is the *sex6*-292 mutation.

[0071] In some embodiments, the genetic variation reducing the level or activity of SSIII produces an increased level of GBSSI protein in the grain.

[0072] In some embodiments, the genetic variation reducing the level or activity of SSIII produces (leads to) an increased level of starch synthase enzyme such as SSI protein in the grain. In some embodiments, the level of GBSSI and SSI is increased. The term "reduced" includes, in some embodiments, substantially no protein or protein with substantially no starch synthase activity.

[0073] In some embodiments, the barley grain has a starch content of at least 41% (w/w).

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[0074] In other embodiments, the barley grain comprises a starch content of at least 43% (w/w), at least 45% (w/w), at least 47% (w/w), at least 50% (w/w), or comprises a starch content of 41-65% (w/w).

[0075] In some embodiments, the barley grain comprises an amylose content of at least 40%, at least 50% or at least 60% as a proportion of the total starch in the grain. In some embodiments, amylose content is determined or expressed on a per caryopsis basis relative to controls.

[0076] In other embodiments, the barley grain comprises a β -glucan content of 5-9% (w/w), or greater than 9% (w/w).

[0077] In some embodiments, the barley grain comprises a fructan content of 2-11%, 3-11%, or 4-11% (w/w).

[0078] In other embodiments, the fructan comprises a degree of polymerization from about 3 to about 12.

[0079] In other embodiments, the barley grain comprises an elevated protein content relative to wild-type barley, or relative to HAG.

[0080] In many embodiments, the barley grain is homozygous for the sex6-292 allele of *SSIIa*.

[0081] The grain may be in any useful form such as, without limitation, wholegrain or cracked, ground, polished, milled, kibbled, rolled or pearled grain.

[0082] The present invention extends to a barley plant capable of producing the grains as described herein having a reduced level or activity of *SSIII* and also to barley wholemeal or flour produced from the grains.

[0083] Grains comprising a reduced level or activity of *SSIII* as described herein are obtained or produced and processed to produce a food or beverage product.

[0084] In some embodiments, the invention is directed to a method of producing a food ingredient or beverage ingredient, wherein the method comprises: (i) obtaining or producing barley grain as described herein; and (ii) processing the grain to produce the ingredient.

[0085] In some embodiments, the ingredient comprises a nucleic acid molecule comprising a genetic variation which is (i) an induced mutation in a gene encoding *SSIII*, or (ii) a transgene which encodes an RNA molecule which reduces

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expression of a gene encoding SSIII, wherein the genetic variation causes said reduced level or activity of SSIII protein.

[0086] In another embodiment, the invention is directed to a method of producing a food or beverage product, wherein the method comprises: (i) obtaining or producing the ingredient produced according to the above method; and (ii) mixing the ingredient with another food ingredient or beverage ingredient to produce the product.

[0087] The ingredient may be conveniently selected from wholemeal, flour, starch, bran, β -glucan, fructan, non-starch polysaccharides, or cracked, ground, polished, milled, kibbled, rolled or pearled grain.

[0088] In another illustrative embodiment, the product is selected from the group consisting of bread, buns, breakfast cereal, cake, biscuit, pastry, crackers, muffins, pizza, croissants, bagels, pretzels, pasta, noodles, baking ingredients, baking mixes, soup, sauce, thickening agent, confectionary, tortillas, granola bars, snacks and other farinaceous goods. The product may be a beverage such as a high energy drink or smoothie.

[0089] The food or beverage product may conveniently be selected from flour, wholemeal, bran, fructan, starch, β -glucan, a low caloric additive, a bulking agent, a dietary fibre, a texturizing agent, a preservative, a probiotic agent or the like or any combination of these.

[0090] In some embodiments, the methods further comprise assessing the level or type of starch, starch content, the level or activity of GBSSI, SSI or SSIIIa or other starch-related enzymes or isoforms thereof, amylose, amylopectin, β -glucan, fructan, non-starch polysaccharides, dietary fibre, resistant starch in the barley grain or the ingredient or product therefrom.

[0091] In some embodiments, the invention provides a method of producing a barley plant capable of producing grain which has a reduced level or activity of SSIII protein and starch wherein the method comprises: (i) introducing into said plant, a nucleic acid which down-regulates the level or activity of endogenous starch synthase III (SSIII) in the plant relative to a control plant, or a mutation in an

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endogenous gene encoding SSIII in the plant, and (ii) selecting the barley plant which produces said grain.

[0092] In some embodiments, the nucleic acid molecule comprises a gene-silencing chimeric gene, an antisense, ribozyme, co-suppression, dsRNA molecule, hairpin RNA or other exogenous nucleic acid molecule that down-regulates endogenous SSIIIa and/or b expression.

[0093] In some embodiments, the method further comprises assessing the level, activity and/or type of starch, starch content, the level or activity of GBSSI, SSI, or SSIIIa or other starch related enzymes, amylose, amylopectin, β -glucan, fructan, non-starch polysaccharide, dietary fibre or resistant starch in the barley grain or a product therefrom, or analyzing the plant with one or more genetic markers including those using the presently disclosed sequences of barley SSIIIa.

[0094] In some embodiments, the reduced level or activity of SSIIIa protein in the grain is less than 25%, less than 10%, less than 5%, or essentially lacking relative to that of a control plant or the plant prior to the introduction of the genetic variation. In some embodiments, the method of producing the barley grain comprises the steps of growing a barley plant and harvesting the grain.

[0095] In another embodiment, the present invention provides for the use of a grain or flour isolated from a plant or grain as described herein in the production of a food product to increase the level of one or two or more of starch, amylose, amylopectin, β -glucan, fructan, non-starch polysaccharide, dietary fibre or resistant starch in the food product.

[0096] In another embodiment, the invention provides a method for preparing a food or beverage, comprising mixing the barley grain or a product obtained therefrom by the presently disclosed methods with another food or beverage ingredient. Thus the method comprises: (i) obtaining or producing barley grain comprising a reduced level or activity of SSIIIa protein; and (ii) processing the grain to produce the product.

[0097] The invention further provides a method for providing one or more starch, amylose, amylopectin, β -glucan, fructan, non-starch polysaccharide, dietary fibre or resistant starch to improve one or more indicators of health in a mammal,

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wherein the method comprises administering, to the mammal, a composition comprising barley grain, wholemeal or flour therefrom or a food or beverage obtained therefrom comprising a reduced level or activity of SSIIIa protein or the food product as described herein.

[0098] In some embodiments, the grain, flour, starch, amylose, amylopectin, β -glucan, fructan, non-starch polysaccharide, dietary fibre or resistant starch is in the form of a food product, a beverage or a pharmaceutical composition. In some embodiments, the one or more indicators of health is an increased number of beneficial intestinal bacteria, reduced number of aberrant crypt foci, increased mineral absorption, reduced level of insulin, reduced glycaemic index, reduced glycaemic load, reduced blood glucose, reduced blood pressure, reduced body weight, reduced blood cholesterol level, increased HDL cholesterol level, increased bone density, increased calcium levels, more frequent bowel movement, or improved blood serum cardiovascular profile.

[0099] In a related embodiment, the invention provides a method for ameliorating one or more symptoms of a condition associated with low levels of one or more dietary starch, starch content, amylose, amylopectin, β -glucan, fructan, non-starch polysaccharide, dietary fibre or resistant starch in a subject, said method comprising administering orally to the subject grain as described herein or a processed product comprising one or more of starch, amylose, amylopectin, β -glucan, fructan, non-starch polysaccharide, dietary fibre or resistant starch obtained therefrom for a time and under conditions sufficient to ameliorate one or more symptoms.

[00100] In some embodiments of the method the condition is selected from the group consisting of diabetes, obesity, heart disease, hypertension, constipation, osteoporosis and cancer. In another in another aspect the present invention provides isolated nucleic acid molecules comprising sequences encoding barley SSIII such as SEQ ID NOs: 2 and 3 or their complements or oligonucleotide fragments thereof for use inter alia in the product of the subject barley grains. In another embodiment, the invention provides isolated barley SSIII proteins having at least about 98% sequence identity to SEQ ID NO: 4 and/or variants thereof and barley grain or products therefrom or ingredients comprising same.

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[00101] Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc. A summary of sequence identifiers is provided in Table 11. A sequence listing is provided after the claims.

[00102] By "gene" is meant a unit of inheritance that occupies a specific locus on a chromosome and consists of transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (i.e., introns, 5' and 3' untranslated (non coding) sequences).

[00103] The term "5' non-coding region" is used herein in its broadest context to include all nucleotide sequences which are derived from the upstream region of an expressible gene, other than those sequences which encode amino acid residues which comprise the polypeptide product of the gene, wherein 5' non-coding region confers or activates or otherwise facilitates, at least in part, expression of the gene.

[00104] Genes and other genetic material (e.g. mRNA, nucleic acid constructs etc) are represented herein in italics while their proteinaceous expression products are represented in non-italicized form. Thus, for example starch synthase III (SSIII) polypeptide is the expression product of *SSIII* nucleic acid sequences.

[00105] Representative examples of the nucleic acid and amino acid sequences of *SSIII* and SSIII molecules are provided in the sequence listing further described in Table 11. In an illustrative embodiment, the SSIII gene is a SSIIIa gene. In some embodiments, barley SSIIIa shares at least about 70% amino acid sequence identity with SSIIIb.

[00106] Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

[00107] The citation of any reference herein should not be construed as an admission that such reference is available as "Prior Art" to the instant application.

[00108] Any subject who could benefit from the present methods or compositions is encompassed. The term "subject" includes, without limitation, humans and non-human primates, livestock animals such as cattle, pigs or chickens,

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or young animals such as calves or piglets, companion animals such as dogs or cats, horses, laboratory test animals, captive wild animals, reptiles and amphibians, fish, and birds. A subject, regardless of whether it is a human or non-human organism may be referred to as a patient, individual, subject, animal, host or recipient. In a particular embodiment the subject is a human.

[00109] The present invention is predicated in part upon the provision of barley starch synthase III (SSIII) genetic and/or proteinaceous mutants which by themselves, or combined with other mutants, provide agronomic advantages.

[00110] As used herein, the terms "modifying", "altering", "increasing", "increased", "reducing", "reduced", "inhibited", "mutant", "variant" or the like are considered relative terms, i.e. in comparison with the wild-type or unaltered or control state. In some embodiments, a wild-type plant is an appropriate "control plant" however in many situations the control plant must be determined by the skilled addressee using their ordinary skill in the art and the information disclosed herein.

[00111] In an illustrative example, a reduced level or activity of SSIII protein or SSII protein expressed from a gene having a genetic variation as described herein is reduced relative to the level or activity of the protein in a barley plant or grain lacking the genetic variation. Illustrative controls include grain from Himalaya and Glacier.

[00112] The terms "wild-type" and "naturally occurring" are used interchangeably to refer to a gene or gene product that has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild type gene or gene product (e.g., a polypeptide) is that which is most frequently observed in a population and is thus arbitrarily designed the "normal" or "wild-type" form of the gene.

[00113] The "level of a protein" refers to the amount of a particular protein, for example SSIII, which may be measured by any means known in the art such as, for example, by Western blot analysis, spectrophotometric, enzymatic or immunological means.

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[00114] The "level of an enzyme activity" refers to the amount of a particular enzyme measured, for example, in an enzyme assay, an incorporation assay or a zymogram.

[00115] The "activity of SSIII protein" refers to the amount of a particular enzyme measured in an enzyme assay. SSIII protein refers to all isolated or non-isolated naturally occurring forms of SSIII and variants thereof. In some embodiments, SSIIIa proteins comprise an amino acid sequence that comprises at least 98% sequence identity to the SSIIIa amino acid sequence of a barley variety such as Himalaya, Himalaya292 or Glacier. In other embodiments, an SSIIIa protein comprises an amino acid sequence having at least 98% sequence identity to SEQ ID NO: 4. Illustrative SSIIIa proteins comprise up to 15 amino acid residue substitutions, insertions or deletions or combinations thereof relative to SEQ ID NO: 4. Thus variants of SEQ ID NO: 4 or SSIIIa proteins encompass amino acid sequences which differ from SEQ ID NO: 4 by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 amino acid residues, or suitably as few as 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acid residue(s).

[00116] SSIIIa genes/alleles encode SSIIIa proteins including a protein comprising at least 98% sequence identity to SEQ ID NO: 4. In some embodiments, an SSIIIa gene of the present invention is expressed as a SSIIIa protein having reduced or no starch synthase activity, or a reduced ability to repress starch synthase expression. In some embodiments, an SSIIIa gene of the barley grain comprising a herein described genetic variant expresses an RNA that is not translated or is only translated at a reduced level relative to SSIIIa nucleic acids encoding a protein having a sequence of SEQ ID NO: 4. In other embodiments, an SSIIIa gene of the barley grain comprising a genetic variant and is not transcribed or is only transcribed at a reduced level relative to SSIIIa genes encoding a protein having a sequence of SEQ ID NO: 4. The activity of isoforms of starch synthases are typically assayed by activity immunostaining or detection after native gel electrophoresis. Thus, for example, starch synthase activity is tested by incubated gels with amylopectin and staining for derived glucans with iodine. A reduction in SSIII activity may alternatively be determined by contacting gel resolved enzymes with ADP-glucose

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and measuring the incorporation of ADP by standard methods. In other embodiments, an SSIIa protein with reduced or partial activity has a reduced activity as a repressor of GBSSI expression or the expression of other starch synthases such as SSI, SSIIa/SBEII genes and activity can be determined indirectly *via* measuring the activity of a non-SSIII starch synthase.

[00117] It would be appreciated that the level of activity of an enzyme might be altered in a mutant if a more or less active protein is produced, but not the expression level (amount) of the protein itself. Conversely, the amount of protein might be altered but the activity (*per* unit protein) remain the same. Reductions in both amount and activity are also possible such as, for example, when the expression of a gene encoding the enzyme is reduced transcriptionally or post-transcriptionally.

[00118] In certain embodiments, the reduction in the level of protein or activity of SSIII is by at least 40% or by at least 60% compared to the level of protein or activity in the grain of unmodified barley, or by at least 75%, at least 90% or at least 95% or at least 99%.

[00119] In some embodiments, a reduction in activity is measured relative to the activity of SSIIa proteins comprising the amino acid sequence set out in SEQ ID NO: 4 that has the activity or the same amino acid sequence (see Figure 3) of SSIIa of Himalaya, Himalaya292 or Glacier barley varieties. In some embodiments, a reduced activity of SSIIa protein includes partial activity such as 5-75% of the wild-type (unmodified) activity of SSIIa protein. In some embodiments, the reduced activity is 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, or 75% of SSIIa protein activity.

[00120] The reduction in the level of the protein or enzyme activity or gene expression may occur at any stage in the development of the leaf, seed or grain, particularly during the daytime when photosynthesis is occurring, or during the grain filling stage while starch is being synthesized in the developing endosperm, or at all

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stages of grain development through to maturity. The term "wild-type" as used herein has its normal meaning in the field of genetics and includes barley cultivars or genotypes which are not modified as taught herein. Some preferred "wild-type" barley varieties are described herein, such as, for example, the cultivar Himalaya.

[00121] In some embodiments, the modified phenotype is achieved by full or partial inhibition of the expression of an *SSIIIa* gene. Techniques well known in the art such as SDS-PAGE and immunoblotting are carried out on hydrolysed and unhydrolysed grains and fractions thereof to identify the plants or grain where modifications have occurred to starch related enzymes. These methods include analysis of plants by methods described herein or further by methods such as such as microarray analysis, electrophoresis, chromatography (including paper chromatography, thin layer chromatography, gas chromatography, gas-liquid chromatography and high-performance liquid chromatography) techniques. Separated components are typically identified by comparison of separation profiles with standards of known identity, or by analytical techniques such as mass spectrometry and nuclear magnetic resonance spectroscopy. For example, reference may be made to Example 9, Robinson, *The Organic Constituents of Higher Plants*, Cordus Press, North Amherst, USA, 1980; Adams *et al.*, *Anal. Biochem.*, 266: 77-84, 1999; Veronese *et al.*, *Enz. Microbial Tech.*, 24: 263-269, 1999; Hendrix *et al.*, *J. Insect Physiol.*, 47: 423-432, 2001; Thompson *et al.*, *Carbohydrate Res.*, 331: 149-161, 2001; and references cited therein. Carbohydrates can be assayed using standard protocols known to persons skilled in the art.

[00122] Alteration in *SSIIIa* level or activity may be achieved by the introduction of one or more genetic variations into the barley plant. That is, the genetic variations lead, directly or indirectly, to the alteration in enzyme activity or level in the plant part during growth or development and consequently to the enzyme, starch and non-starch modifications described herein. The genetic variation may be a heterologous polynucleotide which is introduced into the plant or a progenitor cell, for example by transformation or mutagenesis. The genetic variation and further genetic variation may subsequently be introduced into different genetic backgrounds by crossing, as known in the art of plant breeding. In some embodiments, the level or

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functional activity of *SSIIa* is down regulated to a level less than about 80%, less than 70%, less than 60%, less than 50%, less than 40%, less than 30%, less than 20% or less than 15%, and suitably less than about 10%, less than 9%, less than 8%, less than 7%, less than 6%, less than 5%, less than 4%, less than 3%, less than 2% or less than 1% relative to a corresponding control plant.

[00123] In other embodiments, the level or activity of non-*SSIIa* starch synthase is upregulated to a level more than about 80%, more than 70%, more than 60%, more than 50%, more than 40%, more than 30%, more than 20% or more than 15%, and suitably more than about 10%, more than 9%, more than 8%, more than 7%, more than 6%, more than 5%, more than 4%, more than 3%, more than 2% or more than 1% relative to a corresponding control plant.

[00124] In some embodiments, the level of downregulation of *SSIIa* is modulated to achieve elevated levels of starch or non-starch components of agronomic interest as described herein and elsewhere. The amount of reduced *SSIIa* level or activity required may depend upon other factors such as the variety genetic background and environmental factors. However, it is considered that any optimisation, which may be required in such an event is achievable using routine methods including those described herein. In some embodiments, the variant is a homozygous null mutation.

[00125] Reduced *SSIIa* level or activity may be accomplished in tissues throughout the plant, for example using a constitutive promoter to drive expression of a (transgene) to down regulate *SSIIa*. Preferably, it may be accomplished in sink tissues, more preferably in developing endosperm, using a tissue-specific or developmentally regulated promoter. "Sink cell" and "sink tissue" as used herein, refer to cells, tissues or organs which comprise a net inflow of organic carbon that has entered the cells in a form other than fixation of carbon dioxide i.e. as sugars or other carbohydrates. In plants, sink tissues include all non-photosynthetic tissues, as well as photosynthetic tissues with a net inflow of organic carbon fixed by other photosynthetic cells or otherwise obtained from the surrounding medium or environment by means other than direct fixation of carbon dioxide.

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Genes

[00126] In some embodiments, the present invention involves modification of gene activity and the construction and use of chimeric genes. As used herein, the term "gene" includes any deoxyribonucleotide sequence which includes a protein coding region or which is transcribed in a cell but not translated, as well as associated non-coding and regulatory regions. Such associated regions are typically located adjacent to the coding region or the transcribed region on both the 5' and 3' ends for a distance of about 2 kb on either side. In this regard, the gene may include control signals such as promoters, enhancers, termination and/or polyadenylation signals that are naturally associated with a given gene, or heterologous control signals in which case the gene is referred to as a "chimeric gene". The sequences which are located 5' of the coding region and which are present on the mRNA are referred to as 5' non-translated sequences. The sequences which are located 3' or downstream of the coding region and which are present on the mRNA are referred to as 3' non-translated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene.

[00127] The "starch synthase III gene" "*SSIII*" or the like as used herein refers to a nucleotide sequence encoding starch synthase III (SSIII) in barley, which can readily be distinguished from other starch synthases or other proteins by those skilled in the art. SSIII proteins are described herein as including proteins having an amino acid sequence of at least 98% sequence identity to SEQ ID NO: 4. In a preferred embodiment, a barley SSIIIa gene refers to a nucleic acid molecule or its complement, which may be present in or isolated from barley or derived therefrom, comprising nucleotides having a sequence having at least 80% identity to the cDNA sequence shown in SEQ ID NO: 2 or the genomic sequence of SEQ ID NO: 1. In a preferred embodiment, the *SSIII* gene is an *SSIIIa* gene, or the SSIII protein is an SSIIIa protein, each of which may be applied to any or all of the aspects of the invention disclosed herein. The nucleotide sequence of cDNA of the *SSIIIa* gene from wildtype Himalaya is set out in SEQ ID NO: 2.

[00128] A genomic form or clone of a gene containing the transcribed region may be interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." An "intron" as used herein is a segment of a

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gene which is transcribed as part of a primary RNA transcript but is not present in the mature mRNA molecule. Introns are removed or "spliced out" from the nuclear or primary transcript; introns therefore are absent in the messenger RNA (mRNA). Introns may contain regulatory elements such as enhancers. "Exons" as used herein refer to the DNA regions corresponding to the RNA sequences which are present in the mature mRNA or the mature RNA molecule in cases where the RNA molecule is not translated. An mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide. The term "gene" includes a synthetic or fusion molecule encoding all or part of the proteins of the invention described herein and a complementary nucleotide sequence to any one of the above. A gene may be introduced into an appropriate vector for extrachromosomal maintenance in a cell or for integration into the host genome. Table 3 describes the intron/exon structure of barley SSIIIa gene and SNPs from various varieties (HAG, Glacier, Himalaya292 and Himalaya).

[00129] As used herein, a "chimeric gene" refers to any gene that is not a native gene in its native location. Typically a chimeric gene comprises regulatory and transcribed or protein coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. The term "endogenous" is used herein to refer to a substance that is normally present or produced in an unmodified plant at the same developmental stage as the plant under investigation. An "endogenous gene" refers to a native gene in its natural location in the genome of an organism. As used herein, "recombinant nucleic acid molecule" refers to a nucleic acid molecule which has been constructed or modified by recombinant DNA technology. The terms "foreign polynucleotide" or "exogenous polynucleotide" or "heterologous polynucleotide" and the like refer to any nucleic acid which is introduced into the genome of a cell by experimental manipulations. These include gene sequences found in that cell so long as the introduced gene contains some modification (e.g. a mutation, the presence of a selectable marker gene, etc.) relative to the naturally-occurring gene. Foreign or

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exogenous genes may be genes that are inserted into a non-native organism, native genes introduced into a new location within the native host, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure. The term "genetically modified" includes introducing genes into cells by transformation or transduction, mutating genes in cells and altering or modulating the regulation of a gene in a cell or organisms to which these acts have been done or their progeny.

Polynucleotides

[00130] The present invention including the description, tables and sequence listing, refers to various polynucleotides. As used herein, a "polynucleotide" or "nucleic acid" or "nucleic acid molecule" means a polymer of nucleotides, which may be DNA or RNA or a combination thereof, and includes mRNA, cRNA, cDNA, tRNA, siRNA, shRNA and hpRNA. It may be DNA or RNA of cellular, genomic or synthetic origin, for example made on an automated synthesizer, and may be combined with carbohydrate, lipids, protein or other materials, labelled with fluorescent or other groups, or attached to a solid support to perform a particular activity defined herein, or comprise one or more modified nucleotides not found in nature, well known to those skilled in the art. The polymer may be single-stranded, essentially double-stranded or partly double-stranded. An example of a partly-double stranded RNA molecule is a hairpin RNA (hpRNA), short hairpin RNA (shRNA) or self-complementary RNA which include a double stranded stem formed by basepairing between a nucleotide sequence and its complement and a loop sequence which covalently joins the nucleotide sequence and its complement. Basepairing as used herein refers to standard basepairing between nucleotides, including G:U basepairs. "Complementary" means two polynucleotides are capable of basepairing (hybridizing) along part of their lengths, or along the full length of one or both. A "hybridized polynucleotide" means the polynucleotide is actually basepaired to its complement.

[00131] By "isolated" is meant material that is substantially or essentially free from components that normally accompany it in its native state. As used herein, an "isolated polynucleotide" or "isolated nucleic acid molecule" means a

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polynucleotide which is at least partially separated from, preferably substantially or essentially free of, the polynucleotide sequences of the same type with which it is associated or linked in its native state. For example, an "isolated polynucleotide" includes a polynucleotide which has been purified or separated from the sequences which flank it in a naturally occurring state, e.g., a DNA fragment which has been removed from the sequences which are normally adjacent to the fragment. Preferably, the isolated polynucleotide is also at least 90% free from other components such as proteins, carbohydrates, lipids etc. The term "recombinant polynucleotide" as used herein refers to a polynucleotide formed *in vitro* by the manipulation of nucleic acid into a form not normally found in nature. For example, the recombinant polynucleotide may be in the form of an expression vector. Generally, such expression vectors include transcriptional and translational regulatory nucleic acid operably connected to the nucleotide sequence.

[00132] The present invention contemplates the use of oligonucleotides. As used herein, "oligonucleotides" are polynucleotides up to 50 nucleotides in length. They can be RNA, DNA, or combinations or derivatives of either. Oligonucleotides are typically relatively short single stranded molecules of 10 to 30 nucleotides, commonly 15-25 nucleotides in length. When used as a probe or as a primer in an amplification reaction, the minimum size of such an oligonucleotide is the size required for the formation of a stable hybrid between the oligonucleotide and a complementary sequence on a target nucleic acid molecule. Preferably, the oligonucleotides are at least 15 nucleotides, more preferably at least 18 nucleotides, more preferably at least 19 nucleotides, more preferably at least 20 nucleotides, even more preferably at least 25 nucleotides in length. Various oligonucleotides are described herein (see Table 11).

[00133] Polynucleotides used as a probe are typically conjugated with a detectable label such as a radioisotope, hapten, an enzyme, biotin, a fluorescent molecule or a chemiluminescent molecule. Oligonucleotides based on barley SSIIa sequences are useful in methods of detecting an allele of an SSIIa. Such methods, for example, employ nucleic acid hybridization and in many instances include oligonucleotide primer extension by a suitable polymerase (as used in PCR).

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[00134] A variant of an oligonucleotide of the invention includes molecules of varying sizes of, and/or are capable of hybridizing, for example, to the cereal genome close to that of, the specific oligonucleotide molecules defined herein. For example, variants may comprise additional nucleotides (such as 1, 2, 3, 4, or more), or less nucleotides as long as they still hybridize to the target region. Furthermore, a few nucleotides may be substituted without negatively influencing the ability of the oligonucleotide to hybridize to the target region. In addition, variants may readily be designed which hybridize close to, for example to within 50 nucleotides, the region of the plant genome where the specific oligonucleotides defined herein hybridize. Probes, oligonucleotides and the like are based upon the herein described sequences or corrected versions thereof or variants thereof or functional homologs from other cereal plants.

[00135] The terms "polynucleotide variant" and "variant" and the like refer to polynucleotides or their complementary forms displaying substantial sequence identity with a reference polynucleotide sequence. These terms also encompass polynucleotides that are distinguished from a reference polynucleotide by the addition, deletion or substitution of at least one nucleotide. Accordingly, the terms "polynucleotide variant" and "variant" include polynucleotides in which one or more nucleotides have been added or deleted, or replaced with different nucleotides. In this regard, it is well understood in the art that certain alterations inclusive of mutations, additions, deletions and substitutions can be made to a reference polynucleotide whereby the altered polynucleotide retains the biological function or activity of the reference polynucleotide. Accordingly, these terms encompass polynucleotides that encode polypeptides that exhibit enzymatic or other regulatory activity, or polynucleotides capable of serving as selective probes or other hybridizing agents. In particular, this includes polynucleotides which encode the same polypeptide or amino acid sequence but which vary in nucleotide sequence by redundancy of the genetic code. The terms "polynucleotide variant" and "variant" also include naturally occurring allelic variants.

[00136] By "corresponds to" or "corresponding to" is meant a polynucleotide (a) having a nucleotide sequence that is substantially identical or complementary to all

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or most of a reference polynucleotide sequence or (b) encoding an amino acid sequence identical to an amino acid sequence in a peptide or protein. This phrase also includes within its scope a peptide or polypeptide having an amino acid sequence that is substantially identical to a sequence of amino acids in a reference peptide or protein. Terms used to describe sequence relationships between two or more polynucleotides or polypeptides include "reference sequence", "comparison window", "sequence identity", "percentage of sequence identity", "substantial identity" and "identical", and are defined with respect to a minimum number of nucleotides or amino acid residues or over the full length. The terms "sequence identity" and "identity" are used interchangeably herein to refer to the extent that sequences are identical on a nucleotide-by-nucleotide basis or an amino acid-by-amino acid basis over a window of comparison. Thus, a "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U) or the identical amino acid residue (e.g., Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity.

[00137] The % identity of a polynucleotide can be determined by GAP (Needleman and Wütsch, *J. Mol. Biol.* 48: 444-453, 1970) analysis (GCG program) with a gap creation penalty=5, and a gap extension penalty=0.3. Unless stated otherwise, the query sequence is at least 45 nucleotides in length, and the GAP analysis aligns the two sequences over a region of at least 45 nucleotides. Preferably, the query sequence is at least 150 nucleotides in length, and the GAP analysis aligns the two sequences over a region of at least 150 nucleotides. More preferably, the query sequence is at least 300 nucleotides in length and the GAP analysis aligns the two sequences over a region of at least 300 nucleotides, or at least 400, at least 500 or at least 600 nucleotides in each case. Reference also may be made to the BLAST family of programs as for example disclosed by Altschul *et al.*, *Nucleic Acids Res.* 25:

3389, 1997. A detailed discussion of sequence analysis can be found in Unit 19.3 of Ausubel *et al.*, "Current Protocols in Molecular Biology", John Wiley & Sons Inc, 1994-1998, Chapter 15.

[00138] Nucleotide or amino acid sequences are indicated as "essentially similar" when such sequences have a sequence identity of at least 80 %, particularly at least 85%, quite particularly at least 90%, especially at least 95%, more especially are identical. It is clear that when RNA sequences are described as essentially similar to, correspond to, or have a certain degree of sequence identity with, DNA sequences, thymine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence.

[00139] With regard to the defined polynucleotides, it will be appreciated that % identity figures higher than those provided above will encompass preferred embodiments. Thus, where applicable, in light of the minimum % identity figures, it is preferred that the polynucleotide comprises a polynucleotide sequence which is at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, more preferably at least 93%, more preferably at least 94%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, more preferably at least 99%, more preferably at least 99.1%, more preferably at least 99.2%, more preferably at least 99.3%, more preferably at least 99.4%, more preferably at least 99.5%, more preferably at least 99.6%, more preferably at least 99.7%, more preferably at least 99.8%, and even more preferably at least 99.9% identical to the relevant nominated SEQ ID NO., such as SEQ ID NO: 1, 2, 32 or 33.

[00140] Preferably, a polynucleotide of the invention which encodes a polypeptide with SSIIIa activity is greater than 800, preferably greater than 900, and even more preferably greater than 1,000 or 2000 nucleotides in length.

[00141] Polynucleotides of the present invention may possess, when compared to naturally occurring molecules, one or more mutations which are deletions, insertions, or substitutions of nucleotide residues. Mutants can be either naturally occurring (that is to say, isolated from a natural source) or synthetic (for example, by performing site-directed mutagenesis on the nucleic acid).

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[00142] The present invention refers to the stringency of hybridization conditions to define the extent of complementarity of two polynucleotides. "Stringency" as used herein, refers to the temperature and ionic strength conditions, and presence or absence of certain organic solvents, during hybridization and washing. The higher the stringency, the higher will be the degree of complementarity between a target nucleotide sequence and the labelled polynucleotide sequence (probe). "Stringent conditions" refers to temperature and ionic conditions under which only nucleotide sequences having a high frequency of complementary bases will hybridize. As used herein, the term "hybridizes under low stringency, medium stringency, high stringency, or very high stringency conditions" describes conditions for hybridization and washing. Guidance for performing hybridization reactions can be found in Ausubel *et al.*, (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, NY, 6.3.1-6.3.6., 1989. Aqueous and non-aqueous methods are described in that reference and either can be used. Specific hybridization conditions referred to herein are as follows: 1) low stringency hybridization conditions are for hybridization in 6 X sodium chloride/sodium citrate (SSC) at 45°C, followed by two washes in 0.2 X SSC, 0.1% SDS at 50-55°C; 2) medium stringency hybridization conditions are for hybridization in 6 X SSC at about 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 60°C; 3) high stringency hybridization conditions are for hybridization in 6 X SSC at 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 65°C; and 4) very high stringency hybridization conditions are for hybridization in 0.5 M sodium phosphate buffer, 7% SDS at 65°C, followed by one or more washes at 0.2 X SSC, 1% SDS at 65°C.

Polypeptides

[00143] The terms "polypeptide" and "protein" are generally used interchangeably. The terms "proteins" and "polypeptides" as used herein also include variants, mutants, modifications, analogs and/or derivatives of the polypeptides of the invention as described herein. As used herein, "substantially purified polypeptide" refers to a polypeptide that has been separated from the lipids, nucleic acids, other peptides and other molecules with which it is associated in its native state. Preferably, the substantially purified polypeptide is at least 90% free from other components with

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which it is naturally associated. By "recombinant polypeptide" is meant a polypeptide made using recombinant techniques, i.e., through the expression of a recombinant polynucleotide in a cell, preferably a plant cell and more preferably a cereal plant cell.

[00144] Illustrative proteins having SSIIa activity (unmodified) or partial (reduced) SSIIa activity are set out in the sequence listing and described in Table 11. Accordingly, the present invention proposes without limitation the modification of SSIIa polypeptides having the amino acid sequences set forth in SEQ ID NO: 4 and naturally occurring variants, corrected versions thereof and variants as described herein such as variants having at least about 80% sequence identity or at least 98% sequence identity. The invention extends however to SSIIa proteins having substantially no activity such as substantially no starch synthase activity or no SSIIa activity in the endosperm including the developing endosperm, or starch granule or soluble fraction.

[00145] With regard to a defined polypeptide, it will be appreciated that % identity figures higher than those provided above will encompass particular embodiments. Thus, where applicable, in light of the minimum % identity figures, it is preferred that the polypeptide comprises an amino acid sequence which is at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, more preferably at least 93%, more preferably at least 94%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, more preferably at least 99%, more preferably at least 99.1%, more preferably at least 99.2%, more preferably at least 99.3%, more preferably at least 99.4%, more preferably at least 99.5%, more preferably at least 99.6%, more preferably at least 99.7%, more preferably at least 99.8%, and even more preferably at least 99.9% identical to the relevant nominated SEQ ID NO: 4.

[00146] In some embodiments, the length of a reference sequence aligned for comparison purposes is at least 30%, usually at least 40%, more usually at least 50%, 60%, and even more usually at least 70%, 80%, 90%, 100% of the length of the reference sequence. The comparison of sequences and determination of percent identity or percent similarity between sequences can be accomplished using a

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mathematical algorithm. In some embodiments, the comparison is along the full length protein, preferably using the BLASTP program with default parameters. In certain embodiments, the percent identity or similarity between amino acid sequences is determined using the Needleman and Wunsch, 1970 (*supra*) algorithm which has been incorporated into the GAP program in the GCG software package (available at <http://www.gcg.com>), using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In specific embodiments, the percent identity between nucleotide sequences is determined using the GAP program in the GCG software package (available at <http://www.gcg.com>), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. A non-limiting set of parameters (and the one that should be used unless otherwise specified) includes a Blossum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5.

[00147] In some embodiments, the percent identity or similarity between amino acid or nucleotide sequences can be determined using the algorithm of Meyers and Miller, *Cabios*, 4: 11-17, 1989 which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

[00148] The nucleic acid and protein sequences described herein can be used as a "query sequence" to perform a search against public databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul *et al.*, *J. Mol. Biol.* 215: 403-10, 1990). BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to 53010 nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to 53010 protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, 1997 (*supra*). When utilizing BLAST

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and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used.

[00149] As used herein a "biologically active" fragment of a polypeptide is a portion of a polypeptide of the invention, less than full length, which maintains a defined activity of the full-length polypeptide. In a particularly preferred embodiment, the biologically active fragment is able to elongate long B chains connecting amylopectin structures. Biologically active fragments can be any size as long as they maintain the defined activity, but are preferably at least 200 or at least 250 amino acid residues long.

[00150] Amino acid sequence mutants of the polypeptides of the present invention can be prepared by introducing appropriate nucleotide changes into a nucleic acid of the present invention, or by *in vitro* synthesis of the desired polypeptide. Such mutants include, for example, deletions, insertions or substitutions of residues within the amino acid sequence. A combination of deletion, insertion and substitution can be made to arrive at the final construct, provided that the final peptide product possesses the desired characteristics. In some embodiments, the subject genetic variations encompass mutations that are non-conservative substitutions in conserved regions of SSIIa conserved regions of SSII such as the amino acid motifs set out in SEQ ID NOs: 34 to 41. These motifs are further described in International Publication No. WO/2000/066745 incorporated herein by reference in its entirety.

[00151] The sequence motifs from the WO2000/066745 (wheat SSII) are present at the following positions in the barley SSIIa sequence: (a) 1159-1169; (b) 1178-1188; (c) 1272-1287; (d) 1347-1362; (e) 1395-1410; (f) 1425-1434; (g) 1474-1500; and (h) 1507-1514 (all inclusive).

[00152] The Himalaya (wildtype) barley sequence has one amino acid substitution in each of e, g and h compared to the wheat SSIIa sequence. Those substitutions are conservative amino acid changes. The mutant sequence in amol-38 is at position 1480 within motif (g), and is non-conservative. Accordingly, particular mutants are non-conservative changes in these motifs. Mutant (variant) sequences can be produced using any technique known in the art. Amino acid sequence

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deletions generally range from about 1 to 15 residues, more preferably about 1 to 10 residues and typically about 1 to 5 contiguous residues.

[00153] Substitution mutants have at least one amino acid residue in the polypeptide molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified as the active site(s). Other sites of interest are those in which particular residues obtained from various strains or species are identical. These positions may be important for biological activity. These sites, especially those falling within a sequence of at least three other identically conserved sites, are preferably substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 13 under the heading of "exemplary substitutions".

[00154] Polypeptides of the present invention can be produced in a variety of ways, including production and recovery of natural polypeptides, production and recovery of recombinant polypeptides, and chemical synthesis of the polypeptides.

[00155] The present invention refers to elements which are operably connected or linked. "Operably connected" or "operably linked" and the like refer to a linkage of polynucleotide elements in a functional relationship. Typically, operably connected nucleic acid sequences are contiguously linked and, where necessary to join two protein coding regions, contiguous and in reading frame. A coding sequence is "operably connected to" another coding sequence when RNA polymerase will transcribe the two coding sequences into a single RNA, which if translated is then translated into a single polypeptide having amino acids derived from both coding sequences. The coding sequences need not be contiguous to one another so long as the expressed sequences are ultimately processed to produce the desired protein.

[00156] As used herein, the term "*cis*-acting sequence", "*cis*-acting element" or "*cis*-regulatory region" or "regulatory region" or similar term shall be taken to mean any sequence of nucleotides, which when positioned appropriately and connected relative to an expressible genetic sequence, is capable of regulating, at least in part, the expression of the genetic sequence. Those skilled in the art will be aware that a *cis*-regulatory region may be capable of activating, silencing, enhancing, repressing or otherwise altering the level of expression and/or cell-type-specificity

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and/or developmental specificity of a gene sequence at the transcriptional or post-transcriptional level. In certain embodiments of the present invention, the cis-acting sequence is an activator sequence that enhances or stimulates the expression of an expressible genetic sequence.

[00157] "Promoter" or "promoter sequence" as used herein refers to a region of a gene, generally upstream (5') of the RNA encoding region, which controls the initiation and level of transcription in the cell of interest. A "promoter" includes the transcriptional regulatory sequences of a classical genomic gene, including a TATA box and CCAAT box sequences, as well as additional regulatory elements (i.e., upstream activating sequences, enhancers and silencers) that alter gene expression in response to developmental and/or environmental stimuli, or in a tissue-specific or cell-type-specific manner. A promoter is usually, but not necessarily (for example, some PolIII promoters), positioned upstream of a structural gene, the expression of which it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the gene. Promoters may contain additional specific regulatory elements, located more distal to the start site to further enhance expression in a cell, and/or to alter the timing or inducibility of expression of a structural gene to which it is operably connected.

[00158] "Constitutive promoter" refers to a promoter that directs expression of an operably linked transcribed sequence in many or all tissues of a plant. The term constitutive as used herein does not necessarily indicate that a gene is expressed at the same level in all cell types, but that the gene is expressed in a wide range of cell types, although some variation in level is often detectable. "Selective expression" as used herein refers to expression almost exclusively in specific organs of the plant, such as, for example, endosperm, embryo, leaves, fruit, tubers or root. In one embodiment, a promoter is expressed in all photosynthetic tissue, which may correspond to all aerial parts of the plant, for example a promoter that is involved in expressing a gene required for photosynthesis such as rubisco small subunit promoters. The term may also refer to expression at specific developmental stages in an organ, such as in early or late embryogenesis or different stages of maturity; or to expression that is inducible by certain environmental conditions or treatments. Selective expression may therefore

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be contrasted with constitutive expression, which refers to expression in many or all tissues of a plant under most or all of the conditions experienced by the plant.

[00159] Selective expression may also result in compartmentation of the products of gene expression in specific plant tissues, organs or developmental stages. Compartmentation in specific subcellular locations such as the endosperm, cytosol, vacuole, or apoplastic space may be achieved by the inclusion in the structure of the gene product of appropriate signals for transport to the required cellular compartment, or in the case of the semi-autonomous organelles (plastids and mitochondria) by integration of the transgene with appropriate regulatory sequences directly into the organelle genome.

[00160] A "tissue-specific promoter" or "organ-specific promoter" is a promoter that is preferentially expressed in one tissue or organ relative to many other tissues or organs, preferably most if not all other tissues or organs in a plant. Typically, the promoter is expressed at a level 10-fold higher in the specific tissue or organ than in other tissues or organs. An illustrative tissue specific promoter is the promoter for high molecular weight (HMW) glutenin gene, Bx17 (see Figure 2) which is expressed preferentially in the developing endosperm of cereal plants. Further endosperm specific promoters include the high molecular weight glutenin promoter, the wheat SSI promoter, and the wheat BEII promoter. Other endosperm-specific promoters may readily be obtained from genes which encode starch biosynthetic enzymes or storage proteins in the developing grain.

[00161] The promoters contemplated by the present invention may be native to the host plant to be transformed or may be derived from an alternative source, where the region is functional in the host plant. Other sources include the *Agrobacterium* T-DNA genes, such as the promoters of genes for the biosynthesis of nopaline, octopine, mannopine, or other opine promoters; promoters from plants, such as ubiquitin promoters such as the Ubi promoter from the maize ubi-1 gene, Christensen *et al.*, (1996) (see, e.g., U.S. Patent No. 4,962,028) or actin promoters; tissue specific promoters (see, e.g., U.S. Patent No. 5,459,252 to Conkling *et al.*; WO 91/13992 to Advanced Technologies); promoters from viruses (including host specific viruses), or partially or wholly synthetic promoters. Numerous promoters that are

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functional in mono- and dicotyledonous plants are well known in the art (see, for example, Greve, *J. Mol. Appl. Genet.*, 1: 499-511, 1983; Salomon *et al.*, *EMBO J.*, 3: 141-146, 1984; Garfinkel *et al.*, *Cell*, 27: 143-153, 1983; Barker *et al.*, *Plant Mol. Biol.*, 2: 235-350, 1983; including various promoters isolated from plants and viruses such as the cauliflower mosaic virus promoter (CaMV 35S, 19S). Many tissue specific promoter regions are known. Other transcriptional initiation regions which preferentially provide for transcription in certain tissues or under certain growth conditions, include those from genes encoding napin, seed ACP, zein, or other seed storage proteins. Fruit specific promoters are also known, one such promoter is the E8 promoter, described by Deikman *et al.*, *EMBO J.*, 2: 3315-3320, 1998 and DellaPenna *et al.*, *Plant Cell*, 1: 53-63, 1989. Non-limiting methods for assessing promoter activity are disclosed by Medberry *et al.*, *Plant Cell*, 4: 185-192, 1992; Medberry *et al.*, *Plant J.* 3: 619-626, 1993, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (2nd ed.). Cold Spring Harbour Laboratory, Cold Spring Harbour, NY, 1989, and McPherson *et al.* (U.S. Patent No. 5,164,316).

[00162] Alternatively or additionally, the promoter may be an inducible promoter or a developmentally regulated promoter which is capable of driving expression of the introduced polynucleotide at an appropriate developmental stage of the plant. Other *cis*-acting sequences which may be employed include transcriptional and/or translational enhancers. Enhancer regions are well known to persons skilled in the art, and can include an ATG translational initiation codon and adjacent sequences. The initiation codon must be in phase with the reading frame of the coding sequence relating to the foreign or exogenous polynucleotide to ensure translation of the entire sequence. The translation control signals and initiation codons can be of a variety of origins, both natural and synthetic. Translational initiation regions may be provided from the source of the transcriptional initiation region, or from a foreign or exogenous polynucleotide. The sequence can also be derived from the source of the promoter selected to drive transcription, and can be specifically modified so as to increase translation of the mRNA.

[00163] The nucleic acid construct of the present invention typically comprises a 3' non-translated sequence from about 50 to 1,000 nucleotide base pairs

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which may include a transcription termination sequence. A 3' non-translated sequence may contain a transcription termination signal which may or may not include a polyadenylation signal and any other regulatory signals capable of effecting mRNA processing. A polyadenylation signal is characterized by effecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. Polyadenylation signals are commonly recognized by the presence of homology to the canonical form 5' AATAAA-3' although variations are not uncommon. Transcription termination sequences which do not include a polyadenylation signal include terminators for PolII or PolIII RNA polymerase which comprise a run of four or more thymidines. Examples of suitable 3' non-translated sequences are the 3' transcribed non-translated regions containing a polyadenylation signal from the nopaline synthase (nos) gene of *Agrobacterium tumefaciens* (Bevan *et al.*, *Nucl. Acid Res.*, 11: 369, 1983) and the terminator for the T7 transcript from the octopine synthase gene of *Agrobacterium tumefaciens*. Alternatively, suitable 3' non-translated sequences may be derived from plant genes such as the 3' end of the protease inhibitor I or II genes from potato or tomato, the soybean storage protein genes and the small subunit of the ribulose-1,5-bisphosphate carboxylase (ssRUBISCO) gene, although other 3' elements known to those of skill in the art can also be employed. Alternatively, 3' non-translated regulatory sequences can be obtained *de novo* as, for example, described by An, *Methods in Enzymology*, 153: 292, 1987, which is incorporated herein by reference.

[00164] As the DNA sequence inserted between the transcription initiation site and the start of the coding sequence, i.e., the untranslated 5' leader sequence (5'UTR), can influence gene expression, one can also employ a particular leader sequence. Suitable leader sequences include those that comprise sequences selected to direct optimum expression of the foreign or endogenous DNA sequence. For example, such leader sequences include a preferred consensus sequence which can increase or maintain mRNA stability and prevent inappropriate initiation of translation as for example described by Joshi, *Nucl. Acid Res.* 15: 6643, 1987.

[00165] Additionally, targeting sequences may be employed to target the enzyme encoded by the foreign or exogenous polynucleotide to an intracellular compartment, for example to the chloroplast, within plant cells or to the extracellular

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environment. For example, a nucleic acid sequence encoding a transit or signal peptide sequence may be operably linked to a sequence that encodes a chosen enzyme of the subject invention such that, when translated, the transit or signal peptide can transport the enzyme to a particular intracellular or extracellular destination, and can then be optionally post-translationally removed. Transit or signal peptides act by facilitating the transport of proteins through intracellular membranes, e.g., endoplasmic reticulum, vacuole, vesicle, plastid, mitochondrial and plasmalemma membranes. For example, the targeting sequence can direct a desired protein to a particular organelle such as a vacuole or a plastid (e.g., a chloroplast), rather than to the cytosol. Thus, the nucleic acid construct of the invention can further comprise a plastid transit peptide-encoding nucleic acid sequence operably linked between a promoter region and the foreign or exogenous polynucleotide.

Vectors

[00166] The present invention includes use of vectors for manipulation or transfer of genetic constructs. By "vector" is meant a nucleic acid molecule, preferably a DNA molecule derived, for example, from a plasmid, bacteriophage, or plant virus, into which a nucleic acid sequence may be inserted or cloned. A vector preferably contains one or more unique restriction sites and may be capable of autonomous replication in a defined host cell including a target cell or tissue or a progenitor cell or tissue thereof, or be integrable with the genome of the defined host such that the cloned sequence is reproducible. Accordingly, the vector may be an autonomously replicating vector, i.e., a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a linear or closed circular plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into a cell, is integrated into the genome of the recipient cell and replicated together with the chromosome(s) into which it has been integrated. A vector system may comprise a single vector or plasmid, two or more vectors or plasmids, which together contain the total DNA to be introduced into the genome of the host cell, or a transposon. The choice of the vector will typically depend on the compatibility of the vector with the

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cell into which the vector is to be introduced. The vector may also include a selection marker such as an antibiotic resistance gene, a herbicide resistance gene or other gene that can be used for selection of suitable transformants. Examples of such genes are well known to those of skill in the art.

[00167] The nucleic acid construct of the invention can be introduced into a vector, such as a plasmid. Plasmid vectors typically include additional nucleic acid sequences that provide for easy selection, amplification, and transformation of the expression cassette in prokaryotic and eukaryotic cells, e.g., pUC-derived vectors, pSK-derived vectors, pGEM-derived vectors, pSP-derived vectors, or pBS-derived vectors. Additional nucleic acid sequences include origins of replication to provide for autonomous replication of the vector, selectable marker genes, preferably encoding antibiotic or herbicide resistance, unique multiple cloning sites providing for multiple sites to insert nucleic acid sequences or genes encoded in the nucleic acid construct, and sequences that enhance transformation of prokaryotic and eukaryotic (especially plant) cells.

[00168] By "marker gene" is meant a gene that imparts a distinct phenotype to cells expressing the marker gene and thus allows such transformed cells to be distinguished from cells that do not have the marker. A selectable marker gene confers a trait for which one can "select" based on resistance to a selective agent (e.g., a herbicide, antibiotic, radiation, heat, or other treatment damaging to untransformed cells). A screenable marker gene (or reporter gene) confers a trait that one can identify through observation or testing, i.e., by "screening" (e.g., β -glucuronidase, luciferase, GFP or other enzyme activity not present in untransformed cells). The marker gene and the nucleotide sequence of interest do not have to be linked.

[00169] To facilitate identification of transformants, the nucleic acid construct desirably comprises a selectable or screenable marker gene as, or in addition to, the foreign or exogenous polynucleotide. The actual choice of a marker is not crucial as long as it is functional (i.e., selective) in combination with the plant cells of choice. The marker gene and the foreign or exogenous polynucleotide of interest do not have to be linked, since co-transformation of unlinked genes as, for example,

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described in U.S. Patent No. 4,399,216 is also an efficient process in plant transformation.

[00170] Examples of bacterial selectable markers are markers that confer antibiotic resistance such as ampicillin, kanamycin, erythromycin, chloramphenicol or tetracycline resistance. Exemplary selectable markers for selection of plant transformants include, but are not limited to, a *hyg* gene which encodes hygromycin B resistance; a neomycin phosphotransferase (*npt*) gene conferring resistance to kanamycin, paromomycin, G418 and the like as, for example, described by Potrykus *et al.*, *Mol. Gen. Genet.* 199: 183, 1985; a glutathione-S-transferase gene from rat liver conferring resistance to glutathione derived herbicides as, for example, described in EP-A 256223; a glutamine synthetase gene conferring, upon overexpression, resistance to glutamine synthetase inhibitors such as phosphinothricin as, for example, described in WO 87/05327, an acetyltransferase gene from *Streptomyces viridochromogenes* conferring resistance to the selective agent phosphinothricin as, for example, described in EP-A 275957, a gene encoding a 5-enolshikimate-3-phosphate synthase (EPSPS) conferring tolerance to N-phosphonomethylglycine as, for example, described by Hinchee *et al.*, *Biotech.* 6: 915, 1988, a *bar* gene conferring resistance against bialaphos as, for example, described in WO 91/02071; a nitrilase gene such as *bxn* from *Klebsiella ozaenae* which confers resistance to bromoxynil (Stalker *et al.*, *Science*, 242: 419, 1988); a dihydrofolate reductase (DHFR) gene conferring resistance to methotrexate (Thillet *et al.*, *J. Biol. Chem.* 263: 12500, 1988); a mutant acetolactate synthase gene (ALS), which confers resistance to imidazolinone, sulfonylurea or other ALS-inhibiting chemicals (EP-A-154 204); a mutated anthranilate synthase gene that confers resistance to 5-methyl tryptophan; or a dalapon dehalogenase gene that confers resistance to the herbicide.

[00171] Preferred screenable markers include, but are not limited to, a *uidA* gene encoding a β -glucuronidase (GUS) enzyme for which various chromogenic substrates are known, a β -galactosidase gene encoding an enzyme for which chromogenic substrates are known, an aequorin gene (Prasher *et al.*, *Biochem. Biophys. Res. Comm.* 126: 1259-68, 1985), which may be employed in calcium-sensitive bioluminescence detection; a green fluorescent protein gene (Niedz *et al.*,

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Plant Cell Reports, 14: 403, 1995); a luciferase (*luc*) gene (Ow *et al.*, *Science*, 234: 856, 1986), which allows for bioluminescence detection, and others known in the art. By "reporter molecule" as used in the present specification is meant a molecule that, by its chemical nature, provides an analytically identifiable signal that facilitates determination of promoter activity by reference to protein product.

Methods of modifying gene expression

[00172] In an illustrative embodiment, the level of an SSIIIa protein may be modulated by decreasing the level of expression of a gene encoding the protein in the plant. A plurality of transformants may be selected and screened for those with a favourable level and/or specificity of transgene expression arising from influences of endogenous sequences in the vicinity of the transgene integration site. A favourable level and pattern of transgene expression as disclosed herein is one which results in a substantial decrease in SSIIIa protein of SSIIIa activity or an increase in other starch synthase levels such as GBSSI and/or SSI levels. This may be detected by simple testing of grain from the transformants. Testing may conveniently be performed based upon enzyme activity, e.g. zymogram analysis. Alternatively, a population of mutagenized grain or a population of plants from a breeding program may be screened for individual lines with altered phenotypes.

[00173] Reducing gene expression may be achieved through introduction and transcription of a "gene-silencing chimeric gene" introduced into the plant cell. The gene-silencing chimeric gene may be introduced stably into the plant cell's genome, preferably nuclear genome, or it may be introduced transiently, for example on a viral vector. As used herein "gene-silencing effect" refers to the reduction of expression of a target nucleic acid in a plant cell, which can be achieved by introduction of a silencing RNA. Such reduction may be the result of reduction of transcription, including *via* methylation of chromatin remodeling, or post-transcriptional modification of the RNA molecules, including *via* RNA degradation, or both. Gene-silencing includes an abolishing of the expression of the target nucleic acid or gene and a partial effect in either extent or duration. It is sufficient that the level of expression of the target nucleic acid in the presence of the silencing RNA is lower than in the absence thereof. The level of expression may be reduced by at least

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about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90%, or at least about 95%, or at least about 99%.

Antisense RNA molecules

[00174] Antisense techniques may be used to reduce gene expression according to the invention. The term "antisense RNA" shall be taken to mean an RNA molecule that is complementary to at least a portion of a specific mRNA molecule and capable of reducing expression of the gene encoding the mRNA. Such reduction typically occurs in a sequence-dependent manner and is thought to occur by interfering with a post-transcriptional event such as mRNA transport from nucleus to cytoplasm, mRNA stability or inhibition of translation. The use of antisense methods is well known in the art (see for example, Hartmann and Endres, *Manual of Antisense Methodology*, Kluwer, 1999). The use of antisense techniques in plants has been reviewed by Bourque, *Plant Sci.* 105: 125-149, 1995 and Senior, *Biotech. Genet. Engin. Revs.* 15: 79-119, 1998. Bourque, 1995 (*supra*) lists a large number of examples of how antisense sequences have been utilized in plant systems as a method of gene inactivation. She also states that attaining 100% inhibition of any enzyme activity may not be necessary as partial inhibition will more than likely result in measurable change in the system. Senior, 1998 (*supra*) states that antisense methods are now a very well established technique for manipulating gene expression.

[00175] As used herein, the term "an antisense polynucleotide which hybridizes under physiological conditions" means that the polynucleotide (which is fully or partially single stranded) is at least capable of forming a double stranded polynucleotide with an RNA product of the gene to be inhibited, typically the mRNA encoding a protein such as those provided herein, under normal conditions in a cell. Antisense molecules may include sequences that correspond to the structural genes or for sequences that effect control over the gene expression or splicing event. For example, the antisense sequence may correspond to the coding region of *SSIIIa* gene, or the 5'-untranslated region (UTR) or the 3'-UTR or combination of these. It may be complementary in part to intron sequences, which may be spliced out during or after transcription, but is preferably complementary only to exon sequences of the target

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gene. In view of the generally greater divergence of the UTRs, targeting these regions provides greater specificity of gene inhibition.

[00176] The length of the antisense sequence should be at least 19 contiguous nucleotides, preferably at least 25 or 30 or 50 nucleotides, and more preferably at least 100, 200, 500 or 1000 nucleotides, to a maximum of the full length of the gene to be inhibited. The full-length sequence complementary to the entire gene transcript may be used. The length is most preferably 100-2000 nucleotides. The degree of identity of the antisense sequence to the targeted transcript should be at least 90% and more preferably 95-100%. The antisense RNA molecule may of course comprise unrelated sequences which may function to stabilize the molecule.

[00177] Genetic constructs to express an antisense RNA may be readily made by joining a promoter sequence to a region of the target gene in an "antisense" orientation, which as used herein refers to the reverse orientation relative to the orientation of transcription and translation (if it occurs) of the sequence in the target gene in the plant cell. Accordingly, also provided by this invention is a nucleic acid molecule such as a chimeric DNA coding for an antisense RNA of the invention, including cells, tissues, organs, plants, grain and the like comprising the nucleic acid molecule. Illustrative gene constructs are described in the Examples.

Ribozymes

[00178] The term "ribozyme" as used herein refers to an RNA molecule which specifically recognizes a distinct substrate RNA and catalyzes its cleavage. Typically, the ribozyme contains a region of nucleotides which are complementary to a region of the target RNA, enabling the ribozyme to specifically hybridize to the target RNA under physiological conditions, for example in the cell in which the ribozyme acts, and an enzymatic region referred to herein as the "catalytic domain". The types of ribozymes that are particularly useful in this invention are the hammerhead ribozyme (Haseloff and Gerlach, *Nature* 334: 585-591, 1988; Perriman *et al.*, *Gene*, 113: 157-163, 1992) and the hairpin ribozyme (Shippy *et al.*, *Mol. Biotech.* 12: 117-129, 1999). DNA encoding the ribozymes can be synthesized using methods well known in the art and may be incorporated into a genetic construct or expression vector for expression in the cell of interest. Accordingly, also provided by

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this invention is a nucleic acid molecule such as a chimeric DNA coding for a ribozyme of the invention, including cells, tissues, organs, plants, grain and the like comprising the nucleic acid molecule. Typically, the DNA encoding the ribozyme is inserted into an expression cassette under control of a promoter and a transcription termination signal that function in the cell. Specific ribozyme cleavage sites within any potential RNA target may be identified by scanning the target molecule for ribozyme cleavage sites which include the trinucleotide sequences GUA, GUU and GUC. Once identified, short RNA sequences of between about 5 and 20 ribonucleotides corresponding to the region of the target gene 5' and 3' of the cleavage site may be evaluated for predicted structural features such as secondary structure that may render the oligonucleotide sequence less suitable. When employed, ribozymes may be selected from the group consisting of hammerhead ribozymes, hairpin ribozymes, axehead ribozymes, newt satellite ribozymes, Tetrahymena ribozymes and RNase P ribozymes, and are designed according to methods known in the art based on the sequence of the target gene (for instance, see U.S. Patent No. 5,741,679). The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays.

[00179] As with antisense polynucleotides described herein, ribozymes of the invention should be capable of hybridizing to a target nucleic acid molecule (for example an mRNA encoding a polypeptide provided as SEQ ID NO: 4) or a naturally occurring variant thereof under "physiological conditions", namely those conditions within a cell, especially conditions in a plant cell such as a wheat or barley cell.

RNA interference/duplex RNA

[00180] As used herein, "artificially introduced dsRNA molecule" refers to the introduction of dsRNA molecule, which may e.g. occur endogenously by transcription from a chimeric gene encoding such dsRNA molecule, however does not refer to the conversion of a single stranded RNA molecule into a dsRNA inside the eukaryotic cell or plant cell. RNA interference (RNAi) is particularly useful for specifically reducing the expression of a gene or inhibiting the production of a particular protein. Although not wishing to be limited by theory, Waterhouse *et al.*,

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Proc. Natl. Acad. Sci. U.S.A. 95: 13959-13964, 1998 have provided a model for the mechanism by which dsRNA can be used to reduce protein production. This technology relies on the presence of dsRNA molecules that contain a sequence that is essentially identical to the mRNA of the gene of interest or part thereof. Conveniently, the dsRNA can be produced from a single promoter in a recombinant vector or host cell, where the sense and anti-sense sequences are transcribed to produce a hairpin RNA in which the sense and anti-sense sequences hybridize to form the dsRNA region with an intervening sequence or spacer region forming a loop structure, so the hairpin RNA comprises a stem-loop structure. The design and production of suitable dsRNA molecules for the present invention is well within the capacity of a person skilled in the art, particularly considering Waterhouse *et al.*, 1998 (*supra*), Smith *et al.*, *Nature*, 407: 319-320, 2000, WO 99/53050, WO 99/49029, and WO 01/34815. Accordingly, also provided by this invention is a nucleic acid molecule such as a chimeric DNA coding for a duplex RNA such as a hairpin RNA of the invention, including cells, tissues, organs, plants, grain and the like comprising the nucleic acid molecule.

[00181] In one example, a DNA is introduced that directs the synthesis of an at least partly double stranded RNA product(s) with homology to the target gene to be inactivated. The DNA therefore comprises both sense and antisense sequences that, when transcribed into RNA, can hybridize to form the double-stranded RNA region. In a preferred embodiment, the sense and antisense sequences are separated by a spacer region that comprises an intron which, when transcribed into RNA, is spliced out. This arrangement has been shown to result in a higher efficiency of gene silencing (Smith *et al.*, 2000 (*supra*)). The double-stranded region may comprise one or two RNA molecules, transcribed from either one DNA region or two. The dsRNA may be classified as long hpRNA, having long, sense and antisense regions which can be largely complementary, but need not be entirely complementary (typically forming a basepaired region larger than about 100 bp, preferably ranging between 200-1000 bp). hpRNA can also be smaller with the double-stranded portion ranging in size from about 30 to about 50 bp, or from 30 to about 100 bp (see WO 04/073390, herein incorporated by reference). The presence of the double stranded RNA region is

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thought to trigger a response from an endogenous plant system that processes the double stranded RNA to oligonucleotides of 21-24 nucleotides long, and also uses these oligonucleotides for sequence-specific cleavage of the homologous RNA transcript from the target plant gene, efficiently reducing or eliminating the activity of the target gene.

[00182] The length of the sense and antisense sequences that hybridize should each be at least 19 contiguous nucleotides, preferably at least 27 or 30 or 50 nucleotides, and more preferably at least 100, 200, or 500 nucleotides, up to the full-length sequence corresponding to the entire gene transcript. The lengths are most preferably 100-2000 nucleotides. The degree of identity of the sense and antisense sequences to the targeted transcript should be at least 85%, preferably at least 90% and more preferably 95-100%. The longer the sequence, the less stringent the requirement is for overall sequence identity. The RNA molecule may of course comprise unrelated sequences which may function to stabilize the molecule. The RNA molecule may be a hybrid between different sequences targeting different target RNAs, allowing reduction in expression of more than one target gene, or it may be one sequence which corresponds to a family of related target genes such as a multigene family. The sequences used in the dsRNA preferably correspond to exon sequences of the target gene and may correspond to 5' and/or 3' untranslated sequences or protein coding sequences or any combination thereof.

[00183] The promoter used to express the dsRNA-forming construct may be any type of promoter if the resulting dsRNA is specific for a gene product in the cell lineage targeted for destruction. Alternatively, the promoter may be lineage specific in that it is only expressed in cells of a particular development lineage. This might be advantageous where some overlap in homology is observed with a gene that is expressed in a non-targeted cell lineage. The promoter may also be inducible by externally controlled factors, or by intracellular environmental factors. Typically, the RNA molecule is expressed under the control of a RNA polymerase II or RNA polymerase III promoter. Examples of the latter include tRNA or snRNA promoters.

[00184] Other silencing RNA may be "unpolyadenylated RNA" comprising at least 20 consecutive nucleotides having at least 95% sequence identity to the

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complement of a nucleotide sequence of an RNA transcript of the target gene, such as described in WO 01/12824 or US 6,423,885 (both documents herein incorporated by reference). Yet another type of silencing RNA is an RNA molecule as described in WO 03/076619 (herein incorporated by reference) comprising at least 20 consecutive nucleotides having at least 95% sequence identity to the sequence of the target nucleic acid or the complement thereof, and further comprising a largely-double stranded region as described in WO 03/076619.

[00185] MicroRNA regulation is a specialized branch of the RNA silencing pathway that evolved towards gene regulation, diverging from conventional RNAi/PTGS. MicroRNAs are a specific class of small RNAs that are encoded in gene-like elements organized in a characteristic partial inverted repeat. When transcribed, microRNA genes give rise to partially basepaired stem-looped precursor RNAs from which the microRNAs are subsequently processed. MicroRNAs are typically about 21 nucleotides in length. The released miRNAs are incorporated into RISC-like complexes containing a particular subset of Argonaute proteins that exert sequence-specific gene repression (see, for example, Millar and Waterhouse, *Funct Integr Genomics*, 5: 129-135, 2005; Pasquinelli *et al.*, *Curr Opin Genet Develop* 15: 200-205, 2005; Almeida and Allshire, *Trends Cell Biol.* 15: 251-258, 2005, herein incorporated by reference).

Cosuppression

[00186] Another molecular biological approach that may be used for specifically reducing gene expression is co-suppression. The mechanism of co-suppression is not well understood but is thought to involve post-transcriptional gene silencing (PTGS) and in that regard may be very similar to many examples of antisense suppression. It involves introducing an extra copy of a gene or a fragment thereof into a plant in the "sense orientation" with respect to a promoter for its expression, which as used herein refers to the same orientation as transcription and translation (if it occurs) of the sequence relative to the sequence in the target gene. The size of the sense fragment, its correspondence to target gene regions, and its degree of homology to the target gene are as for the antisense sequences described above. In some instances the additional copy of the gene sequence interferes with the expression of the target plant gene. Reference is made to Patent specification WO 97/20936 and European patent specification 0465572 for methods of implementing

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co-suppression approaches. The antisense, co-suppression or double stranded RNA molecules may also comprise a largely double-stranded RNA region, preferably comprising a nuclear localization signal, as described in WO 03/076619.

[00187] Any of these technologies for reducing gene expression can be used to coordinately reduce the activity of multiple genes. For example, one RNA molecule can be targeted against a family of related genes by targeting a region of the genes which is in common. Alternatively, unrelated genes may be targeted by including multiple regions in one RNA molecule, each region targeting a different gene. This can readily be done by fusing the multiple regions under the control of a single promoter.

Methods of introducing nucleic acids into plant cells/transformation

[00188] A number of techniques are available for the introduction of nucleic acid molecules into a plant host cell, well known to workers in the art. The term "transformation" means alteration of the genotype of an organism, for example a bacterium or a plant, by the introduction of a foreign or exogenous nucleic acid. By "transformant" is meant an organism so altered. As used herein the term "transgenic" refers to a genetically modified plant in which the endogenous genome is supplemented or modified by the integration, or stable maintenance in a replicable non-integrated form, of an introduced foreign or exogenous gene or sequence. By "transgene" is meant a foreign or exogenous gene or sequence that is introduced into the genome of a plant. The nucleic acid molecule may be stably integrated into the genome of the plant, or it may be replicated as an extrachromosomal element. By "genome" is meant the total inherited genetic complement of the cell, plant or plant part, and includes chromosomal DNA, plastid DNA, mitochondrial DNA and extrachromosomal DNA molecules. The term "regeneration" as used herein in relation to plant materials means growing a whole, differentiated plant from a plant cell, a group of plant cells, a plant part such as, for example, from an embryo, scutellum, protoplast, callus, or other tissue, but not including growth of a plant from a seed.

[00189] The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the

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experience and preference of the person practicing the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce a nucleic acid construct into plant cells is not essential to or a limitation of the invention, provided it achieves an acceptable level of nucleic acid transfer. Guidance in the practical implementation of transformation systems for plant improvement is provided by Birch, *Ann Rev Plant Physiol Plant Mol Biol.* 48: 297-326, 1997.

[00190] Introduction and expression of foreign or exogenous polynucleotides may be performed using the T-DNA of the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* (See, for example, Umbeck, U.S. Patent No. 5,004,863, and International application PCT/US93/02480). A construct of the invention may be introduced into a plant cell utilizing *A. tumefaciens* containing the Ti plasmid. In using an *A. tumefaciens* culture as a transformation vehicle, it is most advantageous to use a non-oncogenic strain of the *Agrobacterium* as the vector carrier so that normal non-oncogenic differentiation of the transformed tissues is possible. It is preferred that the *Agrobacterium* harbors a binary Ti plasmid system. Such a binary system comprises (1) a first Ti plasmid having a virulence region essential for the introduction of transfer DNA (T-DNA) into plants, and (2) a chimeric plasmid. The chimeric plasmid contains at least one border region of the T-DNA region of a wild-type Ti plasmid flanking the nucleic acid to be transferred. Binary Ti plasmid systems have been shown effective to transform plant cells as, for example, described by De Framond, *Biotechnology*, 1: 262, 1983 and Hockema *et al.*, *Nature*, 303: 179, 1983. Such a binary system is preferred *inter alia* because it does not require integration into the Ti plasmid in *Agrobacterium*.

[00191] Methods involving the use of *Agrobacterium* include, but are not limited to: (a) co-cultivation of *Agrobacterium* with cultured isolated protoplasts; (b) transformation of plant cells or tissues with *Agrobacterium*; (c) transformation of seeds, apices or meristems with *Agrobacterium*, or (d) inoculation *in planta* such as the floral-dip method as described by Bechtold *et al.*, *C.R. Acad. Sci. Paris*, 316: 1194, 1993 or in wheat (as described in WO 00/63398, herein incorporated by reference). This approach is based on the infiltration of a suspension of

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Agrobacterium cells. Alternatively, the chimeric construct may be introduced using root-inducing (Ri) plasmids of *Agrobacterium* as vectors.

[00192] Methods for transformation of cereal plants such as wheat and barley for introducing genetic variation into the plant by introduction of an exogenous nucleic acid and for regeneration of plants from protoplasts or immature plant embryos are well known in the art, see for example, Wan and Lemaux, *Plant Physiol.* 104: 37-48, 1994, Tingay *et al.*, *Plant Journal* 11: 1369-1376, 1997, Canadian Patent Application No. 2,092,588, Australian Patent Application No 61781/94, Australian Patent No 667939, US Patent No. 6,100,447, International Patent Application PCT/US97/10621, U.S. Patent No. 5,589,617, U.S. Patent No. 6,541,257. Preferably, transgenic plants are produced by *Agrobacterium tumefaciens* mediated transformation procedures. Vectors carrying the desired nucleic acid construct may be introduced into regenerable cereal cells of tissue cultured plants or explants. The regenerable cells are preferably from the scutellum of immature embryos, mature embryos, callus derived from these, or the meristematic tissue. Immature embryos are preferably those from inflorescences about 10-15 days after anthesis.

[00193] The genetic construct can also be introduced into plant cells by electroporation as, for example, described by Fromm *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 82: 5824, 1985 and Shimamoto *et al.*, *Nature*, 338: 274-276, 1989. In this technique, plant protoplasts are electroporated in the presence of vectors or nucleic acids containing the relevant nucleic acid sequences. Electrical impulses of high field strength reversibly permeabilize membranes allowing the introduction of nucleic acids. Electroporated plant protoplasts reform the cell wall, divide and form a plant callus.

[00194] Another method for introducing the nucleic acid construct into a plant cell is high velocity ballistic penetration by small particles (also known as particle bombardment or microprojectile bombardment) with the nucleic acid to be introduced contained either within the matrix of small beads or particles, or on the surface thereof as, for example described by Klein *et al.*, *Nature*, 327: 70, 1987.

[00195] Alternatively, the nucleic acid construct can be introduced into a plant cell by contacting the plant cell using mechanical or chemical means. For

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example, a nucleic acid can be mechanically transferred by microinjection directly into plant cells by use of micropipettes. Alternatively, a nucleic acid may be transferred into the plant cell by using polyethylene glycol which forms a precipitation complex with genetic material that is taken up by the cell.

Mutagenesis

[00196] The plants of the invention can be produced and identified after mutagenesis. This may provide a plant which is non-transgenic, which is desirable in some markets.

[00197] Mutants can be either naturally occurring (that is to say, isolated from a natural source) or synthetic (for example, by performing mutagenesis on the nucleic acid) or induced. Generally, a progenitor plant cell, tissue, seed or plant may be subjected to mutagenesis to produce single or multiple mutations, such as nucleotide substitutions, deletions, additions and/or codon modification. In the context of this application, an "induced mutation" is an artificially induced genetic variation which may be the result of chemical, radiation or biologically-based mutagenesis, for example transposon or T-DNA insertion. In some embodiments, mutations are null mutations such as nonsense mutations, frameshift mutations, insertional mutations or splice-site variants which completely inactivate the gene. Nucleotide insertional derivatives include 5' and 3' terminal fusions as well as intra-sequence insertions of single or multiple nucleotides. Insertional nucleotide sequence variants are those in which one or more nucleotides are introduced into the nucleotide sequence, which may be obtained by random insertion with suitable screening of the resulting products. Deletional variants are characterized by the removal of one or more nucleotides from the sequence. Preferably, a mutant gene has only a single insertion or deletion of a sequence of nucleotides relative to the wild-type gene. Substitutional nucleotide variants are those in which at least one nucleotide in the sequence has been removed and a different nucleotide inserted in its place. The preferred number of nucleotides affected by substitutions in a mutant gene relative to the wild-type gene is a maximum of ten nucleotides, more preferably a maximum of 9, 8, 7, 6, 5, 4, 3, or 2, or only one nucleotide. Such a substitution may be "silent" in that the substitution does not change the amino acid defined by the codon.

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Alternatively, conservative substituents are designed to alter one amino acid for another similar acting amino acid. Typical conservative substitutions are those made in accordance with Table 13 "Exemplary substitutions".

[00198] The term "mutation" as used herein does not include silent nucleotide substitutions which do not affect the activity of the gene, and therefore includes only alterations in the gene sequence which affect the gene activity. The term "polymorphism" refers to any change in the nucleotide sequence including such silent nucleotide substitutions.

[00199] In some embodiments, the barley grain comprises non-conservative substitution deletion of at least part of a *SSII* gene or a frameshift or splice site variation in such gene.

[00200] In some embodiments, one or more mutants are within a conserved region of *SSIIIa* encoding conserved motifs comprising the amino acid sequences set out in SEQ ID NOs: 34 to 41. In some embodiments, one or more mutations are within the region of *SSIIIa* encoding the catalytic domain of *SSIIIa*. Mutagenesis can be achieved by chemical or radiation means, for example EMS or sodium azide (Zwar and Chandler, *Planta* 197: 39-48, 1995).

[00201] Chemical mutagenesis tends to favour nucleotide substitutions rather than deletions. Heavy ion beam (HIB) irradiation is known as an effective technique for mutation breeding to produce new plant cultivars, see for example Hayashi *et al.*, 2007 and Kazama *et al.*, *Plant Biotechnology* 25: 113-117, 2008. Ion beam irradiation has two physical factors, the dose (gy) and LET (linear energy transfer, keV/um) for biological effects that determine the amount of DNA damage and the size of DNA deletion, and these can be adjusted according to the desired extent of mutagenesis. HIB generates a collection of mutants, many of them comprising deletions, that may be screened for mutations in *SSIIIa*. Useful mutants which are identified may be backcrossed with non-mutated plants as recurrent parents in order to remove and therefore reduce the effect of unlinked mutations in the mutagenised genome.

[00202] Biological agents useful in producing site-specific mutants include enzymes that include double stranded breaks in DNA that stimulate endogenous

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repair mechanisms. These include endonucleases, zinc finger nucleases, transposases and site-specific recombinases. Zinc finger nucleases (ZFNs), for example, facilitate site-specific cleavage within a genome allowing endogenous or other end-joining repair mechanisms to introduce deletions or insertions to repair the gap. Zinc finger nuclease technology is reviewed in Le Provost *et al.*, *Trends in Biotechnology* 28(3): 134-141, 2009, See also Durai *et al.*, *Nucleic Acids Research* 33(18): 5978-5990, 2005 and Liu *et al.*, *Biotechnology and Bioengineering*, 106: 97-105, 2010.

[00203] Isolation of mutants may be achieved by screening mutagenized plants or seed. For example, a mutagenized population of barley plants may be screened for low SSIIIa activity in grain starch, mutation of the SSIIIa or by a PCR or heteroduplex based assay, or loss of the SSIII protein by ELISA or loss or gain of enzyme activity or level by starch gel electrophoresis and straining. Alternatively, the mutation may be identified using techniques such as "tiling" in a population mutagenized with an agent such as EMS (Slade and Knauf, *Transgenic Res.* 14: 109-115, 2005) or by deep sequencing of mutagenized pools. Such mutations may then be introduced into desirable genetic backgrounds by crossing the mutant with a plant of the desired genetic background and performing a suitable number of backcrosses to cross out the originally undesired parent background.

[00204] The mutation may have been introduced into the plant directly by mutagenesis or indirectly by crossing of two parental plants, one of which comprised the introduced mutation. The modified plants may be transgenic or non-transgenic. Using mutagenesis, a non-transgenic plant having reduced SSIIIa level or activity or essentially no SSIIIa of may be produced. The invention also extends to the grain or other plant parts produced from the plants and any propagating material of the plants that can be used to produce the plants with the desired characteristics, such as cultured tissue or cells. The invention clearly extends to methods of producing or identifying such plants or the grain produced by such plants.

[00205] Plants of the invention can be produced using the process known as TILLING (Targeting Induced Local Lesions IN Genomes). In a first step, introduced mutations such as novel single base pair changes are induced in a population of plants by treating cells, seeds, pollen or other plant parts with a chemical mutagen or

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radiation, and then advancing plants to a generation where mutations will be stably inherited. DNA is extracted, and seeds are stored from all members of the population to create a resource that can be accessed repeatedly over time.

[00206] For a TILLING assay, PCR primers are designed to specifically amplify a single gene target of interest. Specificity is especially important if a target is a member of a gene family or part of a polyploid genome. Next, dye-labeled primers can be used to amplify PCR products from pooled DNA of multiple individuals. These PCR products are denatured and reannealed to allow the formation of mismatched base pairs. Mismatches, or heteroduplexes, represent both naturally occurring single nucleotide polymorphisms (SNPs) (i.e., several plants from the population are likely to carry the same polymorphism) and induced SNPs (i.e., only rare individual plants are likely to display the mutation). After heteroduplex formation, the use of an endonuclease, such as Cell, that recognizes and cleaves mismatched DNA is the key to discovering novel SNPs within a TILLING population.

[00207] Using this approach, many thousands of plants can be screened to identify any individual with a single base change as well as small insertions or deletions (1-30 bp) in any gene or specific region of the genome. Genomic fragments being assayed can range in size anywhere from 0.3 to 1.6 kb. At 8-fold pooling, 1.4 kb fragments (discounting the ends of fragments where SNP detection is problematic due to noise) and 96 lanes *per* assay, this combination allows up to a million base pairs of genomic DNA to be screened *per* single assay, making TILLING a high-throughput technique. TILLING is further described in Slade and Knauf, 2005 (*supra*), and Henikoff *et al.*, *Plant Physiol.* 135: 630-636, 2004, herein incorporated by reference.

[00208] In addition to allowing efficient detection of mutations, high-throughput TILLING technology is ideal for the detection of natural polymorphisms. Therefore, interrogating an unknown homologous DNA by heteroduplexing to a known sequence reveals the number and position of polymorphic sites. Both nucleotide changes and small insertions and deletions are identified, including at least

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some repeat number polymorphisms. This has been called Ecotilling (Comai *et al.*, *Plant J.* 37: 778-786, 2004).

[00209] Each SNP is recorded by its approximate position within a few nucleotides. Thus, each haplotype can be archived based on its mobility. Sequence data can be obtained with a relatively small incremental effort using aliquots of the same amplified DNA that is used for the mismatch-cleavage assay. The left or right sequencing primer for a single reaction is chosen by its proximity to the polymorphism. Sequencer software performs a multiple alignment and discovers the base change, which in each case confirmed the gel band.

[00210] Ecotilling can be performed more cheaply than full sequencing, the method currently used for most SNP discovery. Plates containing arrayed ecotypic DNA can be screened rather than pools of DNA from mutagenized plants. Because detection is on gels with nearly base pair resolution and background patterns are uniform across lanes, bands that are of identical size can be matched, thus discovering and genotyping SNPs in a single step. In this way, ultimate sequencing of the SNP is simple and efficient, made more so by the fact that the aliquots of the same PCR products used for screening can be subjected to DNA sequencing.

Genetic linkage and Analysis

[00211] As used herein, the term "genetically linked" refers to a marker locus and a second locus being sufficiently close on a chromosome that they will be inherited together in more than 50% of meioses, e.g., not randomly. This definition includes the situation where the marker locus and second locus form part of the same gene. Furthermore, this definition includes the situation where the marker locus comprises a polymorphism that is responsible for the trait of interest (in other words the marker locus is directly "linked" to the phenotype). Thus, the percent of recombination observed between the loci *per* generation (centimorgans (cM)), will be less than 50. In particular embodiments of the invention, genetically linked loci may be 45, 35, 25, 15, 10, 5, 4, 3, 2, or 1 or less cM apart on a chromosome. Preferably, the markers are less than 5 cM or 2 cM apart and most preferably about 0 cM apart.

[00212] As used herein, the "other genetic markers" may be any molecules which are linked to a desired trait barley grain. Such markers are well known to those

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skilled in the art and include molecular markers linked to genes determining traits such as disease resistance, the presence or absence of a null yield, plant morphology, grain quality, other dormancy traits such as grain colour, gibberellic acid content in the seed, plant height, flour colour and the like.

[00213] Marker assisted selection is a well recognized method of selecting for heterozygous plants required when backcrossing with a recurrent parent in a classical breeding program. The population of plants in each backcross generation will be heterozygous for the gene of interest normally present in a 1:1 ratio in a backcross population, and the molecular marker can be used to distinguish the two alleles of the gene. By extracting DNA from, for example, young shoots and testing with a specific marker for the introgressed desirable trait, early selection of plants for further backcrossing is made whilst energy and resources are concentrated on fewer plants.

[00214] Any molecular biological technique known in the art which is capable of detecting alleles of an SSIIa based upon the sequences disclosed herein or other gene can be used in the methods of the present invention. Such methods include, but are not limited to, the use of nucleic acid amplification, nucleic acid sequencing, nucleic acid hybridization with suitably labeled probes, single-strand conformational analysis (SSCA), denaturing gradient gel electrophoresis (DGGE), heteroduplex analysis (HET), chemical cleavage analysis (CCM), catalytic nucleic acid cleavage or a combination thereof (see, for example, Lemieux, *Current Genomics*, 1: 301-311, 2000; Langridge *et al.*, *Aust J Agric Res* 52: 1043-1077, 2001). The invention also includes the use of molecular marker techniques to detect polymorphisms linked to alleles of (for example) an SSII gene which confers altered fructan accumulation. Such methods include the detection or analysis of restriction fragment length polymorphisms (RFLP), RAPD, amplified fragment length polymorphisms (AFLP) and microsatellite (simple sequence repeat, SSR) polymorphisms. The closely linked markers can be obtained readily by methods well known in the art, such as Bulk Segregant Analysis, as reviewed by Langridge *et al.*, 2001 (*supra*).

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[00215] The "polymerase chain reaction" ("PCR") is a reaction in which replicate copies are made of a target polynucleotide using a "pair of primers" or "set of primers" consisting of "upstream" and a "downstream" primer, and a catalyst of polymerization, such as a DNA polymerase, and typically a thermally-stable polymerase enzyme. Methods for PCR are known in the art, and are taught, for example, in "PCR" (McPherson and Moller (Ed), BIOS Scientific Publishers Ltd, Oxford, 2000). PCR can be performed on cDNA obtained from reverse transcribing mRNA isolated from plant cells expressing an SSIII gene or on genomic DNA isolated from a plant.

[00216] A primer is an oligonucleotide sequence that is capable of hybridizing in a sequence specific fashion to the target sequence and being extended during the PCR. Amplicons or PCR products or PCR fragments or amplification products are extension products that comprise the primer and the newly synthesized copies of the target sequences. Multiplex PCR systems contain multiple sets of primers that result in simultaneous production of more than one amplicon. Primers may be perfectly matched to the target sequence or they may contain internal mismatched bases that can result in the introduction of restriction enzyme or catalytic nucleic acid recognition/cleavage sites in specific target sequences. Primers may also contain additional sequences and/or contain modified or labelled nucleotides to facilitate capture or detection of amplicons. Repeated cycles of heat denaturation of the DNA, annealing of primers to their complementary sequences and extension of the annealed primers with polymerase result in exponential amplification of the target sequence. The terms target or target sequence or template refer to nucleic acid sequences which are amplified.

[00217] Methods for direct sequencing of nucleotide sequences are well known to those skilled in the art and can be found for example in Ausubel *et al.* (*supra*) and Sambrook *et al.* (*supra*). Sequencing can be carried out by any suitable method, for example, dideoxy sequencing, chemical sequencing or variations thereof. Direct sequencing has the advantage of determining variation in any base pair of a particular sequence.

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[00218] Many of the above methods are suitable for analyzing DNA and/or RNA to determine whether the barley plant or a part thereof comprises an allele of SSIII which comprise the sequence of SEQ ID NO: 3 or which comprise a nucleic acid other than SEQ ID NO: 3. In some embodiments, a process comprises analysing *SSIIIa* RNA of a barley plant or grain at a position corresponding to nucleobase 4439 of SEQ ID NO: 2 or characterising *SSIIIa* gene of a barley plant or grain at a position corresponding to nucleobase 8610 of SEQ ID NO: 1 or characterising the SSIIIa protein of a barley plant or grain at a position corresponding to amino acid 1480 of SEQ ID NO: 4. In accordance with this aspect of the invention the detection of a nucleobase other than threonine at a position corresponding to nucleobase 4439 of SEQ ID NO: 2 or a position corresponding to nucleobase 8610 of SEQ ID NO: 1 or an amino acid other than leucine at a position corresponding to amino acid 1480 of SEQ ID NO: 4 indicates the presence of an *amo-1* allele/the presence of SEQ ID NO:3.

Plants

[00219] The present invention extends to barley plants that produce or are capable of producing the grain as hereinabove described and as herein described. For example, in some embodiments, the barley plants produce or are capable of producing barley grain comprising: (a) starch, (b) a first genetic variation which is (i) an induced mutation in an endogenous gene encoding a starch synthase III (SSIII), or (ii) a transgene which encodes an RNA molecule which reduces expression of an endogenous gene encoding an SSIII, and (c) a reduced level or activity of SSIII protein relative to the level or activity in barley grain lacking the first genetic variation.

[00220] In some embodiments, the plant or part therefrom comprises an allele of *SSIIIa* other than a allele comprising the nucleic acid sequence of SEQ ID NO: 3.

[00221] In some embodiments, the barley plants are a population of at least 1000 plants growing in a field.

[00222] In some embodiments, the plant is capable of producing grain which further comprises a second genetic variation in a gene encoding a protein involved in

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starch synthesis, starch catabolism, starch phosphorylation or non-starch carbohydrate synthesis. Illustrative proteins are described in the summary. In some embodiments, the second genetic variation is (i) a mutation in a gene encoding a starch synthase II (SSII), in some embodiments a starch synthase IIa (SSIIa), or (ii) a transgene which encodes an RNA molecule which inhibits expression of a gene encoding an SSII, illustratively an SSIIa.

[00223] In some embodiments, the plant is capable of producing grain which comprises (i) an allele of *SSIIa* other than an allele comprising the nucleic acid sequence of SEQ ID NO: 3 and (ii) an allele of *SSIIa* other than an allele comprising the nucleic acid sequence of SEQ ID NO: 33. In another version of this embodiment, the plant produces grain which lacks the allele of *SSIIa* comprising the nucleic acid sequence of SEQ ID NO: 3 and lacks the allele of *SSIIa* comprising the nucleic acid sequence of SEQ ID NO: 33.

[00224] In another embodiments, the description enables a plant capable of producing barley grain comprising: (a) starch, (b) a first genetic variation which is (i) a mutation in a gene encoding an SSIII, or (ii) a transgene which encodes an RNA molecule which inhibits expression of a gene encoding an SSIII, and (c) a reduced level or activity of SSIII protein relative to the level or activity in barley grain lacking the first genetic variation, (d) a second genetic variation which is (i) a mutation in a gene encoding an SSII, or (ii) a transgene which encodes a nucleic acid molecule which inhibits expression of a gene encoding an SSII, (e) a reduced level or activity of SSII protein relative to the level or activity in barley grain lacking the second genetic variation, and wherein the grain comprises an allele of *SSIIa* other than an allele comprising the nucleic acid sequence of SEQ ID NO: 3.

[00225] In exemplary embodiments, the SSIII is SSIIIa. In further illustrative embodiments, the SSII is SSIIa. In further illustrative embodiments the starch content of the grain is at least 43% (w/w), at least 45% (w/w), at least 47% (w/w), at least 50% (w/w), or 41-65% (w/w). In still further illustrative embodiments, the amylose content of the grain is at least 40%, at least 50% or at least 60% as a proportion of the total starch in the grain. In further embodiments, the β -glucan content of the grain is 5-9% (w/w), or greater than 9% (w/w). In still further

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embodiments, the fructan content of the grain as described herein is 2-11%, 3-11%, or 4-11% (w/w). Illustratively, the fructan comprises a degree of polymerization from about 3 to about 12. In yet further embodiments, the grain comprises an elevated protein content relative to wild-type barley, or relative to HAG. In some embodiments, the plant is capable of producing grain which is homozygous for the *sex6-292* allele of *SSIIa*. As described herein, in some embodiments the *SSIII* gene encodes an *SSIII* protein comprising an amino acid sequence having at least 98% sequence identity to SEQ ID NO: 4.

[00226] The term "plant" as used herein as a noun refers to whole plants, but as used as an adjective refers to any substance which is present in, obtained from, derived from, or related to a plant, such as for example, plant organs (e.g. leaves, stems, roots, flowers), single cells (e.g. pollen), seeds, plant cells and the like. Plantlets and germinated seeds from which roots and shoots have emerged are also included within the meaning of "plant". The term "plant parts" as used herein refers to one or more plant tissues or organs which are obtained from a plant and which comprises genomic DNA of the plant. Plant parts include vegetative structures (for example, leaves, stems), roots, floral organs/structures, seed (including embryo, endosperm, and seed coat), plant tissue (for example, vascular tissue, ground tissue, starch granules, starch, grain and the like), cells and progeny of the same. The term "plant cell" as used herein refers to a cell obtained from a plant or in a plant and includes protoplasts or other cells derived from plants, gamete-producing cells, and cells which regenerate into whole plants. Plant cells may be cells in culture. By "plant tissue" is meant differentiated tissue in a plant or obtained from a plant ("explant") or undifferentiated tissue derived from immature or mature embryos, seeds, roots, shoots, fruits, pollen, tumor tissue, such as crown galls, and various forms of aggregations of plant cells in culture, such as calli. Exemplary plant tissues in or from seeds are endosperm, scutellum, aleurone layer and embryo. The invention accordingly includes plants and plant parts and products comprising these, particularly grain.

[00227] As used herein, the term "grain" refers to mature seed of a plant, such as is typically harvested commercially in the field. Thus, the term includes

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harvested seed and seed on a plant that is ready for harvesting. Mature cereal grain such as wheat or barley commonly has a moisture content of less than about 18-20%.

[00228] A "transgenic plant" as used herein refers to a plant that contains a gene construct not found in a wild-type plant of the same species, variety or cultivar. That is, transgenic plants (transformed plants) contain genetic material (a transgene) that they did not contain prior to the transformation. The transgene may include genetic sequences obtained from or derived from a plant cell, or another plant cell, or a non-plant source, or a synthetic sequence. Typically, the transgene has been introduced into the plant by human manipulation such as, for example, by transformation but any method can be used as one of skill in the art recognizes. The genetic material is preferably stably integrated into the genome of the plant. The introduced genetic material may comprise sequences that naturally occur in the same species but in a rearranged order or in a different arrangement of elements, for example an antisense sequence. Plants containing such sequences are included herein in "transgenic plants". A "non-transgenic plant" is one which has not been genetically modified by the introduction of genetic material by recombinant DNA techniques. In a preferred embodiment, the transgenic plants are homozygous for each and every gene that has been introduced (transgene) so that their progeny do not segregate for the desired phenotype.

[00229] As used herein, the term "corresponding non-transgenic plant" refers to a plant which is isogenic relative to the transgenic plant but without the transgene of interest. Preferably, the corresponding non-transgenic plant is of the same cultivar or variety as the progenitor of the transgenic plant of interest, or a sibling plant line which lacks the construct, often termed a "segregant", or a plant of the same cultivar or variety transformed with an "empty vector" construct, and may be a non-transgenic plant. "Wild type", as used herein, refers to a cell, tissue or plant that has not been modified according to the invention. Wild-type cells, tissue or plants may be used as controls to compare levels of expression of an exogenous nucleic acid or the extent and nature of trait modification with cells, tissue or plants modified as described herein.

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[00230] Transgenic plants, as defined in the context of the present invention include progeny of the plants which have been genetically modified using recombinant techniques, wherein the progeny comprise the transgene of interest. Such progeny may be obtained by self-fertilization of the primary transgenic plant or by crossing such plants with another plant of the same species. This would generally be to modulate the production of at least one protein/enzyme defined herein in the desired plant or plant organ. Transgenic plant parts include all parts and cells of said plants comprising the transgene such as, for example, cultured tissues, callus and protoplasts.

[00231] Any of several methods may be employed to determine the presence of a transgene in a transformed plant. For example, polymerase chain reaction (PCR) may be used to amplify sequences that are unique to the transformed plant, with detection of the amplified products by gel electrophoresis or other methods. DNA may be extracted from the plants using conventional methods and the PCR reaction carried out using primers to amplify a specific DNA, the presence of which will distinguish the transformed and non-transformed plants. For example, primers may be designed that will amplify a region of DNA from the transformation vector reading into the construct and the reverse primer designed from the gene of interest. These primers will only amplify a fragment if the plant has been successfully transformed. An alternative method to confirm a positive transformant is by Southern blot hybridization, well known in the art. Plants which are transformed may also be identified i.e. distinguished from non-transformed or wild-type plants by their phenotype, for example conferred by the presence of a selectable marker gene, or conferred by the phenotype of the grain of the plant, or related phenotype such as altered starch related enzymatic activity.

[00232] As used herein, "germination" refers to the emergence of the root tip from the seed coat after imbibition. "Germination rate" refers to the percentage of seeds in a population which have germinated over a period of time, for example 7 or 10 days, after imbibition. A population of seeds can be assessed daily over several days to determine the germination percentage over time. With regard to seeds of the present invention, as used herein the term "germination rate which is substantially the

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same" means that the germination rate of the transgenic seeds is at least 90%, that of isogenic wild-type seeds.

[00233] As used herein, the term "barley" refers to any species of the Genus *Hordeum*, including progenitors thereof, as well as progeny thereof produced by crosses with other species. It is preferred that the plant is of a *Hordeum* species which is commercially cultivated such as, for example, a strain or cultivar or variety of *Hordeum vulgare* or suitable for commercial production of grain.

Food production

[00234] Accordingly, the invention provides barley plants and grain, and products obtained therefrom, that are useful for food or animal feed production. The description provides a process for producing a food ingredient or beverage ingredient, wherein the process comprises (i) obtaining or producing barley grain as described hereinabove or as described herein; and (ii) processing the grain to produce the ingredient.

[00235] Accordingly, in one embodiment, barley grain comprises: (a) starch, (b) a first genetic variation which is (i) an induced mutation in an endogenous gene encoding a starch synthase III (SSIII), or (ii) a transgene which encodes an RNA molecule which reduces expression of an endogenous gene encoding an SSIII, and (c) a reduced level or activity of SSIII protein relative to the level or activity in barley grain lacking the first genetic variation.

[00236] In some embodiments, the barley grain comprises an allele of *SSIIIa* other than a allele comprising the nucleic acid sequence of SEQ ID NO: 3.

[00237] In some embodiments, the grain further comprises a second genetic variation in a gene encoding a protein involved in starch synthesis, starch catabolism, starch phosphorylation or non-starch carbohydrate synthesis. In some embodiments, the second genetic variation is (i) a mutation in a gene encoding a starch synthase II (SSII), in some embodiments a starch synthase IIa (SSIIa), or (ii) a transgene which encodes an RNA molecule which inhibits expression of a gene encoding an SSII, preferably an SSIIa.

[00238] In some embodiments, the grain comprises (i) an allele of *SSIIIa* other than an allele comprising the nucleic acid sequence of SEQ ID NO: 3 and (ii) an

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allele of *SSIIa* other than an allele comprising the nucleic acid sequence of SEQ ID NO: 33. In another version of this embodiment, the grain lacks the allele of *SSIIa* comprising the nucleic acid sequence of SEQ ID NO: 3 and lacks the allele of *SSIIa* comprising the nucleic acid sequence of SEQ ID NO: 33.

[00239] In another embodiments, the barley grain comprises: (a) starch, (b) a first genetic variation which is (i) a mutation in a gene encoding an *SSIII*, or (ii) a transgene which encodes an RNA molecule which inhibits expression of a gene encoding an *SSIII*, and (c) a reduced level or activity of *SSIII* protein relative to the level or activity in barley grain lacking the first genetic variation, (d) a second genetic variation which is (i) a mutation in a gene encoding an *SSII*, or (ii) a transgene which encodes a nucleic acid molecule which inhibits expression of a gene encoding an *SSII*, (e) a reduced level or activity of *SSII* protein relative to the level or activity in barley grain lacking the second genetic variation, and wherein the grain comprises an allele of *SSIIa* other than an allele comprising the nucleic acid sequence of SEQ ID NO: 3.

[00240] In exemplary embodiments, the *SSIII* is *SSIIIa*. In further illustrative embodiments, the *SSII* is *SSIIa*. In further illustrative embodiments the starch content of the grain is at least 43% (w/w), at least 45% (w/w), at least 47% (w/w), at least 50% (w/w), or 41-65% (w/w). In still further illustrative embodiments, the amylose content of the grain is at least 40%, at least 50% or at least 60% as a proportion of the total starch in the grain. In further embodiments, the β -glucan content of the grain is 5-9% (w/w), or greater than 9% (w/w). In still further embodiments, the fructan content of the grain as described herein is 2-11%, 3-11%, or 4-11% (w/w). Illustratively, the fructan comprises a degree of polymerization from about 3 to about 12. In yet further embodiments, the grain comprises an elevated protein content relative to wild-type barley, or relative to HAG. In some embodiments, the grain is homozygous for the *sex6-292* allele of *SSIIa*. As described herein, in some embodiments the *SSIII* gene encodes an *SSIII* protein comprising an amino acid sequence having at least 98% sequence identity to SEQ ID NO: 4.

[00241] The grain of the present invention is, in some embodiments, wholegrain or cracked, ground, polished, milled, kibbled, rolled or pearled grain. In particular embodiments,

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the grain is processed and is unable to germinate. In some embodiments, the ingredient is isolated wholemeal, flour, starch, bran, β -glucan, fructan, non-starch polysaccharides, a baking ingredient, a baking mix, a thickening agent, or cracked, ground, polished, milled, kibbled, rolled or pearled grain, or any combination thereof.

[00242] In further aspects of this embodiment, the description provides for a process for producing a food or animal feed or beverage product, wherein the process comprises: (i) obtaining or producing an ingredient produced according to a process as described herein above or herein ; and (ii) mixing the ingredient with another food ingredient or beverage ingredient to produce the product.

[00243] In some embodiments of the process, the ingredient is used as a bulking agent, a dietary fibre, a texturizing agent, a preservative, a sweetener, a thickening agent, a probiotic agent or any combination of these, in the food or beverage product. In other embodiments, the process further comprises assessing the level or type of starch or starch synthase activity, starch content, amylose, amylopectin, β -glucan, fructan, non-starch polysaccharides, dietary fibre, resistant starch in the barley grain or the ingredient or the product produced therefrom. In some embodiments of the process the food product is a breakfast cereal, biscuit, muffin, muesli bar, noodle, bread, cake, chip, pancake, tortilla, buns, pastry, cracker, pizza, croissants, bagels, pretzels, pasta, soup, sauce, confectionary, and other farinaceous goods.

[00244] In some embodiments, the grain has increased levels of starch and non-starch components compared to corresponding wild-type grains or HAG grains. The plant from which the grain is obtained has a reduced level of SSIIIa activity typically in the endosperm during development. The plant of the present invention is useful for food production and in particular for commercial food production. Such food production might include the making of flour, dough or other products that might be an ingredient in commercial food production. In an embodiment which is desirable for use in food production, the seed or grain of the plant has a starch or non-starch content that is increased relative to the wild-type plant. The grain may have a modified level or activity of degradative enzymes, particularly of one or more amylases such as α -amylase or β -amylase, which is reduced by the presence of a transgene or an introduced mutation which reduces expression of a gene encoding such a degradative enzyme in the grain. Flour or dough from such grain has desirable

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properties for baking or other food production. Amylose content may be increased as described in WO03/94600 by suppressing SBEIIa gene expression or as described in WO04/06751 by suppressing GWD gene expression.

[00245] The desired genetic background of the plant will include considerations of agronomic yield and other characteristics. Such characteristics might include whether it is desired to have a winter or spring types, agronomic performance (yield), disease resistance and abiotic stress resistance. Other varieties will be suited for other growing regions. It is preferred that the plant variety of the invention provide a yield not less than 80% of the corresponding wild-type variety in at least some growing regions, more preferably not less than 85% and even more preferably not less than 90%. The yield can readily be measured in controlled field trials.

[00246] In further embodiments, the starch content of the grain is at least about 42%, at least about 43%, at least about 45%, at least about 47%, at least about 50%, or at least about 55% (w/w), up to 65%, and more preferably not decreased relative to the wild-type. Wild-type barley grain grown commercially usually has a starch content in the range 55-65%, depending somewhat on the cultivar grown. Alternatively, the seed or grain of the invention has a starch content of at least 90% relative to that of grain from a wild-type plant, and preferably at least 95%. Other desirable characteristics include the capacity to mill the grain, in particular the grain hardness. Another aspect that might make a plant of higher value is the degree of fructan or starch extraction from the grain, the higher extraction rates being more useful, or the protein content, the ratio of amylose to amylopectin, or the content of other non-starch polysaccharides such as β -glucan which also contribute to the dietary fibre content of the grain products. Grain shape is also another feature that can impact on the commercial usefulness of a plant, thus grain shape can have an impact on the ease or otherwise with which the grain can be milled.

[00247] Starch is readily isolated from grain of the invention using standard methods, for example the method of Schulman and Kammiovirta, *Starch*, 43: 387-389, 1991. On an industrial scale, wet or dry milling can be used. Starch granule size

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is important in the starch processing industry where there is separation of the larger A granules from the smaller B granules.

Food products

[00248] The invention also encompasses foods, beverages or pharmaceutical preparations produced with products, preferably those comprising increased resistant starch, dietary fibre, amylose, β -glucan, fructan, or other components obtained from the plants or grain of the invention. Such food production might include the making of processed grain, wholemeal, flour, dough or other products that might be an ingredient in commercial food production. The grain of the invention or products derived therefrom containing resistant starch, dietary fibre, amylose, β -glucan or fructan may be used in a variety of food applications for human consumption. As used herein, "humans" refers to *Homo sapiens*. The grain can be used readily in food processing procedures and therefore the invention includes milled, ground, kibbled, pearled or rolled grain or products obtained from the processed or whole grain of the plants of the invention, including flour. These products may be then used in various food products, for example farinaceous products such as breakfast cereals, breads, cakes, biscuits and the like or food additives such as thickeners or binding agents or to make drinks, noodles, pasta or quick soups. The grain or products derived from the grain of the invention are particularly desired in breakfast cereals or as extruded products. The starch or other components may be incorporated into fat or oil products such as margarine or shortening, salad dressing, egg products such as mayonnaise, dairy products such as icecream, yogurt or cheese, cereal products such as wheat flour, fruit juices, other foods or food materials, or the starch or other components may be processed into beverages or foods such as bread, cake, biscuits, breakfast cereals, pasta, noodles or sauces. Fructan is also useful as a low calorie sweetening product.

[00249] In bread, the ingredients may be in the form of flour or wholemeal may substitute for 10% (w/w) or more of unaltered flour or wholemeal, preferably substituting at least 30% and even more preferably at least 50% of the unaltered flour or wholemeal. The formulation might therefore be, for example, flour 90 parts, fat 2 parts, salt 2 parts, improver 1 part, yeast 2.5 parts. The production of the bread may

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be by a rapid dough technique or other techniques as is known by those skilled in the art.

[00250] Alternatively, the product of the invention may be incorporated into a farinaceous based pasta product. The amount of ingredient of the invention employed in the pasta composition may be in the range of 5-20% (w/w) based on the total weight of farinaceous material more particularly in the range of 10 to 20%. Suitable other farinaceous materials will readily be chosen by a person skilled in the art. Other material may also be added to the composition for example dry or liquid eggs (yolks, whites, or both) or high protein substances such as milk protein or fish protein. Vitamins, minerals, calcium salts, amino acids, buffering agents such as disodium hydrogen phosphate, seasoning, gum, gluten or glyceryl monostearate may also be added.

[00251] Other parts of the plants of the invention that are edible may be used as foods for human consumption or as feed for animal use. For example, leaves, stems, or extracts or parts of these comprising cells of the invention from any of these may be used for human or animal consumption. Increased resistant starch, dietary fibre, amylose, β -glucan or fructan content of the plants of the invention and parts thereof may provide advantages for use of these materials as animal feed such as, for example, as feed for pigs, cattle, horses, poultry such as chickens and other animals.

Methods

[00252] The products or compounds of the present invention can be formulated in pharmaceutical compositions which are prepared according to conventional pharmaceutical compounding techniques. See, for example, Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing, Company, Easton, PA, U.S.A. 1990). The composition may contain the active agent or pharmaceutically acceptable derivative active agent. These compositions may comprise, in addition to one of the active substances, a pharmaceutically acceptable excipient, carrier, buffer, stabilizer or other materials well known in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The carrier may take a wide variety of forms depending on the form of preparation desired for administration.

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[00253] For oral administration, the compounds can be formulated into solid or liquid preparations such as capsules, pills, tablets, lozenges, powders, suspensions or emulsions. In preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed, such as, for example, water, glycols, oils, alcohols; flavoring agents, preservatives, coloring agents, suspending agents, and the like in the case of oral liquid preparations (such as, for example, suspensions, elixirs and solutions); or carriers such as starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations (such as, for example, powders, capsules and tablets). Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. If desired, tablets may be sugar-coated or enteric-coated by standard techniques.

[00254] The active agent is preferably administered in a therapeutically effective amount. The actual amount administered and the rate and time-course of administration will depend on the nature and severity of the condition being treated. Prescription of treatment, e.g. decisions on dosage, timing, etc. is within the responsibility of general practitioners or specialists and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of techniques and protocols can be found in Remington's Pharmaceutical Sciences, (*supra*).

[00255] The food or beverage or pharmaceutical preparation may be packaged ready for sale or in bulk form. The invention also provides methods of preparing the food, beverage or pharmaceutical preparation of the invention, and recipes or instructions for preparing such foods or beverages. The methods may comprise the steps of harvesting the plant or plant part, separating grain from other plant parts, crushing, extracting, milling, cooking, canning, packaging or other processing steps known in the art. The methods or recipes or instructions may include the steps of processing the plant product of the invention and/or admixing it with other food ingredients, such as heating or baking the mixture or the product to, for

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example, at least 100°C. The method may include the step of packaging the product so that it is ready for sale.

Industrial use

[00256] The plant products, preferably grain, may be used in production of industrial products such as, for example, ethanol.

[00257] The present invention is further described by the following non-limiting Examples.

EXAMPLE 1: MATERIALS AND METHODS

Plant material

[00258] Barley (*Hordeum vulgare*) lines used in Examples 2-5 were from a back crossed population starting with a cross between parental varieties Himalaya292 (M292, Morell *et al.*, (2003)) which contained the *sex6* (*SSIIa*) mutation designated herein as the *SSIIa-292* allele, and the variety High Amylose Glacier (HAG, also named AC38 or Glacier Pentlandfield) (Banks *et al* 1971) containing the *amol* mutation. These parental varieties are available in the art. The Himalaya292 and HAG varieties, for example, are available from CSIRO or from the Australian Winter Cereals Collection, Tamworth, NSW. Crossing of barley plants was carried out in the greenhouse by standard methods. The back cross populations were generated through 3 back crosses from Himalaya292 (male) to HAG (female), and then 3 generations of single seed descent (SSD). The seeds from the third back cross were named as BC3F1 and from the 3rd SSD were named as BC3F4. To increase the quantity of seeds for each line, 2 or 3 further generations were grown. These were designated as the BC3F6 or BC3F7 generations and used for this study.

[00259] Seventy-one BC3F6 barley lines were grown at CSIRO Plant Industry, Canberra in pots under otherwise natural conditions in 2005. To confirm the selected seed compositions and parameters, a subset of the BC3F7 barley lines which were selected by seed weight, amylose content, and the presence of *SSIIa* and *amol* mutations were grown either at CSIRO Plant Industry, Canberra in a glasshouse, with natural light and at the temperatures 18°C (night) and 24°C (day), or in the field at Yanco, New South Wales, Australia in 2007.

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[00260] Barley spikes were labelled as at anthesis 2 days after the awns first appeared through the top of the flag leaf containing the enclosed spike. Developing seeds were harvested at 15 days post anthesis (DPA) and after removal of the embryo the developing endosperm was extruded through the cut surface and stored at -80°C.

[00261] Other varieties as described herein were obtained commercially or from the Australian Winter Cereals Collection, Tamworth, NSW, Australia.

Genotyping of the BC3F6 population by PCR amplification

[00262] Young barley leaves from the BC3F6 generation of the back crossed population were collected and freeze-dried (FTS systems, Stone Ridge, New York). Genomic DNA was isolated with a fast DNA^R kit according to the supplier's instructions (Q-BIOgene, CA., USA).

[00263] For genotyping for the presence or absence of the *SSIIa* mutation (*SSIIa*-292 allele) in plants derived from crosses with mutant Himalaya292, primers *SSIIaF* (5'-CCTGGAACACTTCAGACTGTACG-3' (SEQ ID NO: 10)) starting at nucleotide 1616 and *SSIIaR* (5'-AGCATCACCAGCTGCACGTCCT-3' (SEQ ID NO: 11)) starting at nucleotide 2044 of the *SSIIa* cDNA (GenBank Accession no: AY133249) were used for the PCR amplification of a 451 bp product spanning the *SSIIa* mutation site at nucleotide 1829 of Himalaya292, as described by Morell *et al.*, (2003). The microsatellite PCR marker EBmac0501 was used in initial experiments for the detection of the *amol* mutation as it located at 68.0 cM on chromosome 1H and was closely linked to the *amol* locus also at 68.0 cM. Primers HHac0501F (5' CACGACGTTGTAAAACGACACTTAAGTGCCATGCAAAG 3' (SEQ ID NO: 12) and HHac0501R (5' AGGGACAAAAATGGCTAAG 3' (SEQ ID NO: 13)) (GrainGenes Database) were used for the amplification of a PCR fragment from the *amol* locus.

[00264] For each PCR reaction of 20 µl, 50 ng genomic DNA, 1.5 mM MgCl₂, 0.125 mM each dNTP, 10 pmol primers, 0.5 M glycine betaine, 1 µl DMSO and 1.5 U of Hotstar Taq polymerase (QIAGEN) were used. The PCR reactions were conducted using a HYBAID PCR Express machine (Integrated Sciences) with 1 cycle of 95°C for 5 minutes, 35 cycles of 94°C for 45 seconds, 58°C for 30 seconds, and 72°C for 1 minute, 1 cycle of 72°C for 10 minutes and 1 cycle of 25°C for 1 minute.

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The PCR products for the detection of the *SSIIa* mutation were digested with the restriction enzyme *NlaIV* at 37°C overnight. Both digested (for *SSIIa* mutation) and non-digested (for *amoI* mutation) PCR fragments were separated on 2% agarose gels and visualized with gel documentary (UVitcc) after GelRed (Biotium) staining.

[00265] For genotyping of barley lines from the back crossed barley population between Himalaya292 x HAG, primers *SSIIaF* and *SSIIaR* were used as described above for the detection of the *SSIIa* mutation from parental line Himalaya292, and the microsatellite marker *EBmac0501* was used for the detection of the *amoI* locus from parental line HAG. For the *SSIIa* mutation, three types of PCR fragment patterns were evident after digestion of the PCR product with *NlaIV* followed by gel electrophoresis, that differentiated the mutated and wildtype *SSIIa* alleles. A single DNA fragment of 347 bp indicated the presence of the mutated *SSIIa* gene (i.e. from Himalaya292), a single 236 bp DNA fragment indicated the presence of the wildtype *SSIIa* gene (i.e. from Himalaya, Glacier and HAG), and the presence of both the 347 bp and 236 bp fragments indicated the heterozygous genotype lines. For the *amoI* mutation from HAG, the *EBmac0501* microsatellite marker also gave three PCR fragmentation patterns for the BC3F6 population. A 167 bp fragment was detected from the *amoI* mutant, a 141 bp fragment was detected from Himalaya292 (wild-type for *amoI*) lines, and both 167 bp and 141 bp fragments were detected in the heterozygous lines. Using the 3130xl Genetic Analyser, the *Bmac0090* microsatellite marker gave three PCR fragmentation patterns for the BC3F6 population. A 234 bp fragment was detected from HAG (*amoI* mutation), a 236 bp fragment was detected from Himalaya292 (wild-type for *amoI*), and both 236 bp and 234 bp fragments were detected in the heterozygous lines.

[00266] For the *SSIIla* gene, the PCR products were digested with the restriction enzyme *EcoRI* and separated on 2% agarose gels as above. Two types of PCR fragment patterns were evident that differentiated the *SSIIla* genes in HAG (mutant *SSIIla*) and Himalaya292 (wild-type *SSIIla*). A 464 bp DNA fragment only indicated the occurrence of the *SSIIla* gene from Himalaya292, while both 303 bp and 161 bp DNA fragments indicated the presence of the *SSIIla* gene from HAG. Three fragments (464 bp, 303 bp and 161 bp) were detected in the heterozygous lines.

Grain characteristics

[00267] Grain was harvested from plants at maturity unless otherwise stated, and average seed weight was determined by weighing 100 seeds with 3 replicates. The seed weight for selected lines was also determined as average seed weight of 500 seeds for 3 replicates for BC3F7 field grown materials at Yanco, NSW. Seed moisture content of grain was measured by standard nuclear magnetic resonance (NMR) methods using an Oxford 4000 NMR Magnet (Oxford analytical instruments Limited). Grain texture was measured using the Single-Kernel Characterization system 4100 (Perten Instruments Inc. Springfield, IL 62707 USA) using the RACI Cereal Chemistry Official testing method 12-01. Seed plumpness was grouped as three categories: shrunken, semi-plumped and plumped as known in the art.

Microscopic examination of barley seed cross sections and scanning electronic microscopy

[00268] Transverse sections approximately 1 mm thick of the middle part of the barley seeds were produced by cutting sections with razor blades, and photographed. They were also coated with gold particles and examined with a JSM-6400 Scanning Electron Microscope (SEM) operating at 15 KV.

Milling of grain

[00269] Grain was ground to wholemeal that would pass through a 0.5 mm sieve, using a cyclonic mill (Cyclotec 1093, Tecator, Sweden). The wholemeal was then used for the analysis below.

 β -glucan and pentosan analysis

[00270] β -glucan content was assayed as described in Megazyme Method (AACC32.23), using 20mg of wholemeal for each of three replicate samples. Pentosan content was measured using the method from Bell, (1985) using 20mg of wholemeal for each of three replicate samples.

Total Starch Content and Starch Extraction

[00271] Barley grains were first ground to wholemeal using a Cyclone mill machine (Cyclote 1093, Tecator, Sweden). Total starch content was assayed using an AACC method 76.13 using 20 mg of wholemeal for each of three replicate samples (Konik-Rose *et al.*, 2007). Starch was isolated from wholemeal by a protease

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extraction method (Morrison *et al.*, 1984) followed by water washing and removal of the tailings. Starch was then washed with acetone and air dried at room temperature (Konik-Rose *et al.*, 2007).

Analysis of starch composition and characteristics

[00272] Amylose and amylopectin contents in the starch of the grain, or the ratio of amylose to amylopectin, was determined by Sepharose CL-2B gel filtration as follows (Gel filtration method). Approximately 10 mg of total starch was dissolved in 3.0 ml of 1M NaOH and fractionated on the basis of molecular weight by chromatography on a Sepharose CL-2B column (Regina *et al.*, 2006). The amount of starch in each of the fractions from the column was measured using the Starch Assay Kit (Sigma) according to the suppliers instructions. The total amount of amylopectin (first peak, higher molecular weight) and amylose (second peak, lower molecular weight) was calculated and the ratio or contents determined.

[00273] Alternatively, amylose content was measured using a small scale (2 mg starch) iodine adsorption method (Morrison and Laignelet, 1983) with some modifications as described by Konik-Rose *et al.*, 2007).

Chain length distribution

[00274] Amylopectin chain length distribution was measured, after debranching of the starch samples, by the method of O'Shea *et al.*, 1998 using a P/ACE 5510 capillary electrophoresis system (Beckman Coulter, NSW Australia) with argon laser-induced fluorescence (LIF) detection. Molar difference plots were generated by subtracting the normalized chain length distribution for modified starch from the normalized distribution for starch from an isogenic non modified control.

[00275] The gelatinization temperature profiles of starch samples were measured using a Pyris 1 differential scanning calorimeter (Perkin Elmer, Norwalk CT, USA). The viscosity of starch solutions was measured on a Rapid-Visco-Analyser (RVA, Newport Scientific Pty Ltd, Warriewood, Sydney), using conditions as reported by Batey *et al.*, 1997. The parameters that were measured included peak viscosity (the maximum hot paste viscosity), holding strength, final viscosity and pasting temperature. Pasting properties were measured using the Rapid Visco Analyser as follows. Starch (3.0 g) was added to distilled water (25.0 ml) in the DSC

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pan and the RVA run profile was: 2 mins at 50°C, heat for 6 mins to 95°C, hold at 95°C for 4 mins, cool for 4mins to 50°C, hold at 50°C for 4mins. The measured parameters were: Peak viscosity at 95°C, Holding strength at end of 95°C holding period, Breakdown = Peak Viscosity – Holding strength, Final viscosity at end of 50°C holding period, Setback = Final Viscosity – Holding strength. The software Thermocline for Windows version 2.2 (Newport Scientific Pty Ltd, NSW Australia) was used for collection and analysis of data.

[00276] The swelling volume of flour or starch was determined according to the method of Konik-Rose *et al.*, 2001. The uptake of water was measured by weighing the sample prior to and after mixing the flour or starch sample in water at defined temperatures (for example, 90°C) and following collection of the gelatinized material.

Starch granule morphology, birefringence and granule size distribution

[00277] Granule morphology was examined by SEM (JSM-6400) and light microscopy with polarized light. The shapes and birefringence of the starch granules were examined as described by Yamamori *et al.*, (2000). Granule size distribution (by volume) of the starch slurries was determined using a laser diffraction particle size analyser (Mastersizer 2000, Malvern Instruments, Malvern, England). The percentage of small B-type starch granules was determined using a cut-off diameter of 7 µm.

Lipid analysis

[00278] Total lipid content was assayed by NMR using an Oxford 4000 NMR Magnet, Oxford Analytical Instruments Limited, UK. For each sample, 1 g of seeds was dried at 38.8°C for 64 hours. The dried seeds were then measured using NMR and compared against a pure barley oil extracted from cv. Himalaya or M292 grain.

Protein content, lipid content, moisture content and ash content

[00279] Protein content was determined by measurement of nitrogen content using Mass Spectrometer Method using a Europa 20-20 isotope ratio mass spectrometer with an automated nitrogen and carbon analyzer preparation system. Three to 8 mg of barley wholemeal was used. A nitrogen to protein conversion factor

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of 6.25 was used for the calculation of the protein content in barley seeds (Mosse 1990). Lipid content, moisture content and ash content were measured using the AOAC 983.23 method, AACC Method 44-19 and AACC Method 08-01 (AOA Chemists, 1990).

Total dietary fibre assay

[00280] The gravimetric method of Prosky *et al.* (1985; AOAC 985.29) was used to determine total dietary fibre (TDF) of the wholemeal. Duplicate samples were assayed.

Non starch polysaccharide assay

[00281] Total neutral non-starch polysaccharides (NSP) were measured by a modification of the gas chromatographic procedure of Theander *et al.* (1995). The modification involved a 2-hour hydrolysis with 1 M sulphuric acid followed by centrifugation to remove insoluble NSP and a further hydrolysis of the supernatant using 2 M trifluoroacetic acid for soluble NSP.

Resistant starch assay

[00282] An *in vitro* procedure was used to determine resistant starch (RS) content. The method has two sections: firstly, starch in each sample was hydrolysed under simulated physiological conditions; secondly, by-products were removed through washing and the residual starch determined after homogenization and drying of the sample. Starch quantitated at the end of the digestion treatment represented the resistant starch content of the sample. Typically, triplicate samples of whole meal along with appropriate standards were mixed with artificial saliva and the resultant bolus incubated with pancreatic and gastric enzymes at physiological pH and temperature. The amount of residual starch in the digesta was determined using conventional enzymatic techniques and spectrophotometry and the resistant starch content of the sample expressed as a percentage of sample weight or total starch content.

[00283] On day 1, an amount of sample representing up to 500 mg of carbohydrate was weighed into a 125 ml Erlenmeyer flask. A carbonate buffer was prepared by dissolving 121 mg of NaHCO_3 and 157 mg of KCl in approximately 90 ml purified water, adding 159 μL of 1 M $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ solution and 41 μL of 0.49 M

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MgCl₂·6H₂O, adjusting the pH to 7 to 7.1 with 0.32 M HCl, and adjusting the volume to 100 ml. This buffer was stored at 4°C for up to five days. An artificial saliva solution containing 250 units of α-amylase (Sigma A-3176 Type VI-B from porcine pancreas) per ml of the carbonate buffer was prepared. An amount of the artificial saliva solution, approximately equal to the weight of food, was added to the flask. About 15-20 sec after adding the saliva, 5 ml of pepsin solution in HCl (1 mg/ml pepsin (Sigma) in 0.02 M HCl, pH 2.0, made up on day of use) was added to each flask. The mixing of the amylase and then pepsin mimicked a human chewing the sample before swallowing it. The mixture was incubated at 37°C for 30 min with shaking at 85 rpm. The mixture was then neutralised with 5 ml of 0.02M NaOH. 25 ml of acetate buffer (0.2 M, pH 6) and 5 ml of pancreatin enzyme mixture containing 2 mg/ml pancreatin (Sigma, porcine pancreas at 4x USP activity) and 28U of amyloglucosidase (AMG, Sigma) from *Aspergillus niger* in acetate buffer, pH6, were added per flask. Each flask was capped with aluminium foil and incubated at 37°C for 16 hours in a reciprocating water bath set to 85 rpm.

[00284] On day 2, the contents of each flask was transferred quantitatively to a 50 ml polypropylene tube and centrifuged at 2000 x g for 10 min at room temperature. The supernatants were discarded and each pellet washed three times with 20 ml of water, gently vortexing the tube with each wash to break up the pellet, followed by centrifugation. 50 uL of the last water wash was tested with Glucose Trinder reagent for the absence of free glucose. Each pellet was then resuspended in approximately 6 ml of purified water and homogenised three times for 10 seconds using an Ultra Turrax TP18/10 with an S25N-8G dispersing tool. The contents were quantitatively transferred to a 25 ml volumetric flask and made to volume. The contents were mixed thoroughly and returned to the polypropylene tube. A 5 ml sample of each suspension was transferred to a 25 ml culture tube and immediately shell frozen in liquid nitrogen and freeze dried.

[00285] On day 3, total starch in each sample was measured using reagents supplied in the Megazyme Total Starch Procedure kit. Starch standards (Regular Maize Starch, Sigma S-5296) and an assay reagent blank were prepared. Samples, controls and reagent blanks were wet with 0.4 ml of 80% ethanol to aid dispersion,

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followed by vortexing. Immediately, 2 ml of DMSO was added and solutions mixed by vortexing. The tubes were placed in a boiling water bath for 5 min, and 3 ml of thermostable α -amylase (100 U/ml) in MOPS buffer (pH 7, containing 5mM CaCl_2 and 0.02% sodium azide added immediately. Solutions were incubated in the boiling water bath for a further 12 min, with vortex mixing at 3 min intervals. Tubes were then placed in a 50°C water bath and 4 ml of sodium acetate buffer (200 mM, pH 4.5, containing 0.02% sodium azide) and 0.1 ml of amyloglucosidase at 300 U/ml added. The mixtures were incubated at 50°C for 30 min with gentle mixing at 10 min intervals. The volumes were made up to 25 ml in a volumetric flask and mixed well. Aliquots were centrifuged at 2000 x g for 10 min. The amount of glucose in 50 μL of supernatant was determined with 1.0 ml of Glucose Trinder reagent and measuring the absorbance at 505 nm after incubation of the tubes at room temperature in the dark for a minimum of 18 min and a maximum of 45 min.

Quantification of water-soluble carbohydrate contents

[00286] Total water soluble carbohydrates (WSC) were extracted from wholemeal following the method of Lunn and Hatch, (1995) with the following modifications. Wholemeal is defined herein as the product obtained by milling mature grain, without subsequent fractionation (e.g. sieving) to remove the bran. Therefore wholemeal contains all of the components in the grain.

[00287] Barley wholemeal (100 mg) was extracted three times with 10 ml each time of 80% ethanol (v/v) in a boiling water bath for 10 minutes. The supernatants from each extraction were pooled, freeze dried and re-suspended in 2 ml milliQ water. The quantities of sucrose, glucose, fructose, maltose and fructo-oligosaccharides (fructans) were analysed by high performance anion exchange chromatography (HPAEC) as described in Ruuska *et al.*, (2006).

[00288] To determine maltose levels, total sugars extracted from barley whole meal were assayed essentially as described by Bernfeld, (1955), using maltose standard solutions for comparison, as follows. Total sugars were diluted 10 to 100-fold. Maltose standards (10 tubes) were prepared as 0.3 to 5 micromoles per ml. One ml of each dilution of maltose (in total sugars or maltose dilutions) was mixed with 1 ml of dinitrosalicylic acid colour reagent. The sugar solution was then incubated at

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100°C for 5 minutes and cooled to room temperature. Ten ml reagent grade water was added to each tube and mixed well. The samples were measured at A_{540} with a spectrophotometer. Maltose was also determined by HPAEC as described above.

Analysis of starch granule bound proteins and SSIIIa enzymatic activity

[00289] Starch granule bound proteins were isolated and separated on SDS-PAGE gel as described (Rahman *et al.*, 1995). The proteins were then stained by silver staining (Li *et al.*, 1999a). The protein gels were scanned (Epson Perfection 2450 PHOTO; Epson America Inc., CA, USA).

[00290] For zymogram analysis, developing endosperms at 15 dpa were isolated and ground in a mortar and a pestle with 3 volumes of extraction buffer (20 mM Tris-HCl, pH 7.5, 5 mM DTT, and 1 mM Pefabloc) at 4°C. The homogenate was then centrifuged at 10,000g for 20 min at 4°C and samples of the supernatants (containing 20 µg proteins) were used for analysis of SSIIIa activity and SSI activity by zymogram analysis (Abel *et al.*, 1996).

Enzyme assays

[00291] Total starch synthase activity in samples such as developing endosperm of cereals are measured by extraction of proteins and assay by the methods described in Libessart *et al.* 1995 or Cao *et al.*, 1999. The assays use ^{14}C labeled ADPG substrate and measure incorporation of the monomer into starch polymers. Individual isoforms of starch synthase in extracts were separated by gel electrophoresis and assayed in-gel (zymogram) as follows. Extracts from samples such as developing seeds were prepared using 50 mM potassium phosphate buffer, pH7.5, 5 mM EDTA, 20% glycerol, 10 µM Pefabloc and 0.05 mM dithiothreitol (DTT). After grinding the seeds to a pulp in the buffer or homogenizing the sample, the mixtures were centrifuged at 14,000 g for 15 min at 4°C and the supernatants drawn off. The protein concentration in the supernatants was measured using Coomassie Protein Reagent. Extracts were stored at -80°C if the protein extracts were to be run on native gels later on. For denaturing gel electrophoresis, 100 µl of extract was mixed with SDS and β-mercaptoethanol and the mixtures are incubated in boiling water for 4 min to denature the proteins. Electrophoresis was carried out in standard denaturing polyacrylamide gels using 8% polyacrylamide separating gels overlaid

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with 4.5% polyacrylamide stacking gels. After electrophoresis, the proteins were renatured by soaking the gels in 40 mM Tris-HCl buffers for a minimum of 2 hr, changing the buffer every 30 min and using at least 100 ml of buffer for each buffer change. For non-denaturing gels, the denaturing step with SDS and β -mercaptoethanol was omitted and SDS omitted from the gels. A starch synthase assay buffer including Tris-glycine (25 mM Tris, 0.19 M glycine), 0.133 M ammonium sulphate, 10 mM $MgCl_2$, 670 $\mu g/ml$ BSA and 1 mM ADPG substrate was used to detect starch synthase bands, followed by staining with 2% KI, 0.2% I_2 iodine solution to detect the starch product.

[00292] Alternatively, starch synthase or other starch biosynthetic enzymes are detected in samples using specific antibodies (ELISA).

Statistical analyses of the relationship between genotypes and seed components or starch properties

[00293] Statistical analyses were performed using Genstat version 9. Analysis of variance was performed for grain weight, total starch content, amylose content, amylopectin content, protein content, lipid content, β -glucan content, pentosan content, WSC content, starch granule size and amylopectin chain length distribution to obtain the least significant difference (LSD, $P < 0.05$), looking at variation between the genotypes.

EXAMPLE 2: GENOTYPING PLANTS

[00294] A population of barley lines segregating for the presence or absence of mutations at the *SSIIa* and *amo1* loci was generated by performing three backcrosses from a *SSIIa*-292 donor line (Himalaya292) into an *amo1* recurrent parent (High Amylose Glacier, HAG) as described in Example 1 (Plant Material). Three generations of single seed descent were performed from the BC3F2 lines in order to generate sufficient fixed genotypes to investigate the relative impact of the *sex6* and *amo1* loci, alone and in combination, on starch synthesis, grain composition and morphology. The ability to accurately assign the progeny lines to genotypes was central to this study. Because the genetic change underpinning the *sex6* phenotype had previously been demonstrated to be a lesion in the *SSIIa* gene (Morell *et al.*, 2003),

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the status of all lines at the *sex6* locus could be unambiguously defined. However, the *amo1* mutation was defined by phenotype alone given that the causal gene had not previously been defined. In order to assign lines to the four possible genotype classes (wild type, *SSIIa* mutant, *amo1* mutant, *SSIIa-amo1* double mutant), markers linked to *amo1* were required.

[00295] Given that the causal gene at the *amo1* locus had not been identified, the closest linked markers available were sought. SSR markers identified from barley mapping populations within approximately 10 cM of the *amo1* locus were examined for polymorphism in this population and the two parental varieties. Of the 12 markers tested (see Example 4), EBmac0501 located at 58.0 cM, and Bmac0090 located at 58.0 cM gave clear polymorphisms between the Himalaya292 and *amo1* parents. Therefore, all of the barley BC3F6 lines were initially genotyped using the marker for the causal mutation in the *SSIIa* gene as described in Example 1 and the microsatellite marker EBmac0501 which was tightly linked and therefore useful as a surrogate marker of *amo1*-38 or wild-type status.

[00296] Among the 71 BC3F6 lines genotyped, 13 lines were homozygous for both *SSIIa*-292 and *amo1*-AC38 alleles (*SSIIa*-292/*amo1*-AC38) and were therefore considered as *SSIIa-amo1* double mutants, 13 lines were homozygous for the *SSIIa*-292 and wild type *amo1*-alleles (*SSIIa*-292/*amo1*-wt) and therefore designated as *SSIIa* single mutants, 9 lines were homozygous for wild type *SSIIa* and mutant *amo1*-AC38 alleles (*SSIIa*-wt/*amo1*-AC38) and were therefore designated as *amo1* single mutants, while 13 lines were wild type for both *SSIIa* and *amo1* (*SSIIa*-wt/*amo1*-wt) and designated as wild type. Other lines were heterozygous for either the *SSIIa* mutation or for the EBmac0501 marker. In each of these lines, there was no recombination seen between either of the markers EBmac0501 or Bmac0090 and the *amo1* alleles (mutant and wild-type). A further 5 lines were identified that were homozygous at the *sex6* locus and homozygous for the 3 *amo1* markers but had recombined between these three markers. The remaining 18 lines were heterozygous at one of the 4 markers used and were excluded from the phenotypic analysis. These genotypes are summarized in Table 1.

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[00297] The parental varieties were also different in the hulled or hullless phenotypes- HAG is a hulled variety of barley while Himalaya292 was hullless. The *SSIIa-amol* double mutants were segregating for this characteristic, and therefore they could be classed in two subgroups- hulled or hullless. Therefore, the four genotypes of barley lines distinguished as described above were categorized into five groups, namely: wildtype lines, *SSIIa* single mutants, *amol* single mutants, hullless double mutants and hulled double mutants. Four lines were used from each of the five groups for the analysis of starch granule distribution, WSC, CE and seed morphology. One line from each genotype was used for endosperm structure and starch granule morphology. Eleven wildtype lines, 9 lines of *amol* mutants, 13 lines of *SSIIa* mutants, 4 lines of hullless double mutants and 6 lines of hulled double mutants were used for the analysis of grain composition, amylose content and seed weight as follows.

EXAMPLE 3: PHENOTYPING PLANTS

Grain Weight

[00298] Average seed weight (average of 100 seed weight) was measured for homozygous lines from the BC3F6 population. Average seed weight was 52.7 ± 5.0 mg for 11 wildtype lines, 52.8 ± 2.8 mg for 9 *amol* lines, 38.7 ± 2.5 mg for 13 *SSIIa* mutant lines, and 47.6 ± 4.5 mg for the 6 hulled double mutant lines and 44.7 ± 1.0 mg for the 4 hullless double mutant lines. There were no statistically significant differences between seed weights of the *amol* mutant lines and the wildtype lines ($P < 0.05$), showing that the *amol* mutation did not affect seed weight. However, there were statistically significant differences ($P < 0.05$) between each of the *SSIIa* single mutants and double mutant (hulled and hullless) and each of the three other genotypes. Similar observations on the seed weights of the 4 genotypes were also obtained for BC3F7 populations in separate glasshouse and field trials of the lines in 2007. One surprising and unexpected result was that the reduced seed weight caused by the presence of the *SSIIa* mutation, known to be due to the reduced amylopectin synthesis in the absence of *SSIIa* activity, was partly offset by the combination with the *amol* mutation.

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Grain morphology

[00299] Intact grains from four representative lines for each genotype were examined by stereoscopic microscope on both dorsal and crease sides. The *SSIIa* single mutant lines produced shrunken seeds while the wildtype and *amo1* single mutant lines produced plump well filled seeds. The double mutant seeds, both hulled and hulless, were observed to have an intermediate phenotype, plumper than *SSIIa* mutant seeds, yet not as well filled as *amo1* and wildtype seeds (semi-plump phenotype). These observations were consistent with the grain weight data.

[00300] To further illustrate the nature of plumpness of the seeds from these genotypes, transverse sections of the middle part of seeds across the largest diameter were examined. Transverse sections from wildtype and *amo1* mutant lines showed fully filled endosperms while *SSIIa* mutant lines showed incompletely filled (shrunken) seeds with a considerable reduction in endosperm packing density. The *SSIIa-amo1* double mutant lines showed an intermediate phenotype with an endosperm that was more filled than the *SSIIa* mutant endosperm and yet less filled than wildtype or *amo1* mutant lines.

Grain Composition and Starch Analysis

[00301] The analysis of starch and grain composition of BC3F6 population was conducted using all homozygous lines for both *SSIIa* and three markers for *amo1* locus lacking recombinations at the *amo1* locus. These lines included all wildtype lines, lines containing the *amo1* locus alone, lines containing the mutant *SSIIa* locus alone, and lines containing both the *SSIIa* and *amo1* mutations. For the analysis of starch granule distribution, water soluble carbohydrates (WSC), starch chain length distribution and grain morphology, a subset of 4 lines per genotype was used.

Total starch content.

[00302] Total starch content was measured as described in Example 1 on BC3F6 seed for the four genotypes. Starch content averaged $64.3 \pm 2.4\%$ for wildtype lines, $57.2 \pm 2.8\%$ for *amo1* mutant lines, $34.9 \pm 4.0\%$ for *SSIIa* mutant lines, $50.8 \pm 2.8\%$ for hulless double mutant lines and $47.6 \pm 2.3\%$ for hulled double mutant lines. Compared to the wildtype lines, *amo1* mutants, hulless double mutants, hulled double mutants and *SSIIa* mutant lines contained 7.1%, 13.5%, 16.7% and 29.4%

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respectively less total starch. These values were statistically different among the five groups ($P < 0.05$) except that the values for the hulless and hulled groups were not significantly different. Consistent relationships between the grain weights of the five groups were also obtained ($P < 0.05$) for BC3F7 grain from separate glasshouse and field trials in 2007. These data showed that the increased grain weights observed for the *SSIIa-amo1* double mutant seeds compared to the *SSIIa* single mutant seeds were due to increased starch content.

Amylose content

[00303] Amylose content was measured for all lines from the four genotypes by an iodine method and calculated as a percentage of the starch extracted from the grain (w/w). Amylose content ranged from $32.0 \pm 3.2\%$ for wildtype grain, $49.5 \pm 2.7\%$ for *amo1* mutant grain, $57.6 \pm 10.0\%$ for *SSIIa* mutant grain, $62.2 \pm 4.1\%$ for hulless double mutant grain and $59.8 \pm 2.3\%$ for hulled double mutant grain. Statistical analysis showed that the *SSIIa* mutant grain and double mutant grain contained significantly higher amylose contents in the seed than those from *amo1* mutants and wildtype lines. However, the amylose contents of *SSIIa* mutant and double mutants were not significantly different ($P < 0.05$), showing that the *SSIIa* mutation was increasing the proportion of amylose in the total starch of the grain but addition of the *amo1* mutation did not further significantly increase the proportion of amylose. These differences in amylose content between genotypes were consistent in BC3F7 lines grown in 2007.

[00304] Figure 1 shows the relationship between amylose content and starch content for the 53 homozygous BC3F6 lines. For lines containing the wildtype *SSIIa* gene (triangular symbols) a clear separation into two phenotypic groupings could be made. The grouping with elevated amylose consistently contained the three markers from the *amo1* locus (solid triangles) while the group with lower amylose content were wildtype for all three *amo1* markers (open triangles). The lines with the *SSIIa* genotype (non-functional *SSIIa* lines) also revealed the presence of two phenotypic groupings, separated in this case not by amylose content but by starch content. The lower starch content group contained the *SSIIa* mutant allele and the wild type *amo1* locus (open diamonds), while the higher starch content group contained both the *SSIIa*

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and *amo1* mutant loci (solid diamonds). Five lines exhibiting recombinations between the *amo1* markers (3 with wild type alleles at *SSIIa*, 2 with the mutant *SSIIa* allele) were included in the phenotypic analysis. Alignment of the phenotypic and genotypic data for the 5 recombinant lines provided evidence suggesting that, of the three markers, the *SSIIa* SNP (single nucleotide polymorphism) marker was more tightly linked to the *amo1* locus than either of the EBmac 0501 or Bmac0090 markers.

Starch Chain length distribution

[00305] To examine the effects of the genotypes on starch chain length distribution, starch was isolated from grain from four lines from each group of the BC3F6 cross population and analysed by Fluorophore Assisted Carbohydrate Electrophoresis (FACE). The percentage of chains were pooled into bins consisting of DP 6-8, DP 9-14, DP 15-24, DP 35-34, DP 35-44, and DP >45. There were no statistically significant differences ($P < 0.05$) among the bins for *SSIIa* mutants, hullless double mutants and hulled double mutants. However, there was a major difference ($P < 0.05$) between groups containing the wild type *SSIIa* allele compared to groups containing the mutant *SSIIa* allele. Those genotypes with the *SSIIa* mutant allele contained an increased proportion of chains of DP6-8, with greater than 10% of chains of this size, and also an increased proportion of chains of DP 9-14. They also exhibited a decreased proportion of chains with DP15-24. The wildtype lines had less than 5% DP6-8 chain lengths. The *amo1* mutants contained a statistically significantly decreased amount of DP 9-14 and an increased amount of DP 15-24 relative to the wild-type chain length distribution.

Starch granule size distribution

[00306] To investigate the effects of *SSIIa* and *amo1* mutant genotypes on starch granule size in endosperm starch, the starch granule size distribution was examined for four selected lines from each group of the BC3F6 backcross population. The results showed that B starch granule (defined as $< 7 \mu\text{m}$ diameter) contents in the wildtype, *amo1* mutants, *SSIIa* mutants, hullless double mutants and hulled double mutants were $20.2 \pm 6.4\%$, $30.7 \pm 3.6\%$, $17.5 \pm 1.8\%$, $19.7 \pm 3.6\%$ and $18.3 \pm 7.2\%$ of total starch in each line, respectively. The *amo1* mutant seeds contained significantly more B starch granules than seeds from the other four groups.

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[00307] The mean granule sizes of the distribution peaks larger than 10 μm in diameter (A starch granules) were also evaluated. The mean size of A starch granules was $18.9 \pm 0.5 \mu\text{m}$ for wildtype lines, $10.9 \pm 0.3 \mu\text{m}$ for *amo1* mutants, $16.4 \pm 2.6 \mu\text{m}$ for *SSIIa* mutants, $18.7 \pm 0 \mu\text{m}$ for hulless double mutants and $17.5 \pm 0.6 \mu\text{m}$ for hulled double mutants. Statistical analysis showed that *amo1* mutant seeds contained significantly smaller A starch granules than seeds from each of other four groups of barley ($P < 0.05$). There were no statistically significant differences ($P < 0.05$) among the A granules in seeds of wildtype, *SSIIa* mutants, hulless double mutants and hulled double mutants, as for the B granules.

Starch granule morphology

[00308] Purified barley starches from lines from the five groups were stained with iodine and examined under normal light microscopy. Consistent with their amylose content, starch granules from all genotypes gave a purple color after staining with iodine. Under polarized light microscopy, more than 90% of the starch granules from wildtype seeds and *amo1* seeds showed the "maltose cross" birefringence signature of crystalline starch granules. However, less than 10% of the starch granules from *SSIIa* mutant or double mutant seeds exhibited such birefringence.

[00309] When observed under SEM, grain from the wildtype lines exhibited normal spherical starch granules, while the *amo1* mutant genotype gave smaller spherical A starch granules that matched the results from the analysis described above. Starches from the *SSIIa* and double mutant seeds showed predominantly smaller deformed starch granules. Of the two mutants giving deformed starch granules, the *SSIIa* mutant line produced tubular, elongated A granules ($26 \mu\text{m} \times 12 \mu\text{m}$) while the hulless double mutant seeds exhibited more pronounced tubular elongations of the A granules ($28 \mu\text{m} \times 21 \mu\text{m}$).

[00310] The location of starch granules in the endosperm matrix was examined in transverse sections of the barley seeds. Wildtype lines contained multiple flat spherical starch A granules surrounding multiple small B starch granules while the *amo1* mutant line contained multiple loosely packed starch granules surrounding smaller B starch granules. Starch granules could not be clearly identified for the *SSIIa*

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mutant seeds in transverse sections. Hulless double mutant lines contained lenticular shaped starch A granules tightly packed in the endosperm cells.

Previous analysis of the *SSIIa* mutation in Himalaya292 showed that the mutation was associated with pleiotropic effects on grain composition (Clarke *et al.*, 2008) which were thought to result from redirection of carbohydrate in the grain as a result of alterations in total starch synthesis (Morell *et al.*, 2003). Therefore, an analysis of the major carbohydrate, protein and lipid fractions of the grain for each of the 4 genotype groups was conducted.

Beta-glucan content

[00311] Beta-glucan content in grain was measured for all lines from the BC3F6 population. β -glucan content was $6.0 \pm 0.5\%$ (ranging from 5.3% to 7.0%) for wildtype lines, $8.2 \pm 0.5\%$ (ranging from 7.6% to 8.4%) for *amo1* mutant lines, $7.6 \pm 1.4\%$ (ranging from 5.9% to 11.3%) for *SSIIa* mutants, $7.1 \pm 0.4\%$ for hulless double mutants and $6.5 \pm 0.8\%$ (ranging from 5.5% to 7.7%) for hulled double mutants. Statistical analysis showed that *amo1* mutant, *SSIIa* mutant and hulless double mutant seeds contained significantly more β -glucan than seeds from wildtype lines and hulled double mutant lines ($P < 0.05$), but there was no statistically significant difference among *amo1* mutants, *SSIIa* mutants and hulless double mutants, or between wildtype and hulled double mutants seeds in β -glucan content, respectively.

[00312] The statistical analysis for these selected F7 lines from five groups grown under glasshouse or field conditions showed that for each trial, seed from *amo1* mutant lines contained more β -glucan than seed from double mutant lines. There were no significant differences in β -glucan content between *SSIIa* mutant or double mutant seeds.

Pentosan content

[00313] Pentosan content in grain was measured for lines from the five groups. Pentosan content was $4.9 \pm 0.6\%$ for wildtype lines, $4.9 \pm 1.1\%$ for *amo1* mutant lines, $7.3 \pm 1.4\%$ for *SSIIa* mutants, $5.0 \pm 0.3\%$ for hulless double mutants and $6.5 \pm 1.0\%$ for hulled double mutants. Statistical analysis showed that both *SSIIa* mutant lines and hulled double mutants significantly contained more pentosan in grain than that from wildtype lines, *amo1* mutant and hulless double mutant lines ($P < 0.05$),

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but, there was no significant difference between *SSIIa* mutant lines and hulled double mutants or among wildtype lines, *amo1* mutant and hullless double mutants in pentosan content, respectively.

Water soluble carbohydrates

[00314] To determine the effect of the mutations singly or in combination on water soluble carbohydrate contents in barley grain, four lines were analysed from each group. Compared to the water soluble carbohydrate composition in wildtype seeds, *amo1* seeds did not contain significantly different levels of total WSC, free glucose, sucrose or maltose, or fructan. However, *SSIIa* mutant and double mutant seeds contained significantly greater amounts of each of these carbohydrates ($P < 0.05$). The seeds of the *SSIIa* single mutants contained significantly more fructose, sucrose and total WSC.

Protein content

[00315] Protein content was measured in grains of the five groups. Protein content was $10.3 \pm 0.8\%$ for wildtype seeds, $10.4 \pm 1.1\%$ for *amo1* mutant seeds, $12.6 \pm 0.9\%$ for *SSIIa* mutant seeds, $14.6 \pm 0.6\%$ for hullless double mutant seeds and $13.8 \pm 1.4\%$ for hulled double mutant seeds. Both hullless and hulled double mutant seeds contained significantly more protein than *SSIIa* mutant seeds, wildtype seeds or *amo1* mutant seeds, but there were no significant differences between hullless and hulled double mutant seeds or between *amo1* mutants and wildtype seeds in protein content.

Lipid content

[00316] Lipid content was measured for grains of the five groups. Total lipid content was $2.9 \pm 0.2\%$ for wildtype seeds, $3.5 \pm 0.3\%$ for *amo1* mutant seeds, $6.4 \pm 0.9\%$ for *SSIIa* mutant seeds, $4.9 \pm 0.3\%$ for hullless double mutant seeds and $5.0 \pm 0.3\%$ for hulled double mutant seeds. *SSIIa* mutant seeds contained significantly more lipid than hullless and hulled double mutant seeds, wildtype seeds and *amo1* mutant seeds, but there were no significant differences between hullless and hulled double mutant seeds or between *amo1* mutant and wildtype seeds.

Contents of Starch and Other Grain Components on a Per Caryopsis Basis

[00317] Expressing grain composition data on a percentage basis can be misleading when the major grain constituent, starch, differs in content significantly between genotypes, leading to apparent increases in grain constituents that do not reflect underlying synthesis rates. In order to examine the absolute levels of synthesis of the various grain components in each of the genotypes, Table 2 presents the composition data on a per caryopsis basis. This analysis confirmed that, as expected, modification in starch synthesis levels was the major driver of grain weight differences between these genotypes. However, analysis of the data on this basis indicated that there were major changes in grain starch synthesis among the genotypes. Each of the mutant genotypes had decreased grain amylopectin synthesis, with the *SSIIa* mutant genotype having a severe suppression of amylopectin synthesis. In contrast, the *amo1* mutant genotypes (*amo1* mutants and *SSIIa-amo1* double mutants) had significant increases in amylose synthesis compared to the *amo1* wild-type genotype, while the *SSIIa* genotype (*SSIIa* mutants) had a small decrease in amylose synthesis. The data however demonstrated that the major cause of a restoration of starch content in the *SSIIa-amo1* double mutant genotype relative to the *SSIIa* single mutant genotype was due to an increase in both amylose (79% increase) and amylopectin content (61% increase).

[00318] Analysis of the levels of other grain constituents on a per caryopsis basis suggested that protein content was increased by at least 5% in the *SSIIa-amo1* double mutant genotype relative to the other genotypes, lipid content was specifically increased in both the *SSIIa* and *SSIIa-amo1* mutant genotypes, β -glucan and pentosan synthesis levels remained unaltered in all genotypes with the potential exception of β -glucan synthesis in the *amo1* genotype, and water soluble carbohydrates levels were inversely proportional to starch contents.

Discussion

[00319] The interaction between recessive mutations at the *SSIIa* and *amo1* loci was examined. These mutations both resulted in an elevated amylose phenotype relative to wildtype, with amylose contents typically 60-70% in the starch of *SSIIa* mutant grains and 35-45% in *amo1* mutant grains. Determination of the 4 possible

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genotypes for the *SSIIa* (*SSIIa*-wt and *SSIIa*-292) and *amo1* (*amo1*-wt and *amo1*-AC38) loci was an important aspect of this study. The mutant and wildtype alleles of the *SSIIa* locus were able to be unambiguously distinguished using a marker based on the causal mutation in the starch synthase IIa gene. Initially, a closely linked marker (Bmac0501, consensus map location 58.0 cM) was used to assay for the presence of the chromosome region containing the *amo1* locus.

[00320] The impact of combining these mutations on amylose content was examined. The data showed that there was no statistically significant difference in the proportion of amylose (as a weight percentage of the total starch content) when the *SSIIa* mutant and *amo1* mutant loci were combined relative to lines carrying the *SSIIa* mutant locus alone. However, the combination of the *SSIIa* and *amo1* mutations did have unexpected consequences on starch synthesis and grain weight, increasing starch content and seed weight relative to the *SSIIa* mutation alone by virtue of increasing both amylose and amylopectin contents per grain.

[00321] Barley *SSIIa* mutants contained starch with a high percentage of amylose. The data showed that *SSIIa* single mutant grain on average contained only 40% as much starch as the wildtype grain on a per grain basis. The high amylose phenotype of *SSIIa* mutant seed was thus due to a preferential reduction of amylopectin, which was decreased by 75%, compared to amylose which was only decreased by 25%. In the case of the *SSIIa*-*amo1* double mutant grain, there was also a decrease in amylopectin synthesis compared to wildtype (31% reduction) but an increase in amylose content (increased by 37%) per seed. These results were intriguing, suggesting that the *amo1*-wt (wild-type) gene product not only participated in amylopectin synthesis but also repressed amylose synthesis.

[00322] The impact of the *amo1* mutant allele on starch chain length distribution was subtle. In a wildtype background, the presence of the *amo1* mutant allele caused a slight decrease in short chain lengths (DP 9-14) and an increase in the DP 15-24 fraction. In *SSIIa* mutant backgrounds, the impact of the *amo1* mutant locus on chain length distribution was negligible. In contrast, the *SSIIa* mutant allele had a major affect on amylopectin structure and thus chain length distribution, increasing

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the proportion of short chains (DP 6-8 and DP 9-14) and decreasing chains with DP15-25.

[00323] The combination of the *SSIIa* and *amo1* mutant alleles provided an unexpected phenotype in which starch content and seed weight were partially restored compared to the properties of lines containing only the *SSIIa* mutant allele.

EXAMPLE 4: GENETIC MARKERS LINKED TO THE BARLEY AMO1 LOCUS

[00324] In order to identify genetic markers linked closely to the *amo1* locus, 12 SSR (Simple Sequence Repeat) markers (Ramsay *et al.*, 2000) located between 56.00 cM and 64.60 cM on the short arm of chromosome 1H of barley were selected. These SSR markers were EBmac0405, Bmag0105, Bmac0063, HVM20, Bmac0090, EBmac0560a, EBmac0501, Bmac0044, Bmac0032, Bmag0113, Bmag0211, and Bmag0350. The primers for these SSR markers were synthesized according to the sequences listed in the GrainGenes Database. These markers were tested in PCR reactions using DNA from each of two parental barley varieties, High Amylose Glacier (HAG) and Himalaya292 and the amplification products compared between the parents by gel electrophoresis.

[00325] For each PCR reaction (20 µl), 50 ng genomic DNA, 1.5 mM MgCl₂, 0.125 mM each dNTP, 10 pmol primers, 0.5 M glycine betaine, 1 µl DMSO and 1.5 U of Hotstar Taq polymerase (QIAGEN, Australia) were used. The PCR conditions for the amplifications for the SSR markers were: 1 cycle of 95°C for 4 minutes, 15 cycles of 94°C for 30 seconds, 65°C to 50°C with decreasing 1°C each cycle for 30 seconds, and 72°C for 1 minute 20 seconds, 30 cycles of 94°C for 15 seconds, 50°C for 15 seconds, and 72°C for 45 seconds, and 1 cycle of 25°C for 1 minute. The PCR reactions were assembled in identical fashion except for the primer pairs. The PCR products were separated by electrophoresis on 2% agarose gels and visualized with gel documentary (UVitec) after GelRed (Biotium) staining, or by using a 3130xl Genetic Analyser (Applied Biosystems) according to the manufacturer's instructions.

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[00326] When the 12 SSR markers were tested, 3 of 12 markers, namely Bmac0032 located at 64.6 cM, EBmac0501 located at 58.0 cM and Bmac0090, gave different sized PCR products amplified from DNA from the HAG and Himalaya292 parental plants. That is, these markers showed polymorphism between the two parental varieties. These SSR markers were then used for the genotyping of 71 BC3F6 lines. All of the lines genotyped with the Bmac0032 marker gave the same sized fragments as HAG, which showed that all of the lines had recombined between the Bmac0032 and *amol* loci and the SSR marker Bmac0032 was not tightly linked with the *amol* locus. In contrast, of the 71 BC3F6 lines genotyped with the EBmac0501 marker, 56 were homozygous for one or the other of the fragment patterns. Of the 56 homozygous lines, 25 displayed the EBmac0501 marker from HAG and 31 exhibited the EBmac0501 marker from Himalaya292. These results showed that the EBmac0501 marker did not have a high frequency of genetic recombination with *amol* locus. Therefore, the SSR marker EBmac0501 was a tightly linked microsatellite marker for the *amol* locus.

Markers based on the *SSIHa* gene of barley

[00327] SSI, SSIIa and SSIII are thought to be primarily involved in amylopectin synthesis involved in the extension of specific subsets of available non-reducing ends within the starch molecule. Studies of *Arabidopsis* and rice SSI null mutants showed that SSI is involved in biosynthesis of the small outer chains of the amylopectin cluster (8 - 12 dp) in leaf starch of *Arabidopsis* and in the endosperm starch of rice (Li *et al.*, *J. of Exp. Botany*, 62(14), 5217-5213, 2011 and reference referred to herein). Starch from barley and wheat *SSIHa* mutants had an increase in chains of DP3-8, indicating that the *SSIHa* enzyme played a role in extending shorter glucan chains of DP3-8 to longer glucan chains of DP12-35 (Yamamori *et al.*, *Theoretical and Applied Genetics*, 101: 21-29, 2000; Morrell *et al.*, *The Plant Journal*, 34: 173-185, 2003; Konik *et al.*, *Theoretical and Applied Genetics*, 115: 1053-1065, 2007). Loss of SSIIIa in maize and rice conferred an increased amylase phenotype, with a reduction in the proportion of very long chains (DP >50 in maize or DP >30 in rice), and slightly reduced gelatinization temperature Janc *et al.*, *Cereal Chemistry*, 76: 629-637, 1999; Fujita *et al.*, *Plant Physiol.*, 144: 2009-2023, 2007).

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Arabidopsis mutants, defective for SSIV, appear to have fewer, larger starch granules within the plastid and a role in priming starch granule formation has been postulated for the SSIV protein (Roldan *et al.*, *The Plant Journal*, 49: 492-504, 2007).

[00328] Based on the data described above, it was thought that the *amol* locus might be near to the *SSIIa* gene of barley. To test this possibility and to develop a DNA marker based on the *SSIIa* gene in barley, portions of the *SSIIa* gene were first isolated from the two parental varieties. DNAs from HAG and Himalaya292 were used for the amplification of PCR fragments using primers based on the wheat *SSIIa* genomic DNA sequence (Li *et al.*, 2000). The oligonucleotide primers SSIIaF (5'-GGAGGTCTCGGGGATGT-3' (SEQ ID NO: 14)) locating in exon 7 and SSIIaR (5'-GCTCCAGGAAGTAAACGGTCAGG-3' (SEQ ID NO: 15)) locating in exon 8 of the wheat *SSIIa* gene were used for the PCR amplification of a 464 bp product. For each PCR reaction (20 μ l), 50 ng genomic DNA, 1.5 mM MgCl₂, 0.125 mM each dNTP, 10 pmol primers, 0.5 M glycine betaine, 1 μ l DMSO and 1.5 U of Hotstar Taq polymerase were used. The PCR reactions were conducted using 1 cycle of 95°C for 5 minutes, 35 cycles of 94°C for 45 seconds, 58°C for 30 seconds, and 72°C for 1 minute, 1 cycle of 72°C for 10 minutes and 1 cycle of 25°C for 1 minute. A 464bp fragment was produced in each amplification. The PCR fragments were treated with 0.5 units of Shrimp Alkaline Phosphatase (USB Corporation, USA), 2.5 units of Exonuclease I and 1x PCR buffer (QIAGEN, Australia) according to the protocol from USB Corporation and sequenced using the automated ABI system with dye terminators as described by the manufacturers.

[00329] The 464 bp fragments had a sequence difference which provided an *Nla*IV restriction site in one fragment but not the other. Therefore, treatment of the PCR products with this enzyme followed by electrophoresis on 2% agarose gels provided a convenient way to distinguish the *SSIIa* genes from the two parental varieties. The production of only the 464 bp DNA fragment indicated the presence of the *SSIIa* gene from Himalaya292 (i.e. wild-type *SSIIa* gene), and the production of both 303 bp and 161 bp DNA fragments indicated the presence of the *SSIIa* gene from HAG.

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[00330] The DNA marker for the *SSIIa* gene which was polymorphic between the HAG and Himalaya292 parental lines (Example 4) was also used to genotype the homozygous lines. This analysis showed the presence of the *SSIIa* gene from HAG in 26 lines, and the *SSIIa* gene from Himalaya292 in 30 lines. Among the 56 lines genotyped with the EBmac0501 and *SSIIa* gene markers, 5 lines showed recombinant genotypes. When the genotypes and phenotypes including the plumpness of seeds and the starch contents were correlated, 4 lines genotyped with EBmac0501 and 1 line genotyped with the *SSIIa* gene marker initially gave recombinant phenotypes (PCT/AU2010/000968), suggesting that there was some recombination between the *SSIIa* marker and the *amol* locus and that the *amol* gene and *SSIIa* genes were genetically distinct even if closely linked in barley. However, repeated tests showed that the presumed recombinant between the *SSIIa* marker and the *amol* gene had been mis-classified, and indeed there were no recombinants between the *SSIIa* marker and the *amol* gene in the segregating barley population.

[00331] The *SSIIa* gene sequences and *SSIIa* activities in the barley varieties and mutants were therefore examined more closely, in particular between HAG and its wild-type parent Glacier, as described in the following Examples.

EXAMPLE 5: DETECTION OF *SSIIa* ACTIVITY BY ZYMOGRAM ANALYSIS.

[00332] The presence of *SSIIa* activity in endosperms from the barley varieties Himalaya and Glacier, the mutants Himalaya292 and HAG and from selected lines from each of the 4 BC3F6 genotypes was examined by zymogram analysis, which detects enzymatic activity, as described in Example 1 and summarized as follows. Developing endosperms at 15 DPA were isolated and ground in a mortar and a pestle with 3 volumes of extraction buffer (20 mM Tris-HCl, pH 7.5, 5 mM DTT, and 1 mM Pefabloc SC (Roche)) at 4°C. Each homogenate was then centrifuged at 10,000g for 20 min at 4°C and aliquots of the supernatants, each containing 20 µg of extracted protein, were used for analysis of *SSIIa* activity by zymogram analysis according to Abel *et al.*, 1996).

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[00333] Two starch synthase activities were detected on the zymogram gels, a faster migrating band which was starch synthase I (SSI) and a slower migrating band which was SSIIa, possibly a combination of SSIIa and SSIIb. The zymograms did not show any consistent change in the SSIIa band among lines from the 4 BC3F6 genotypes. In particular, the *amol* mutant endosperms contained an active SSII protein. However, surprisingly, three out of 5 *SSIIa-amol* double mutants showed a substantially higher level of SSI activity compared to the *amol* and *SSIIa* single mutants. Wildtype lines had consistently low SSI activity.

EXAMPLE 6: THE *amol* LOCUS NEGATIVELY REGULATES THE EXPRESSION OF GBSSI, SSI AND SSIIa/SBEII PROTEINS IN THE ENDOSPERM

[00334] To understand the cause of the observed increase in amylose content on a per caryopsis basis (Example 3, Table 1), the level of granule bound starch synthase I (GBSSI) protein in the starch granules in barley grains from each of the 4 genotypes was analysed. GBSSI is the major starch synthase enzyme involved in amylose synthesis in cereal grains including barley and is primarily localized in starch granules within the endosperm. Starch granules from *amol* mutants contained significantly higher levels on a weight basis of GBSSI, SSI, SSIIa, SBEIIa and SBEIIb proteins compared to wild-type starch granules. Starch granules from *SSIIa-amol* double mutants also contained more GBSSI protein than granules from *SSIIa* single mutants, but had similar amounts of GBSSI protein compared to wildtype granules. However, starch granules from *SSIIa-amol* double mutant granules did not detectably contain any SSI protein or any SSIIa/SBEIIa/SBEIIb proteins, consistent with an altered localization of these proteins from the granules to the soluble fraction in the developing endosperms. Starch granules from *SSIIa* single mutants contained significantly less GBSSI protein than the other 3 genotypes as well as no detectable SSI protein or SSIIa/SBEIIa/SBEIIb proteins.

Discussion

[00335] The description in Examples 2-6 illustrates the impact on amylose content of combining recessive mutations at the *SSIIa* and *amol* loci. Both of these

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mutations alone yielded increased amylose content in barley grains and reduced starch content. By combining them, it would have been reasonable to expect a more severe phenotype such as a further increase in amylose content and a further reduction in starch content. Unexpectedly however, combining the *amo1* mutation with the *SSIIa* mutation significantly restored starch synthesis in the endosperm of *SSIIa* mutants through parallel increases in both amylose and amylopectin content, and without further raising the proportion of amylose in the starch.

[00336] The Examples also provided a further description of the effects of the *SSIIa* and *amo1* mutations on starch synthesis. The effect of the *SSIIa* mutation was to predominantly decrease amylopectin synthesis, yielding a 75% reduction on a per caryopsis basis (Table 1), while amylose synthesis was decreased by 28%. Given that amylose synthesis requires the granular matrix to be present, the impact on amylose synthesis of the loss of starch synthase IIa through the *SSIIa* mutation might be a secondary consequence of the major decrease in amylopectin synthesis in this mutant. It was evident that the high amylose content of the grain starch of *SSIIa* mutants was a consequence of the greater inhibition of amylopectin synthesis than amylose synthesis. In contrast, the data presented in Table 1 suggested that the *amo1* mutation promoted a shift from amylopectin synthesis to amylose synthesis, resulting in the elevated amylose phenotype of the *amo1* mutant. When combined with the *SSIIa* mutation, the impact of *amo1* was to increase the synthesis of both amylose and amylopectin, resulting in the significant restoration of starch synthesis and therefore grain weight in the *SSIIa-amo1* double mutant, but no further increase in amylose content on a percentage basis was observed. However, the amylose content on a per seed basis was significantly increased in the *SSIIa-amo1* double mutant compared to the *SSIIa* mutant alone (Table 1). Therefore, the *amo1* mutation in both *amo1* mutant endosperms and *SSIIa-amo1* mutant endosperms promoted the synthesis of more amylose than in wildtype endosperms. This result suggested that the function of the gene at the *amo1* locus is to negatively regulate amylose synthesis.

[00337] As GBSSI is essential for the biosynthesis of amylose in the endosperm (Nelson & Rines, 1962) and also contributes to the synthesis of the long chains of amylopectin (Maddolein *et al.*, 1994; Denyer *et al.*, 1996), the expression

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level of GBSSI in the starch granules in the endosperm was tested. This showed that the levels of expressed GBSSI protein in both *amo1* mutant grains and *SSIIa-amo1* double mutant grains were significantly increased compared to the wildtype grains and *SSIIa* single mutant grains, respectively. This suggested that the function of the gene at the *amo1* locus was to negatively regulate expression of GBSSI, which has a primarily role in synthesizing amylose.

[00338] The data that the *SSIIa* gene mapped close to the *amo1* locus in barley suggested that the *ssIIa* gene was a candidate for the gene containing the *amo1* mutation. This conclusion was strengthened by further mapping data presented above and the sequencing data in Example 7. The *SSIIa* gene was very tightly linked to the *amo1* locus and we did not see any recombination between this gene and *amo1* in more than 190 lines. Secondly, sequencing of the entire *SSIIa* gene (Example 7) showed that there was a non-conservative difference in the amino acid sequence of the *SSIIa* protein in the *amo1* mutant compared to either the reference Himalaya or parent Glacier proteins and this substitution (from leucine to arginine) was in conserved motif 7 within the catalytic domain of *SSIIa* proteins (Li *et al.*, 2000). Activity of *SSIIa* as assayed by zymograms was similar, however, in *amo1* and wild type endosperms, so either the *amo1* variant of *SSIIa* retained its starch synthase activity or the band of activity observed in the zymograms was from the co-migrating isoform *SSIIb*. It is proposed without limitation that the *SSIIa* gene in HAG comprises the *amo1* mutation, and that *SSIIa* is a negative regulator of GBSSI in barley although *SSIIa* is expressed in developing endosperm.

[00339] The role of starch synthase IIa in the elongation of amylopectin chains of DP15-24 has been previously demonstrated in barley (Morell *et al.*, 2003) and extensively reviewed elsewhere for other species (Konik-Rose *et al.*, 2007). As described herein the *amo-1* mutation had only a subtle effect on chain length distribution, with minor but statistically significant effects in the chain length ranges DP9-14 (and DP15 to 24). The chain length distributions of the *SSIIa* and *SSIIa-amo1* mutant amylopectins did not show statistically significant differences across the entire distribution.

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[00340] The analysis of mutations affecting starch biosynthesis in a wide range of systems (including *Chlamydomonas*, rice, maize, pea, barley, *Arabidopsis*) has been highly informative in defining the key genes involved in the synthetic process and understanding their respective roles. However, it has frequently been noted that when combinations of mutations are generated, the effects of the mutations are not additive, suggesting interactions between genes and gene products are important.

EXAMPLE 7: Cloning and Sequencing of barley *SSIIIa* genomic DNA and cDNA

[00341] Whilst the zymogram evidence showed that complete inactivation of the *SSIIIa* enzymatic activity was not responsible for the *amol* phenotype, the zymogram data did not exclude the possibility of a more subtle effect in the protein such as a change in kinetic properties of the enzyme, perhaps caused by a change in the amino acid sequence of the *SSIIIa* protein in the *amol* mutants. In order to investigate whether other polymorphisms in the *SSIIIa* gene could cause the *amol* phenotype, the cDNA sequences from wildtype barley Himalaya, and genomic DNA sequences from wildtype barley (Himalaya), *SSIIIa* mutant (Himalaya292), *amol* mutant (HAG) and wildtype barley (Glacier, a parent line for HAG) were obtained by PCR cloning and sequenced as follows.

[00342] In order to obtain genomic *SSIIIa* DNA sequences, genomic DNA preparations isolated from barley varieties Himalaya, Himalaya292, Glacier and HAG were used for the PCR amplification of fragments using 3 pairs of primers. These primers were based on the cDNA and genomic DNA sequences of two wheat *SSIIIa* genes (Li *et al.*, 2000), GenBank Accession Nos: AF258608 and AF258609). The three pairs of primers were ZLSSIIIa-P1F (5'ATGGAGATGTCTCTCTGGCCA 3' (SEQ ID NO: 16), locating at nucleotide 29 of wheat *SSIIIa* cDNA) and ZLSSIIIa-P1R (5' TCTGCATACCACCAATCGCCGT 3' (SEQ ID NO: 17), locating at nucleotide 3806 of wheat *SSIIIa* genomic DNA); ZLSSIIIa-P2F (5'ATCGTGACCTAACAGCT TTGGCG 3' (SEQ ID NO: 18), locating at nucleotide 3189 of wheat *SSIIIa* genomic DNA) and ZLSSIIIa-P2R (5'

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GACAGAAGAACCCAAATCTGCGGTC 3' (SEQ ID NO: 19) locating at nucleotide 7189 of wheat *SSIIa* genomic DNA); ZLSSIIa-P3F (5' GGAGGTCTCGGGGATGTTGTTAC 3' (SEQ ID NO: 20), locating at nucleotide 6038 of wheat *SSIIa* genomic DNA) and ZLSSIIa-P3R (5' CCACAAATGTAAATA TCATTGATGTAT 3' (SEQ ID NO: 21), locating at nucleotide 9524 of wheat *SSIIa* genomic DNA).

[00343] For obtaining *SSIIa* cDNA sequences, total RNA was extracted from the developing endosperm (15 days post anthesis, (dpa)) of the barley varieties. The procedures for the RNA extraction were as detailed in Clarke and Rahman, 2005. First strand cDNA was synthesized and used for PCR amplification of *SSIIa* cDNAs. Primers used for amplification of full length of cDNA sequence were ZLSSIIa-P1F and ZLSSIIa-P4R (5'-ACGTCACCTGCGGTTCTTATCTCG-3' (SEQ ID NO: 22), located at nucleotide 9403, after the translation stop codon of the genomic DNA sequence of wheat *SSIIa*).

[00344] For each PCR reaction (20 µl), 50 ng cDNA or genomic DNA, 1.5 mM MgCl₂, 0.125 mM each dNTP, 10 pmol primers, 0.5 M glycine betaine, 1 µl DMSO and 1.5 U of Advantage 2 Taq polymerase mix (Clontech) were used. The PCR reactions were conducted using a HYBAID PCR Express (Integrated Sciences) with 1 cycle of 95°C for 5 minutes, 35 cycles of 94°C for 45 seconds, 59°C for 30 seconds, and 72°C for 3 minute, 1 cycle of 72°C for 10 minutes and 1 cycle of 25°C for 1 minute. The PCR fragments were cloned into a pCR2.1 TOPO cloning vector (Invitrogen). DNA sequencing was performed at JCMRS, ANU, Australia using an automated ABI system with dye terminators as described by the manufacturers. DNA sequences were analysed using the GCG suite of programs (Devereaux *et al.*, 1984) to detect single-nucleotide polymorphisms (SNPs) in genomic DNAs of the *SSIIa* genes from Himalaya292, Himalaya, Glacier and HAG.

[00345] The *SSIIa* genomic DNA sequences from Himalaya, Himalaya292, HAG and Glacier lines beginning at the translation initiation codon (ATG) in each case were each 9550 nucleotides long and contained 16 exons and 15 introns (Table 3). The *SSIIa* cDNA sequence from Himalaya was 5088 nucleotides long, with a protein coding region from nucleotides 1 to 4770 which encoded a polypeptide with

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1593 amino acid residues (SEQ ID NO: 4). Comparison between the *SSIIa* genomic nucleotide sequences from Himalaya292 (and its parent Himalaya) and HAG (and its parent Glacier) showed that there were 8 single nucleotide polymorphisms (SNPs) for HAG (and 7 SNPs for Glacier) (Table 3). All of these were nucleotide substitutions, not deletions or insertions. Among them, 4 SNP variations for HAG (and 3 SNPs for Glacier) were in exons (Table 3). Three SNPs were in exon 3 (for HAG and Glacier) and one was in exon 14 of *SSIIa* only from HAG. Two SNP variations at nucleotide 1084 (for HAG and Glacier) and 4439 (for HAG only) of the cDNA sequences produced a change in the amino acid sequence of the encoded protein. One change was conservative, changing a hydrophobic amino acid methionine (present in *SSIIa* in Himalaya and Himalaya292) to another hydrophobic amino acid valine (in HAG and Glacier) at amino acid position 362 of the protein, whereas the other was a non-conservative change from a hydrophobic amino acid leucine (in Himalaya, Himalaya292 and Glacier) to a basic amino acid arginine (HAG) at position 1480 of the protein (Table 3). This last amino acid variation present only in the *amo1* mutant HAG but not its parental variety Glacier was a good candidate for the genetic variation that caused the *amo1* phenotype.

[00346] In the 5 lines that exhibited a broken linkage between the *amo1* locus and either the EBmac0501 or Bmac0090 marker, all showed a linkage association between the *SSIIa* marker and *amo1* locus (Table 1) indicating that the *SSIIa* gene was the closest marker to the *amo1* locus of these markers. We therefore attempted to identify lines containing a recombination between the *SSIIa* marker used and the gene underpinning the *amo1* phenotype, by genotyping progeny from each of the 18 heterozygous lines comprising the *SSIIa* mutation and which were not used for the genotypic grouping analysis described above. Grains harvested from those plants were morphologically phenotyped and all 190 grains analysed showed no linkage breakdown between the *SSIIa* gene and the *amo1* locus, confirming that the *SSIIa* gene was very tightly linked to the casual gene for the *amo1* phenotype and a strong candidate as the gene causing the *amo1* phenotype. Further studies such as down-regulation of the expression of the *SSIIa* gene and complementation of the

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amol mutation by over-expression of a wild type *SSIIIa* gene were aimed at confirming this and are described in Examples 8-10 as follows.

[00347] A CAPS marker (Cleaved Amplified Polymorphic Sequence) was designed based on one SNP at nucleotide 6323 of the barley *SSIIIa* genomic DNA between two parental lines. This polymorphism created an *EcoRI* site in the *SSIIIa* gene from HAG, but not from Himalaya292. Primers *SSIIIa*-P5F (5' GGAGGTCTCGGGGATGT 3', (SEQ ID NO: 14)) located at nucleotide 7442 bp and *SSIIIa*-P5R (5' GGTTCAGGAAGTAAACGGTCAGG 3' (SEQ ID NO: 23)) located at nucleotide 7893 of the barley *SSIIIa* genomic DNA were used for the PCR amplification of the CAPS marker for *SSIIIa* gene, a 464 bp product which was then digested with *EcoRI*.

EXAMPLE 8: Reducing *SSIIIa* and *SSIIIb* gene expression in barley with transgenes encoding inhibitors of gene expression.

[00348] Two starch synthase III enzymes (*SSIIIa*, also known as *SSIII-2*, and *SSIIIb* also known as *SSIII-1*) are expressed in the developing wild-type barley endosperm from the *SSIIIa* and *SSIIIb* genes, respectively. In order to confirm the specific roles of these isoforms on starch synthesis and particularly on their role in negatively regulating amylose content in the grain, hairpin RNA constructs were made targeting these two genes.

[00349] Barley *SSIIIa* and *SSIIIb* share approximately 70% amino acid sequence identity. The wheat *SSIIIa* sequence is approximately 90% identical to the barley *SSIIIa* protein.

Constructs to inhibit *SSIIIa* and *SSIIIb* gene expression in barley.

[00350] A plasmid vector (pBx17IRcasNOT) was first constructed as a backbone vector for making constructs for the specific suppression of gene expression in barley endosperm by hairpin RNA technology i.e. using duplex RNA. The vector contained the following elements in order: an endosperm-specific promoter which included a 1895 bp sequence from a gene encoding the high molecular weight (HMW) glutenin Bx17 subunit in wheat (Reddy and Appels, 1992), a forward oriented cassette of attR-*ccdB* of 1447 bp in length (Gateway cloning technology

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GibcoBRL/Life Technologies) with a *Bam*HI restriction enzyme site at the 5' end and an *Eco*RI site at the 3' end, a rice starch branching enzyme I intron 4 which was a 467 bp sequence corresponding to nucleotide 6202 to nucleotide 6668 of Genbank Accession No. D10838 in reverse orientation to the promoter, a rice branching enzyme I intron 9 which was a 495 bp sequence from nucleotide 9112 to nucleotide 9606 of the sequence D10838 in forward orientation, a reverse cassette of attR-*ccdB* (1435 bp) with a *Spe*I site at its 5' end and a *Kpn*I site at its 3' end, and lastly a *nos* 3' polyadenylation sequence of 267 bp long (Chamberlain *et al.*, 1994). The promoter was known to be expressed strongly in a tissue specific manner in wheat and barley endosperm in developing seeds.

[00351] To make the hairpin RNA construct targeting expression of the barley *SSIIIa* gene, a targeting sequence of 399 bp was amplified by PCR from a wheat *SSIIIa* cDNA (GenBank Accession No: AF258608 from nucleotide 4514 to nucleotide 4912) using the primers *SSIIIa*-F 5'-AAAAGGATCCGGTACCGTTCTAACCTATGATGAGCCTCT-3' (SEQ ID NO: 24), containing *Bam*HI and *Kpn*I sites towards its 5' end, and *SSIIIa*-R 5'-AAAAGAATTCACTAGTGAATTTTCGAGCGGCATGGTAC-3' (SEQ ID NO: 25), containing *Eco*RI and *Spe*I sites towards its 5' end. A wheat sequence could be used in the inhibitory construct because of the degree of similarity of the wheat and barley genes. PCR amplification was carried out under standard conditions using Hotstar polymerase (Qiagen, Australia) on a Hybaid PCR Express thermal cycler (Hybaid, UK). The thermal profile was 4 min at 94°C followed by 35 cycles of 30 sec at 94°C, 30 sec at 58°C and 1 min at 72°C. Two copies of the resultant PCR fragment were inserted into the backbone vector pBx17IRcasNOT, one copy in reverse orientation into the *Spe*I and *Kpn*I sites and a second copy in the forward orientation into the *Bam*HI and *Eco*RI sites i.e to form an inverted repeat structure. The resultant plasmid was designated as pwSSIIIaRNAi. This construct is shown schematically in Figure 2. The *Hind*III/*Not*I fragment from pwSSIIIaRNAi was then cloned into an *Agrobacterium* Ti binary expression vector, pWBVec8, which contains a hygromycin resistance gene driven by a cauliflower mosaic virus (CaMV) 35S promoter (Wang *et al.*, 1998). The resultant construct designated pwSSIIIaRNAi-vec8 was suitable for transformation of plant cells using

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Agrobacterium. The binary vector containing the pwSSIIIaRNAi-vec8 sequence was introduced into *Agrobacterium tumefaciens* strain AGL1 by electroporation and transformants containing the binary vector selected and grown using LB broth supplemented with 50 µg/ml each of rifampicin and spectinomycin as a growth medium.

[00352] To make the hairpin RNA construct targeting expression of the barley *SSIIIb* gene, the targeting sequence of 428 bp was amplified by PCR from a wheat *SSIIIb* cDNA (GenBank Accession No: EU333946.2 from nucleotide 3414 to nucleotide 3841) using the primers SSIIIb-F 5'-AAAAGGATCCGGTACCGGTGAATTTGATGGTCCCGTGTAG-3' (SEQ ID NO: 26), containing *Bam*HI and *Kpn*I sites towards its 5' end, and SSIIIb-R 5'-AAAAGAATTCACTAGTCAGTTCTTGC GCGTGGATGGTAC-3' (SEQ ID NO: 27), containing *Eco*RI and *Spe*I sites towards its 5' end. PCR amplification and cloning into the backbone vector were analogous to the SSIIIa construct (above) to form the plasmid designated pwSSIIIbRNAi (Figure 2). The *Hind*III/*Nor*I fragment from the pwSSIIIbRNAi construct was then cloned into vector pWBVec8 to form the construct designated pwSSIIIbRNAi-vec8. This binary vector was introduced into *Agrobacterium tumefaciens* strain AGL1 and transformants grown as for the *SSIIIa* construct, above.

Transformation of barley with the gene constructs

[00353] *Agrobacterium*-mediated transformation of barley (cv. Golden Promise) was carried out using the method of Tingay *et al.*, 1997, modified by the addition of 1.25 mg CuSO₄.5H₂O into the plant tissue culture media. Regenerated hygromycin-resistant plants were acclimatised for one week inside a moist growth chamber and then planted individually in 8 cm pots with soil supplemented with 1 g/kg Osmocote (Scotts, Australia). The plants were then grown in a glasshouse with a temperature regime of 24°C (day) and 18°C (night).

Analysis for the presence of transgenes in the plants

[00354] Genomic DNA was extracted from leaves of the plants after about one month of growth using a FastDNA Kit (Q-BIOgene) according to the manufacturer's instructions. Initial screening to confirm that plants are transformed with the hygromycin gene of the constructs was done using primers ZLhph253 5'-

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CGACGTCTGTCGAGAAGTTT CTG-3' (SEQ ID NO: 28) and ZLhph915 5'-CTCCAGAAGAAGATGTTGGCGAC-3' (SEQ ID NO: 29). The plants were further verified as transformants and the presence of the hairpin RNA encoding genes detected using gene-specific primers that amplified a fragment containing a portion of the HMW glutenin Bx17 promoter and the forward *wSSIIa* or the forward *wSSIIb* fragment. The PCR primers for this were Bx17-3' 5'-CAACCATGTCCTGAACCTTCACC-3' (SEQ ID NO: 30), and SSIIa-R 5'-AAAAGAATTCAGTGAATTTTCGAGCGGCATGGTAC-3' (SEQ ID NO: 25) for *pwSSIIaRNAi* transgenic plants, and Bx17-3' and SSIIb-R 5'-AAAAGAATTCAGTGAATTTTCGAGCGGCATGGTAC-3' (SEQ ID NO: 19) for the *pwSSIIbRNAi* transgenic plants. PCR reactions were carried out using Go Taq Flexi DNA Polymerase (Promega) and amplification products were resolved in 1% agarose in 1X TBE buffer using 1 Kb Plus DNA Ladder (Invitrogen) as molecular weight standards. A 550 bp fragment specific to the *pwSSIIaRNAi* construct and a 570 bp fragment specific to *pwSSIIbRNAi* construct was amplified from the transgenic plants, including in each case about 150 bp from the Bx17 promoter region and about 400 bp of the *SSIIa* or *SSIIb* fragments.

[00355] Southern blot hybridisation analysis to determine the copy number of the introduced transgenes is carried out as described by Lagudah *et al.*, 1991 with the modification that 6 M ammonium acetate is used to precipitate protein contaminants prior to the final precipitation of the DNA with isopropanol. A total of 10 µg DNA per sample is digested with the restriction enzymes *HindIII* or *NorI* and resolved in 1.3% agarose, using the Bx17 promoter digested with *BamHI/HindIII* as molecular weight and positive control. Hybridisation and wash conditions are as previously described (Rahman *et al.*, 1997) using 25% formamide in the hybridization solution.

Analysis of the expression level of *SSIIa* and *SSIIb* genes in the transformants

[00356] For detection of the expression levels of endogenous barley *SSIIa* and *SSIIb* genes or transgenes encoding *SSIIa* or *SSIIb*, the RNA products were detected by quantitative RT-PCR. Proteins such as *SSIIa* and *SSIIb* are detected by Western blot methods or zymogram analysis. Total RNA from 15 dpa barley grains

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was extracted using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. Long and short RNAs were fractionated using a Nucleospin miRNA extraction kit (Macherey-Nagel) and quantified using the Nanodrop 1000 kit (Thermo Scientific). A total of 5 µg long RNA template was used to synthesise cDNA using the SuperScript III reverse transcriptase kit (Invitrogen). Quantitative real time PCR (qRT-PCR) was done in a Rotor-Gene 6000 (Corbett) using 100 ng cDNA template and primers for the wSSIIIaRNAi or wSSIIIbRNAi fragments. Real-time PCR amplification was conducted using Platinum Taq DNA polymerase (Invitrogen) and Sybr Green I (Invitrogen) reporter dye. Quantitation was normalised using a tubulin reference gene which was constitutively expressed (Toyota *et al.*, 2006) and data validation and melt curve analysis was done using Rotor-Gene Series Real Time Rotary Analyzer Software (Corbett).

[00357] Among about 84 regenerated plants for each construct, five confirmed transgenic barley plants transformed with pwSSIIIaRNAi-vec8 and 10 plants transformed with pwSSIIIbRNAi-vec8 were selected and analysed by RT-PCR for expression levels of the endogenous *SSIII* genes. Each of the transformed plants exhibited reductions in the levels of the *SSIIIa* or *SSIIIb* genes, respectively, with some plants exhibiting less than 50% of the wild-type levels: for *SSIIIa* (pwSSIIIaRNAi-vec8) transformed lines 3, 4, 19, 9 and 21, and for *SSIIIb* (pwSSIIIbRNAi-vec8) lines 23, 30, 40, 5, 20, 26, 36, 76, 9, 53 showed reductions of at least 50%.

Analysis of starch granule bound proteins and *SSIIIa* enzymatic

[00358] Developing and mature grains are obtained from each transformed plant and assayed for *SSIII* proteins by zymogram analysis, and for total starch content, amylose content, starch chain length distribution, β-glucan content, pentosan content and other parameters as described above.

EXAMPLE 9. Constructs to express barley *SSIII* genes.

[00359] In order to confirm and compare the functionality of the cloned *SSIIIa* sequences, expression constructs were made to express the sequences in barley or in heterologous plant species such as rice or *Arabidopsis*. These constructs also allowed complementation analysis of the *amo1* mutation in barley, to provide further

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evidence that the *amo1* mutation was in the barley *SSIIIa* gene. A cDNA including the full length *SSIIIa* protein coding sequence from barley, isolated as described in Example 7, was inserted into an expression vector as an *EcoRI* fragment in the sense orientation between the Bx17 promoter and nos3' polyadenylation sequence/transcription terminator. The *NofI* fragment containing the Bx17-*SSIIIa*-NOS expression cassette was then excised from this vector and inserted into the *NofI* site of the pWBVec8 derivative vector pVec8SJ to create pSJ101. pVec8SJ differed from pWBVec8 in that the orientation of the hygromycin selectable marker cassette was reversed by cutting with *Clal* and religation. In pVec8SJ, the *NofI* and *HindIII* sites for insertion of expression cassettes were therefore closer to the right border and the gene of interest would therefore be transferred before the selectable marker gene which conferred hygromycin resistance.

[00360] Barley transformation and analysis of the plants, including for the presence of the transgenes were performed as described in Examples 1 and 8. The presence of transgenes in the transformants was confirmed using gene-specific primers that amplified a fragment containing a portion of the HMW glutenin Bx17 promoter and a part of the *SSIIIa* coding region. The PCR primers for this were Bx17-3' 5'-CAACCATGTCCTGAACCTTCACC-3' (SEQ ID NO: 30), and ZLSSIIIa-IR_RM 5'-GCTTGTTCAACAATGAGTCTTGTCG-3' (SEQ ID NO: 31)

[00361] Regenerated T₀ barley plants from the transformation process were planted in soil and screened by PCR. Among 52 T₀ transgenic lines containing the hygromycin resistance gene from pSJ101, 27 contained the exogenous *SSIIIa* gene. Immature seeds (15 dpa) were collected from the lines containing gene of interest and stored at -80°C.

[00362] Total RNAs were isolated from the immature seeds and used in real-time PCR amplifications. Six transgenic barley lines (lines 19, 6, 17, 2, 13 and 16) were selected which were transformed with pSJ101, and shown to have *SSIIIa* transcript levels at least 2-fold higher than in the wild-type immature seeds at the same stage. The enzymatic activities for *SSIIIa* in the 15 dpa immature seeds are examined using the zymogram assay.

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[00363] The transformed plants were grown in soil and, when flowering, are crossed with an *SSIIa* mutant (line 871), double mutants (HH4 or HH29) and an *amol* mutant (HH130) for transgenic plants containing *pwSSIIaRNAi-vec8* and *pwSSIIbRNAi-vec8*, or with an *amol* mutant (HH130) or double mutant (HH4 or HH29) for transgenic plants transformed with pSJ101.

EXAMPLE 10. Complementation of *SSIII* mutants of plants other than barley.

[00364] Functionality of the *SSIII* sequences can also be tested in heterologous species, including in *SSIII* mutants in species such as *Arabidopsis thaliana*. Two mutant *Arabidopsis* lines each containing a T-DNA insertion (SALK_065732 and SALK_102605) were ordered from the Arabidopsis Biological Resource Center (ABRC stock center). The T-DNA insertions were in exon 13 of *SSIII* for mutant line SALK_065732, located at nucleotide 34865 of the sequence of Genbank Accession No. AC007296.2 and in the exon 9 of *SSIII* for SALK_102605, located at nucleotide 35724 of AC007296.2. Transcripts for the 5' part of the endogenous *SSIIa* gene could be detected in these mutant *Arabidopsis* lines, but not for the 3' part of the gene. There was no *SSIII* protein expression detected in either mutant. Detailed analyses of both mutants were published in Zhang *et al.*, 2005.

Construction of expression constructs for dicot plants

[00365] The CaMV 35S promoter was cloned into the *Agrobacterium* vector pORE02 (Coutu *et al.*, 2007) as described by Wood *et al.*, 2009. The promoter, multiple cloning sites and *nos3'* polyA regions were excised as an *SphI*-*ZraI* fragment and ligated into pVec8SJ vector cut with *EcoRI*, Klenow blunted-*SphI*. This deleted the hygromycin selectable marker cassette from the vector. The duplicated region of the 35S promoter was then removed by cutting with *ScaI* and self ligation and the hygromycin marker was inserted from pJP3303 (obtained from James Petrie, CSIRO, Canberra, Australia) as a *KpnI*, T4 DNA polymerase blunt-*SphI* fragment between the *NcoI*, Klenow blunt-*SphI* sites to create the plasmid vector designated as pVec8SJ35SHYG.

[00366] The full length *SSIIa* cDNA from the barley variety High Amylose Glacier (HAG) was cloned in analogous fashion as described for the Himalaya *SSIIa*

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cDNA (Example 7) and both cDNAs were excised from the TOPO vectors as *NofI*-*SacI* fragments and ligated into the pVec8SJ35SHYG plasmid cut with the same enzymes to create plasmids pSJ119 and pSJ120.

[00367] Plants of *Arabidopsis* lines SALK-065732 and SALK-10260 were each separately transformed with constructs pSJ119 and pSJ120 by the dipping method as described by Clough and Bent, (1998). T₀ seeds were harvested from the four transformation combinations, SALK-065732/pSJ119, SALK-065732/pSJ120, SALK-10260/pSJ119 and SALK-10260/pSJ120. T₀ seeds were grown on agar plates containing MS basic medium supplemented with 50 mg/l Hygromycin and 150 mg/l Timentin to select T₁ transgenic plants. Fast growing T₁ transgenic plants that were resistant to hygromycin were transferred to and grown in soil. PCR amplification analysis confirmed that 7 T₁ plants for SALK102605/pSJ119, 10 for SALK102605/pSJ120, 6 for SALK065732/pSJ119 and 6 for SALK065732-pSJ120 were transformed.

[00368] The starch properties of the *Arabidopsis* mutant and overexpression transformed lines are compared to determine if the *SSIIa* gene can complement the *Arabidopsis* mutation in the SALK lines. It is predicted that overexpression of the barley cv. Himalaya *SSIIa* gene (wild-type) will complement the *Arabidopsis* mutation whereas overexpression of the *SSIIa* gene isolated from HAG will not complement the mutation or only partially complement the mutation. This procedure therefore can be used to screen for the functionality of isolated *SSIIa* genes.

EXAMPLE 11. Mutagenesis of barley and isolation of mutants

[00369] Barley mutagenesis was performed as described by Zwar and Chandler, 1995 using grain from the *SSIIa* mutant, line 871, which has a mutant *SSIIa* (*SSIIa*-292 allele) gene in a barley cv. Tantarara genetic background. Barley grain was washed several times until the water was clean. Approximately 1.5 kg of grain was imbibed at 4°C overnight without surface-sterilization using about 4 L water in a bucket. Air was bubbled through the water with several air stones and an air pump. Air was bubbled through the mixture for 8 hours and the water changed several times during this period. The barley grains were then treated with 1 mM Na azide (0.13 g)

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dissolved in 2 litre 0.1 M KH_2PO_4 (27.2 g) buffer pH 3.0 for 2 h, with air bubbling as above, then washed extensively for 2 hours by running tap water into the bucket. Treated grains were air dried and were stored at 4°C before sowing in flats in the greenhouse or in the field within a week.

[00370] Line 871 M1 mutagenised seeds (1.5 kg) were grown in the field (Ginninderra Experimental Station, Canberra, Australia) and approximately 2.1 kg of 871 M2 seeds were bulk harvested. Five 871 M2 seeds which showed a plump morphology in comparison with the shrunken phenotype of the parental 871 seeds (named as 871MP M2) were identified by visual inspection. These were sown into soil to produce progeny plants (M2 plants), grown to maturity and seeds harvested (M3 seeds). Three of the 5 plump 871MP M2 seeds produced plumped 871MP M3 seeds, i.e. the altered phenotype was heritable.

[00371] Total leaf DNAs were isolated from the young leaves of 20 cm tall 871MP M3 plants. DNA fragments were amplified using 15 pairs of primers which spanned the full length of the genomic DNA sequence of the *SSIIIa* gene, spanning a region of 9550 bp. The primer pairs used were pair 1: ZLwSSIII5'F (5' ATGGAGATGTCTCTCTGGCCA 3'), locating at nucleotide 1 of barley *SSIIIa* genomic DNA, and ZLBSSIII-2G17R (5'CTTCTGACCTCATCTAAGCAAGG 3'), locating at nucleotide 818 of barley *SSIIIa* genomic DNA, pair 2: ZLwSSIIIN-1F (5' CTGGTCTTTGG CTGCCATATAGC 3'), locating at nucleotide 710 of barley *SSIIIa* genomic DNA, and ZLBSSIIIE3-2R (5' TCTCAATGTCTCTTGCACAGTCC 3'), locating at nucleotide 1604 of barley *SSIIIa* genomic DNA, pair 3: ZLSSIII-IR-FM (5' GCATTTGAGCTGGATTGACCAGC 3'), locating at nucleotide 1559 of barley *SSIIIa* genomic DNA, ZLBSSIIIE3-1R (5' TCCTGACGAATCCATCTCAAACC 3'), locating at nucleotide 1955 of barley *SSIIIa* genomic DNA, pair 4: ZLBSSIII-2TLF1 (5' GCAGTTGAT GAAGCTGGGAATGATC 3'), locating at nucleotide 1858 of barley *SSIIIa* genomic DNA, and ZLBSSIII-2R12 (5' TCTCCCTCCTTAAGTTCC AGTCC 3'), locating at nucleotide 2641 of barley *SSIIIa* genomic DNA, pair 5: ZLBSSIII-2G19F (5' GGATCTTTTCGGCTGTTGATCTCC 3'), locating at nucleotide 2548 of barley *SSIIIa* genomic DNA, and ZLBSS3E3R7 (5' GCCATTGTTCTCATAGACCGTGC

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3'), locating at nucleotide 3350 of barley *SSIIIa* genomic DNA, pair 6: ZLSS3P21 (5' ATCGT GA CCTAACAGCTTTGGCG 3'), locating at nucleotide 3189 of barley *SSIIIa* genomic DNA, and ZLwSS3P2 (5' TCTGCATACCACCA ATCGCCGT 3'), locating at nucleotide 3806 of barley *SSIIIa* genomic DNA, pair 7: ZLBSSIII-2TLF2 (5' GTTGAGTTTGGCCAGAACATGTG 3'), locating at nucleotide 3625 of barley *SSIIIa* genomic DNA, and ZLBSSIII-2TLR2 (5' GTGCCATCACTCAACTGATTAGAAC 3'), locating at nucleotide xxx of barley *SSIIIa* genomic DNA, pair 8: ZLSS3P3 (5' AAAGGCACTTGTGTTGGACTGGG 3'), locating at nucleotide 4062 of barley *SSIIIa* genomic DNA, and ZLBSSIII-2R14 (5' CAGTGATGTAAGAACGCAAGCTC 3'), locating at nucleotide 4867 of barley *SSIIIa* genomic DNA, pair 9: ZLBSSIII-2G17F (5' CTTCTGACCTC ATCTAAGCAAGG 3'), locating at nucleotide 4803 of barley *SSIIIa* genomic DNA, and ZLBSSIII-2R9 (5' CTCGACGGCAATATGGATA ATACGC 3'), locating at nucleotide 5857 of barley *SSIIIa* genomic DNA, pair 10: ZLBSSIII-2F5 (5' TCTCCGAGTCGGATGAAAATGGG 3'), locating at nucleotide 5785 of barley *SSIIIa* genomic DNA, and ZLBSSIII-2SNPR4 (5' TAAACCAGTACAAGGCTCT AGCC 3'), locating at nucleotide 6555 of barley *SSIIIa* genomic DNA, pair 11: ZLBSSIII-2F6 (5' CAAAGTTTTTCTTGGAGTGGCACAG 3'), locating at nucleotide 6425 of barley *SSIIIa* genomic DNA, and ZLSS3P16 (5' CAGAAGAACCCAAATCTGCGGTC 3'), locating at nucleotide 7189 of barley *SSIIIa* genomic DNA, pair 12: ZLBSSIII-2F15 (5' TAGGATGTTTGGCGTCGGATGTG 3'), locating at nucleotide 7171 of barley *SSIIIa* genomic DNA, and ZLBSSIII-2R15 (5' GTGTACGGATCC CAGATATCTGG 3'), locating at nucleotide 7919 of barley *SSIIIa* genomic DNA, pair 13: ZLSS3P14 (5' CGCCATTGCTCCTCATCGTGAC 3'), locating at nucleotide 7888 of barley *SSIIIa* genomic DNA, and ZLBSSIII-2R (5' CTCACCCCTACCGTG GTAAACACC 3'), locating at nucleotide 8417 of barley *SSIIIa* genomic DNA, pair 14: ZLBSSIII-2P4F (5' TGGATTTT AGGTGGTTTTGCTTGG 3'), locating at nucleotide 8358 of barley *SSIIIa* genomic DNA, and ZLSS3P4 (5' CTCCATGACCCTCTTACACAGG 3'), locating at nucleotide 9030 of barley *SSIIIa* genomic DNA, pair 15: ZLBSSIII-2F7 (5'

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GCTCTGTCTCTTGGTCTTGAACC 3'), locating at nucleotide 8849 of barley *SSIIIa* genomic DNA, and ZLSS3P11 (5' CCACAAATGTAAATATCATTGATGTAT 3'), locating at nucleotide 9550 of barley *SSIIIa* genomic DNA.

[00372] Conditions for PCR amplification of DNA fragments from barley genomic DNA, DNA sequencing and sequence analysis were as described in the Example 7 above. The *SSIIIa* gene sequences from the mutants are determined and compared to the wild-type and mutant sequence in the *amo1* barley.

[00373] The zymogram method described above is used on progeny from the mutagenesis to identify mutants having altered mobility of the *SSIIIa* protein in its grain, or lacking *SSIIIa* altogether. Two starch synthase activities were seen on zymogram gels for wild-type barley endosperm extracts, a faster migrating activity band which was starch synthase I (SSI) and a slower migrating activity band which was *SSIIIa*. The zymogram method also allowed detection of grains having increased levels of SSI, as for the *amo1* mutant grain. The visual inspection method described above followed by zymogram analysis on selected grains allows the screening of large numbers (>100,000) of grains from mutagenized populations.

[00374] Sodium azide mutagenesis induces mainly single nucleotide substitutions in the genomic DNA of G bases to A bases. In some cases, the changes form a premature stop codon in the protein coding sequences, resulting in mutants with little or no *SSIII* activity. Although DNA based screening methods or TILLING methods can also be used to detect mutants in a population mutagenized with sodium azide, in most cases the single nucleotide polymorphisms that are identified will not have formed premature stop codons. Moreover, each set of PCR reactions in such methods would screen fragments of the *SSIIIa* genes of about 800 nucleotides, not the whole 9550 nucleotides of the barley *SSIIIa* genomic DNA, so multiple reactions would need to be set up to cover the whole gene. In contrast, zymogram-based screening will detect stop codons formed by mutation in any part of the amino acid sequence of *SSIIIa* cDNA and therefore single assays can be performed for each mutant line.

[00375] Mutants are analysed for total starch content, amylose content, starch chain length distribution, β -glucan content and other parameters as described in

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Example 1. A range of mutants is obtained, from ones with only partial loss of *SSIIa* activity through to complete loss (null mutants).

EXAMPLE 12: COMBINATION OF *SSIIa* MUTATIONS AND OTHER STARCH GENETIC VARIATIONS

[00376] The barley *amo1* mutant variety HAG was crossed with barley plants transformed with a hairpin RNA construct targeting the *SBEIIa* gene or the *SBEIIb* gene, or both transgenes, in order to combine the *SSIIa* mutation from HAG with the transgene(s). Transformants containing the transgenes that suppressed *SBEIIa* gene expression, in particular, yielded a dramatic increase in amylose content (WO03/94600). The combination of the *SSIIa* mutation with the transgene did not increase the grain amylose content on a per caryopsis basis relative to the grain having the transgene(s) but not the *SSIIa* mutation.

[00377] In similar fashion, the barley *SSIIa* mutants are crossed with barley plants containing a transgene expressing a hairpin RNA targeting the glucose, water di-kinase (GWD) gene (WO2009/067551). Suppression of GWD gene expression in the endosperm caused reduced grain starch phosphorylation, increased grain α -amylase and increased plant production characteristics such as increased grain yield and increased seedling vigour. Plants and grain containing both of these genetic variations are generated.

EXAMPLE 13: FIELD TRIALS OF *SSIIa*-*AMO1* DOUBLE MUTANTS

[00378] To evaluate the yield performance of the *SSIIa*-*SSIIa* double mutants when growing in the field, 3 hulless double mutant lines, 2 hulled double mutant lines, 4 hulless *SSIIa* mutants, 1 hulled *SSIIa* mutant line, 1 hulless wildtype barley line (cultivar Torrens) and 2 hulled wildtype barley lines (cultivars Tantangara, Sloop) were grown at Narrandera and Moree, NSW, Australia. Each of the barley lines was grown under both irrigated and non-irrigated (dryland) conditions at both sites. Two plots for each line were grown under each condition at both sites in a randomized pattern. Barley seeds (120 g) were sown in each plot (19 m²).

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[00379] The grain weight obtained after harvesting each plot in December 2008 was measured. At Narrandera, under irrigation, the double mutant, *SSIIa* mutant and hulless wildtype lines produced 2.23 ± 0.16 kg, 1.14 ± 0.57 kg and 1.65 ± 0.79 kg of grain, respectively, per plot. Under dryland conditions, the double mutant, *SSIIa* mutant and hulless wildtype lines produced 0.55 ± 0.34 kg, 0.11 ± 0.12 kg and 0.41 ± 0.16 kg of grain, respectively, per plot.

[00380] At Moree, under irrigation, the double mutant, *SSIIa* mutant and hulless wildtype lines produced 1.62 ± 0.72 kg, 0.54 ± 0.40 kg and 2.11 ± 0.08 kg of grain, respectively. Under dryland conditions, the double mutant, *SSIIa* mutant and hulless wildtype lines produced 0.88 ± 0.33 kg, 0.38 ± 0.27 kg and 1.14 ± 0.34 kg of barley grains, respectively.

[00381] Therefore, under both irrigated and non-irrigated conditions at both sites, hulless double mutant and hulless wildtype lines produced similar yields of grain. The grain yields were significantly greater than the yield from hulless *SSIIa* mutants.

Grain yield of hulled barley lines:

[00382] At Narrandera, under irrigation, the double mutant, *SSIIa* mutant and hulled wildtype lines produced 2.77 ± 0.37 kg, 2.09 ± 0.76 kg and 4.39 ± 2.59 kg of grain, respectively, per plot. Under dryland conditions, the double mutant, *SSIIa* mutant and hulled wildtype line produced 0.60 ± 0.06 kg, 0.35 ± 0.14 kg and 0.59 ± 0.46 kg of grain, respectively.

[00383] At Moree, under irrigation condition, the double mutants, *SSIIa* mutants and hulled wildtype lines produced 2.15 ± 0.81 kg, 1.24 ± 0.12 kg and 2.73 ± 0.96 kg of grain, respectively, per plot. Under dryland conditions, the double mutants, *SSIIa* mutants and hulled wildtype lines produced 1.19 ± 0.40 kg, 0.76 ± 0.60 kg and 2.13 ± 0.23 kg of grain, respectively, per plot.

[00384] Therefore, under both irrigated and non-irrigated conditions at both sites, hulled wildtype lines yielded more grain than hulled double mutant and hulled *SSIIa* mutant lines, and hulled double mutants produced more grain than hulled *SSIIa* mutants. These experiments showed the benefit of combining the *SSIIa* and *SSIIla* mutations to increase field-grown grain yield relative to the *SSIIa* mutation alone.

EXAMPLE 14. PRODUCTION OF FOOD PRODUCTS AND INGREDIENTS

[00385] Grain was harvested from eleven barley lines grown in the field at Yanco, NSW, Australia in 2008, and milled to produce flour. The lines were 3 hulless double mutants, 2 hulled double mutants, 3 *SSIIa* mutants including Himalaya292, 2 wildtype (cultivars Tantangara and Himalaya) and 1 *amo1* mutant (HAG). The grain harvested from these lines was milled using a Quadrumat Jnr. mill (Brabender Quadrumat Jnr. Mill, Cyrulla's Instruments, Sydney, NSW Australia) to produce flour that was then sieved to 300 μ m diameter. No tempering regime was applied before Quadrumat milling.

[00386] Two types of small-scale (10g) breads were baked for each of 11 barley lines. Small-scale loaves were baked for these test purposes, but the method can be readily scaled up to commercial quantities. One type of bread was made with 100% barley flour as an ingredient, milled as described above, while the other type of bread was made with a blend of 30% flour and 70% commercial wheat flour as the flour ingredient. Flour (13.02 g) and the other ingredients were mixed into a dough, to peak dough development time in a 35-g mixograph. The recipe used, based on the 13.02 g of flour in each case was: flour 100%, salt 2%, dry yeast 1.5%, vegetable oil 2%, and improver 1.5%. The water addition level was based on the micro Z-arm water absorption values that were adjusted for the full formula. The moulding and panning were done in two-stage proofing steps at 40°C and 85% room humidity. Baking was done in a Rotel oven for 14 min at 190°C.

[00387] After baking, the 10g bread loaves were stored at -80°C for three weeks for the batch of 100% barley breads, or for 1 week for the batch of 30% barley breads, and then analysed for RS content as described in Example 1, and GI levels. For the RS content, the *in vitro* procedure determined resistant starch content. Duplicated samples from the 10g bread loaves, along with appropriate standards, were mixed with artificial saliva, and the resultant bolus was incubated with pancreatic and gastric enzymes at physiological pH and temperature. The amount of residual starch in the digesta was determined using conventional enzymatic and spectrophotometric

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techniques, and the resistant starch content of the sample was expressed as a percentage of sample weight.

RS content of barley wholemeals

[00388] RS content and GI levels were first determined for the wholemeal milled from each of the groups of barley genotypes. The RS contents were 0.9%, $3.5 \pm 0.3\%$, $3.4 \pm 0.1\%$, 1.9% and $0.5 \pm 0.1\%$ for the *amo1* mutant, hulless double mutant, hulled double mutant, *SSIIa* mutant and wildtype wholemeal, respectively. Unexpectedly, both the hulless and hulled double mutant wholemeal contained approximately 3.5-, 2.3- and 10-fold higher RS content than the *amo1* mutant, *SSIIa* mutant and wildtype wholemeal, respectively (Table 4). Importantly, wholemeals from both hulless and hulled double mutants contained significantly more RS than wholemeal from the *SSIIa* mutant. There were no statistically significant differences in RS content between hulless double mutant and hulled double mutant wholemeal, or between *amo1* mutant and wildtype wholemeal. Although GI levels differed among wholemeals from 5 groups of barley, they were not statistically significantly different.

RS content of breads containing 100% barley flour

[00389] The RS contents for breads that contained 100% barley wholemeal were determined and are given in Table 5. The analyses showed that the RS contents in breads made with 100% barley wholemeal as the flour ingredient were $2.2 \pm 0.3\%$, $5.5 \pm 0.1\%$, $5.6 \pm 0.3\%$, $2.1 \pm 0.4\%$ and $0.8 \pm 0.3\%$ for *amo1* mutant, hulless double mutant, hulled double mutant, *SSIIa* mutant and wildtype grain (Table 6). The statistical analysis indicated that breads made from wholemeal from both hulless and hulled double mutant barley yielded significantly higher RS contents than that from *SSIIa* mutants, *amo1* mutant and normal barley lines (Table 6). There were no significant differences in RS content from breads containing 100% flour of hulless and hulled double mutants. The breads from hulless and hulled double mutants produced 2.5-fold, 2.5-fold and 6.7-fold higher RS content than bread made from *SSIIa* mutant, *amo1* mutant and normal barley, respectively (Table 6).

RS content of breads containing 30% barley flour

[00390] The RS contents of breads that contained 30% barley flour were determined and are given in Table 7. RS contents of breads made from *amo1* mutant,

hulless double mutant, hulled double mutant, *SSIIa* mutant and wildtype wholemeal were $1.9 \pm 0.3\%$, $3.1 \pm 0.2\%$, $3.0 \pm 0.1\%$, $2.0 \pm 0.3\%$, $0.9 \pm 0.1\%$, respectively. Breads from both hulless and hulled double mutant barley yielded significantly higher RS contents than bread from *SSIIa* mutant, *amo1* mutant and normal barley grain (Table 6). There were no significant differences in RS content between breads containing 30% flour of hulless and hulled double mutants. The breads from hulless and hulled double mutants produced 1.6-, 1.6- and 3.3-fold higher RS content than bread from *SSIIa* mutants, *amo1* mutant and normal barley lines, respectively (Table 6).

[00391] The calculation of RS content as mg RS per gram of starch was performed to analyse the nature of the increase of RS in breads made from the double mutants. These data were analysed to see whether the increase of RS content was due to the increase of total starch or due to the changes of starch structure. The results showed that bread produced with 100% barley flour from *amo1* mutant, hulless double mutant, hulled double mutant, *SSIIa* mutant and wildtype grain had 41.7, 105.1 ± 2.8 , 106.9 ± 3.3 , 75.0 ± 8.1 and 16.3 ± 4.5 mg RS per g of starch of breads (Figure 4.6). Both hulless double mutants and hulled double mutant breads yielded approximately 2.5-, 1.4- and 6.5-fold higher RS than breads from *amo1* mutant, *SSIIa* mutant and wildtype grain. The statistical analysis showed that although breads from all 4 groups of barley contained more RS than that from the wildtype lines, the RS content (mg RS per g of starch) of breads from both double mutants were statistically significantly higher than that from *amo1* mutants, *SSIIa* mutants ($P < 0.05$). Bread from *SSIIa* mutant grain contained statistically significantly more RS than that from *amo1* mutants.

GI level of 100% barley breads

[00392] The GI level of breads that contained 100% barley wholemeal from all barley lines were determined and are given in Table 5. GI levels of breads from *amo1* mutant, hulless double mutant, hulled double mutant, *SSIIa* mutant and wildtype grain were 68.5 ± 2.1 , 63.5 ± 4.5 , 60.8 ± 4.1 , 63.9 ± 10.3 , 80.3 ± 2.9 , respectively (Table 10). The statistical analysis indicated that breads from both hulless and hulled double mutant, and *SSIIa* mutant grain produced significantly lower values of GI than that from *amo1* mutant and normal barley lines (Table 10). There were no significant differences for GI values for breads containing 100% flour of hulless and hulled double mutant, and *SSIIa* mutant grain. The breads from hulless and hulled

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double mutant, and *SSIIa* mutant grain yielded approximately 80% of GI level as that from *amo1* mutant and normal barley lines, respectively (Table 10).

GI level of 30% barley breads

[00393] The GI level of breads that contained 30% barley flour from all barley lines were determined and are given in Table 7. GI levels of breads from *amo1* mutants, hullless double mutants, hulled double mutants, *SSIIa* mutants and wildtype lines were 84.5 ± 3.5 , 83.2 ± 2.1 , 83.5 ± 0.8 , 82.3 ± 3.9 , 87.8 ± 4.5 , respectively (Table 10). The GI values for breads made from the 5 groups of barley were not statistically significantly different.

Conclusions

[00394] Wholemeal from both hullless and hulled double mutant barley grain contained significantly higher RS contents than wholemeal from *amo1* mutant, *SSIIa* mutant and wildtype grain. Wholemeal from both double mutants contained approximately 3.5-, 1.8- and 7.0-fold higher RS content comparing to wholemeal from *amo1* mutants, *SSIIa* mutants and wildtype lines. In a similar pattern, bread made from the double mutant barley grain contained significantly higher RS content than bread made from *SSIIa* mutants, *amo1* mutant and wildtype barley. The increase of RS content was not only due to the increase of amount of high amylose starch, but also the changes of starch structure as the increase of RS content was observed per g of starch.

[00395] Breads from both double mutant and *SSIIa* mutant grain yielded significantly lower GI values than that from both *amo1* mutant and wildtype grain. The GI values of breads containing *SSIIa-amo1* double mutant grain and *SSIIa* mutant grain were approximately 7% and 20% lower than of breads containing *amo1* mutant and wildtype grain when the breads were made from 100% barley flour.

[00396] Many modifications will be apparent to those skilled in the art without departing from the scope of the present invention.

[00397] The disclosure of every patent, patent application, and publication cited herein is hereby incorporated herein by reference in its entirety.

Table 1. Genotypes and phenotypes of BC3F6 lines of barley

Genotype	Line No.	Seed Weight (mg)	Starch Content (%)	Amylose Content (%)	Sex6 Locus Genotype	EBmac0501 Genotype	Bmac0090 Genotype	SS/IIa Genotype	Phenotype Assignment
Wild type	HH18	60.10	62.49	28.56	Wt*	Wt	Wt	wt	Wildtype
Wild type	HH21	49.33	62.77	34.80	Wt	Wt	Wt	wt	Wildtype
Wild type	HH28	55.85	62.61	35.50	Wt	Wt	Wt	wt	Wildtype
Wild type	HH60	48.19	66.25	32.73	Wt	Wt	Wt	wt	Wildtype
Wild type	HH61	44.34	61.42	33.96	Wt	Wt	Wt	wt	Wildtype
Wild type	HH71	44.43	58.75	33.02	Wt	Wt	Wt	wt	Wildtype
Wild type	HH73	56.13	64.93	32.59	Wt	Wt	Wt	wt	Wildtype
Wild type	HH93	57.20	66.66	28.82	Wt	Wt	Wt	wt	Wildtype
Wild type	HH94	57.80	66.3	35.20	Wt	Wt	Wt	wt	Wildtype
Wild type	HH103	51.82	65.9	30.07	Wt	Wt	Wt	wt	Wildtype
Wild type	HH109	49.59	64.5	29.31	Wt	Wt	Wt	wt	Wildtype
Wild type	HH110	48.72	63.72	29.04	Wt	Wt	Wt	wt	Wildtype
Wild type	HH126	53.88	60.64	35.93	Wt	Wt	Wt	wt	Wildtype
amo1	HH17	55.52	53.81	50.15	Wt	amo1	amo1	amo1	amo1
amo1	HH30	53.59	58.01	48.40	Wt	amo1	amo1	amo1	amo1
amo1	HH56	45.99	54.97	51.23	Wt	amo1	amo1	amo1	amo1
amo1	HH63	51.60	54.24	48.26	Wt	amo1	amo1	amo1	amo1
amo1	HH79	53.47	57.96	51.32	Wt	amo1	amo1	amo1	amo1
amo1	HH81	54.38	60.5	51.75	Wt	amo1	amo1	amo1	amo1
amo1	HH82	54.80	60.79	52.86	Wt	amo1	amo1	amo1	amo1
amo1	HH125	53.22	55	44.00	Wt	amo1	amo1	amo1	amo1

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Genotype	Line No.	Seed Weight (mg)	Starch Content (%)	Anylose Content (%)	Sex6 Locus Genotype	EBmac0501 Genotype	Bmac0090 Genotype	SSIIa Genotype	Phenotype Assignment
amo1	HH130	52.56	59.5	46.00	Wt	amo1	amo1	amo1	amo1
SSIIa M	HH2	33.68	36.27	53.30	SSIIa M	Wt	Wt	wt	SSIIa M
SSIIa M	HH35	34.85	35.48	70.63	SSIIa M	Wt	Wt	wt	SSIIa M
SSIIa M	HH39	36.98	35.86	68.90	SSIIa M	Wt	Wt	wt	SSIIa M
SSIIa M	HH42	36.07	33.02	59.66	SSIIa M	Wt	Wt	wt	SSIIa M
SSIIa M	HH43	33.22	33.27	43.51	SSIIa M	Wt	Wt	wt	SSIIa M
SSIIa M	HH48	36.35	33.71	56.77	SSIIa M	Wt	Wt	wt	SSIIa M
SSIIa M	HH50	40.88	32.42	46.09	SSIIa M	Wt	Wt	wt	SSIIa M
SSIIa M	HH51	38.72	31.22	47.56	SSIIa M	Wt	Wt	wt	SSIIa M
SSIIa M	HH69	40.14	32	52.86	SSIIa M	Wt	Wt	wt	SSIIa M
SSIIa M	HH83	41.20	35.71	79.90	SSIIa M	Wt	Wt	wt	SSIIa M
SSIIa M	HH84	39.57	32.18	63.53	SSIIa M	Wt	Wt	wt	SSIIa M
SSIIa M	HH97	38.27	33.77	60.56	SSIIa M	Wt	Wt	wt	SSIIa M
SSIIa M	HH127	40.73	34.22	49.29	SSIIa M	Wt	Wt	wt	SSIIa M
SSIIa-amo1	HH4	43.79	53.42	57.21	SSIIa M	amo1	amo1	amo1	SSIIa-amo1
SSIIa-amo1	HH6	46.17	54.06	60.57	SSIIa M	amo1	amo1	amo1	SSIIa-amo1
SSIIa-amo1	HH7	44.20	48.83	66.27	SSIIa M	amo1	amo1	amo1	SSIIa-amo1
SSIIa-amo1	HH19	52.21	43.88	59.03	SSIIa M	amo1	amo1	amo1	SSIIa-amo1
SSIIa-amo1	HH29	44.52	48.25	64.53	SSIIa M	amo1	amo1	amo1	SSIIa-amo1

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Genotype	Line No.	Seed Weight (mg)	Starch Content (%)	Amylose Content (%)	Sex6 Locus Genotype	EBmac0501 Genotype	Bmac0090 Genotype	SSIIa Genotype	Phenotype Assignment
SSIIa-amo1	HH133	49.34	49.85	55.78	SSIIa M	amo1	amo1	amo1	SSIIa-amo1
SSIIa-amo1	HH174	43.39	46.3	63.84	SSIIa M	amo1	amo1	amo1	SSIIa-amo1
SSIIa-amo1	HH188	54.22	48.4	53.82	SSIIa M	amo1	amo1	amo1	SSIIa-amo1
SSIIa-amo1	HH198	48.13	48.43	61.11	SSIIa M	amo1	amo1	amo1	SSIIa-amo1
SSIIa-amo1	HH199	47.62	49.81	61.99	SSIIa M	amo1	amo1	amo1	SSIIa-amo1
SSIIa-amo1	HH122	49.04	47.03	59.68	SSIIa M	amo1	amo1	amo1	SSIIa-amo1
SSIIa-amo1	HH140	39.04	46.84	61.24	SSIIa M	amo1	amo1	amo1	SSIIa-amo1
SSIIa-amo1	HH177	35.54	48.2	68.24	SSIIa M	amo1	amo1	amo1	SSIIa-amo1
amo1 Recombinant	HH116	48.11	50.39	52.94	SSIIa M	Wt	amo1	amo1	SSIIa-amo1
amo1 Recombinant	HH146	39.05	35.59	52.20	SSIIa M	amo1	wt	wt	SSIIa-
amo1 Recombinant	HH23	56.33	54.05	46.67	wt	Wt	wt	amo1	amo1
amo1 Recombinant	HH24	52.62	63.11	38.69	wt	amo1	amo1	wt	Wild type
amo1 Recombinant	HH118	49.68	65.65	38.00	wt	Wt	amo1	wt	Wild type

*wt: wildtype.

SSIIa M: SSIIa mutation.

Table 2. Grain constituents of barley genotypes on a per seed basis

	Starch (mg)	Amylose (mg)	Amylopectin (mg)	Protein
Wildtype	34.1 ± 3.9 ^a	10.7 ± 1.5 ^b	23.4 ± 2.8 ^a	5.4 ± 0.7 ^b
<i>amol</i>	30.2 ± 2.5 ^b	15.0 ± 1.7 ^a	15.3 ± 1.3 ^b	5.5 ± 0.6 ^b
<i>SSIIa M</i>	13.2 ± 1.7 ^d	7.7 ± 2.1 ^c	5.5 ± 1.2 ^d	4.9 ± 0.4 ^c
<i>SSIIa-amol</i>	22.7 ± 1.9 ^c	13.8 ± 1.1 ^a	8.9 ± 1.2 ^c	6.5 ± 0.5 ^a
LSD P<0.05)	2.4	1.5	1.7	0.5
	Lipid (mg)	α-glucan (mg)	Pentosan (mg)	WSC (mg)
Wildtype	1.6 ± 0.2 ^c	3.2 ± 0.5 ^b	2.6 ± 0.5 ^a	1.4 ± 0.2 ^b
<i>amol</i>	1.9 ± 0.2 ^b	4.3 ± 0.3 ^a	2.6 ± 0.6 ^a	2.2 ± 0.1 ^b
<i>SSIIa M</i>	2.5 ± 0.3 ^a	3.0 ± 0.4 ^b	2.8 ± 0.4 ^a	3.8 ± 1.3 ^a
<i>SSIIa-amol</i>	2.3 ± 0.2 ^a	3.1 ± 0.3 ^b	2.7 ± 0.5 ^a	3.3 ± 0.4 ^a
LSD P<0.05)	0.2	0.3	0.5	0.9

All data are from the BC3F6 population and expressed on a mg per seed basis

Data represent the mean of samples assayed ±SD.

Data marked with the same letter are not significantly different at p=0.05

Table 3. Intron and exon structure of barley *SSIIIa* gene and SNPs among barley *SSIIIa* genes from HAG, Glacier, Himalaya292 and Himalaya

Nucleotide No.	Exon No.	Intron No.	Size of exon or intron in barley	Nucleotide No.	SNP in HAG, Glacier genomic DNA	SNP in Himalaya-292, Himalayagenomic DNA
1 to 90	1		90			
91 to 1019	1	1	929	330	T	C
1020 to 1083	2		64			
1084 to 1171	2	2	88			
1172 to 3829	3		2658	2101 2693 3273	G (V)* T C	A (M) C T
3830 to 4026		3	197			
4027 to 4244	4		218			
4245 to 4591		4	347			
4592 to 4862	5		271			
4863 to 5723		5	861	5610	G	A
5724 to 5899	6		176			
5900 to 6012	6		113			
6013 to 6120	7		108			
6121 to 6379		7	259	6323	C (EcoRI)#	T
6380 to 6489	8		110			
6490 to 7151		8	662			
7152 to 7254	9		103			
7255 to 7564		9	310			

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Nucleotide No.	Exon No.	Intron No.	Size of exon or intron in barley	Nucleotide No.	SNP in HAG, Glacier genomic DNA	SNP in Himalaya-292, Himalayagenomic DNA
7565 to 7735	10		171			
7736 to 7828		10	93			
7829 to 7957	11		129			
7958 to 8042		11	85			
8043 to 8225	12		183			
8226 to 8344		12	119	8338	A	T
8345 to 8476	13		132			
8477 to 8575		13	99			
8576 to 8687	14		112	8602	T (L) for Glacier G (R) for HAG	T (L)
8688 to 8777		14	90			
8778 to 8906	15		129			
8907 to 8986		15	80			
8987 to 9498	16		128			

* Letters in the brackets are amino acid residues encoded. V: Valine; M: Methionine; L: Leucine; R: Arginine.

DNA sequences at nucleotide 6323 from HAG and Glacier can be digested by restriction enzyme, *EcoRI*

Table 4. RS content and GI level of barley wholemeal

Genotype	Line name	GI	GI average	SD	RS wholemeal (g/100g)	RS average	SD
<i>amol</i> mutant	HAG	64.6	64.6 ^a		0.9	0.9 ^c	
Hulled double mutant	HH88_F7	81.0	79.0 ^a	2.8	3.5	3.4 ^a	0.1
Hulled double mutant	HH122_F7	77.0			3.3		
Hulless double mutant	HH4_F7	77.3	78.0 ^a	0.9	3.3	3.5 ^a	0.3
Hulless double mutant	HH29_F7	79.1			3.4		
Hulless double mutant	HH29_F7-	77.8			3.8		
<i>SSIIa</i> mutant	292	68.2	68.2 ^a		1.9	1.9 ^b	
wildtype barley	Himalaya	76.6	70.8 ^a	9.6	0.6	0.5 ^c	0.1
wildtype barley	Glacier	76.0			0.4		
wildtype barley	Tantangara	59.7			0.4		
LSD (5%)			22.6			0.7	

L.S.D.: it is the least significant difference; differences greater than this are significant ($P < 0.05$).

a, b and c: based on LSD, mean values with the same letter are not significantly different, and with the different letter are significantly different at significant difference ($P < 0.05$).

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Table 5. RS content and GI level of bread produced using 100% barley wholemeal

Sample ID	Genotype	Line name	RS content (g/100 g)	GI level
ZL2.9.1	<i>amoi</i> mutant	HAG	2.39	70
ZL2.9.1	<i>amoi</i> mutant	HAG	2.01	67
10.1	Hulless double mutant	HH29_F7	5.46	57
10.1	Hulless double mutant	HH29_F7	5.6	62
6.1	Hulless double mutant	HH4_F7	5.53	66
6.1	Hulless double mutant	HH4_F7	5.32	61
2.1	Hulless double mutant	HH7_F7	5.61	70
2.1	Hulless double mutant	HH7_F7	5.65	65
11.1	hulled double mutant	HH88_F7	5.76	66
11.1	hulled double mutant	HH88_F7	5.96	62
1.1	hulled double mutant	HH122_F7	5.29	57
1.1	hulled double mutant	HH122_F7	5.2	58
ZL1.8.1	<i>SSIIa</i> mutant	871	1.96	49
ZL1.8.1	<i>SSIIa</i> mutant	871	2.08	53
ZL1.1.1	<i>SSIIa</i> mutant	Himalaya292	1.49	57
ZL1.1.1	<i>SSIIa</i> mutant	Himalaya292	1.58	60
4.1	<i>SSIIa</i> mutant	HH50_F7	2.22	74
4.1	<i>SSIIa</i> mutant	HH50_F7	2.26	74
3.1	wildtype	Himalaya	0.65	82
3.1	wildtype	Himalaya	0.52	82
9.1	wildtype	Tantangara	1.04	76
9.1	wildtype	Tantangara	1.05	81

Table 6. Statistical analysis of the effects of genotype on RS contents of bread produced with 30% or 100% barley flour

Genotype	No Sample	RS content 100% (g/100g)	SD	RS content 30% (g/100g)	SD
<i>amo1</i> mutant	2	2.2 ^b	0.3	1.9 ^b	0.3
hulless double mutant	6	5.5 ^a	0.1	3.1 ^a	0.2
hulled double mutant	4	5.4 ^a	0.4	3.0 ^a	0.1
<i>SSIIa</i> mutant	6	1.9 ^b	0.4	1.8 ^b	0.3
Wildtype	4	0.7 ^c	0.3	1.0 ^c	0.1
L.S.D. (P<0.05)		0.5		0.3	

Note:

RS content 30%: RS content of the breads that contained 30% barley flour.

RS content 100%: RS content of the breads that contained 100% barley flour.

L.S.D.: it is the least significant difference; differences greater than this are significant (P < 0.05).

a, b and c: based on LSD, mean values with the same letter are not significantly different, and with the different letter are significantly different at significant difference (P < 0.05).

Table 7. RS content and GI level of breads produced with 30% barley flour

Sample ID	Genotype	Line name	RS (g/100 g)	GI level
ZL1.4.1	<i>amoi</i> mutant	HAG	2.15	82
ZL1.4.1	<i>amoi</i> mutant	HAG	1.72	87
ZL1.5.1	Hulless double mutant	HH29_F7	3.01	83
ZL1.5.1	Hulless double mutant	HH29_F7	3.07	82
ZL2.7.1	Hulless double mutant	HH4_F7	3.03	83
ZL2.7.1	Hulless double mutant	HH4_F7	3.27	83
ZL2.6.1	Hulless double mutant	HH7_F7	3.15	84
ZL2.6.1	Hulless double mutant	HH7_F7	3.03	84
ZL1.3.1	Hulled double mutant	HH88_F7	3.04	83
ZL1.3.1	Hulled double mutant	HH88_F7	3.09	86
ZL1.10.1	Hulled double mutant	HH122_F7	3.01	81
ZL1.10.1	Hulled double mutant	HH122_F7	2.71	84
ZL2.1.1	<i>SSIIa</i> mutant	871	1.87	77
ZL2.1.1	<i>SSIIa</i> mutant	871	1.82	81
ZL2.3.1	<i>SSIIa</i> mutant	Himalaya292	1.7	80
ZL2.3.1	<i>SSIIa</i> mutant	Himalaya292	1.87	82
ZL2.2.1	<i>SSIIa</i> mutant	HH50_F7	2.15	86
ZL2.2.1	<i>SSIIa</i> mutant	HH50_F7	1.69	84
ZL1.2.1	Wildtype	Himalaya	0.96	93
ZL1.2.1	Wildtype	Himalaya	1.02	94
ZL2.8.1	Wildtype	Tantangara	0.84	83
ZL2.8.1	Wildtype	Tantangara	0.89	85

Table 8. Statistical analysis of the effects of genotype on RS content (mg RS per g starch) of bread produced with 100% barley flour

Genotype	No of samples	RS content 100% (g/100g)	SD	Total starch in breads (%)	SD	mg RS per g starch	SD
<i>amol</i> mutant	1	2.2 ^b	0.3	57.4 ^a	-	41.7 ^o	
hulless double mutant	3	5.5 ^a	0.1	52.7 ^a	1.8	105.1 ^a	2.8
hulled double mutant	2	5.4 ^a	0.4	51.8 ^a	4.7	106.9 ^a	3.3
<i>SSIIa</i> mutant	3	1.9 ^b	0.4	25.1 ^b	3.0	75.0 ^b	8.1
Wildtype	2	0.7 ^c	0.3	51.6 ^a	2.6	16.3 ^d	4.5
L.S.D. (P<0.05)		0.5		8.9		16.3	

Table 9. Statistical analysis of the effects of genotype on RS content (mg RS per g starch) of breads produced with 30% barley flour

Genotype	No Sample	RS content 30% (g/100g)	SD	Total starch in breads (%)	SD	mg RS per g starch	SD
<i>amol</i> mutant	1	1.9 ^b	0.3	61.9 ^a	-	30.7 ^b	
hulless double mutant	3	3.1 ^a	0.2	65.3 ^a	2.2	47.5 ^a	0.8
hulled double mutant	2	3.0 ^a	0.1	63.7 ^a	1.1	47.1 ^a	3.0
<i>SSIIa</i> mutant	3	1.8 ^b	0.3	58.0 ^b	2.3	31.6 ^b	0.3
Wildtype	2	1.0 ^c	0.1	65.1 ^a	0.1	14.6 ^c	1.1
L.S.D. (P<0.05)		0.3		5.6		4.2	

Table 10. Statistical analysis of the effects of genotype on GI level of the 10g breads produced with 30% or 100% barley flour

Genotype	No Sample	GI level 100%	SD	GI level 30%	SD
<i>am1</i> mutant	2	68.5 ^a	2.1	84.5 ^a	3.5
hulless double mutant	6	63.5 ^b	4.5	83.2 ^a	2.1
hulled double mutant	4	60.8 ^b	4.1	83.5 ^a	0.8
<i>SSIIa</i> mutant	6	63.9 ^b	10.3	82.3 ^a	3.9
Wildtype	4	80.3 ^a	2.9	87.8 ^a	4.5
L.S.D. (P<0.05)		12.1		5.8	

GI level 30%: GI level of the breads that contained 30% barley flour.

GI level 100%: GI level of the breads that contained 100% barley flour.

L.S.D.: it is the least significant difference; differences greater than this are significant ($P < 0.05$).

a and b: based on LSD, mean values with the same letter are not significantly different, and with the different letter are significantly different at significant difference ($P < 0.05$).

Table 11. Summary of sequence identifiers

SEQUENCE ID NO.	DESCRIPTION
1	Barley <i>ssIIa</i> gene from Himalaya, genomic sequence beginning from the translation start codon
2	Barley <i>SSIIa</i> cDNA from a wildtype barley cv. Himalaya, beginning at the translation initiation codon
3	Barley <i>SSIIa</i> cDNA from an <i>am1</i> mutant (High Amylose Glacier, <i>am1-38</i> allele), beginning at translation initiation codon
4	Barley <i>SSIIa</i> amino acid sequence from a wildtype barley cv. Himalaya
5	Barley <i>SSIIa</i> amino acid sequence from an <i>am1</i> mutant (High Amylose Glacier, <i>am1-38</i> allele)
6	Wheat <i>SSIIa</i> cDNA (GenBank Accession No: AF258608)
7	Wheat <i>SSIIb</i> cDNA (GenBank Accession No:)

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SEQUENCE ID NO.	DESCRIPTION
	EU333946.2)
8	Wheat SSIIa amino acid sequence (GenBank Accession No: AF258608)
9	Wheat SSIIb amino acid sequence (GenBank Accession No: EU333946.2)
10	Primer SSIIaF
11	Primer SSIIaR
12	Primer HHac0501F
13	Primer HHac0501R
14	Oligonucleotide primers SSIIaF and SSIIa-P5F
15	Oligonucleotide primer SSIIaR
16	Primer ZLSSIIa-P1F
17	Primer ZLSSIIa-P1R
18	Primer ZLSSIIa-P2F
19	Primer ZLSSIIa-P2R
20	Primer ZLSSIIa-P3F
21	Primer ZLSSIIa-P3R
22	Primers ZLSSIIa-P1F and ZLSSIIa-P4R
23	Primer SSIIa-P5R
24	Primer SSIIa-F
25	Primer SSIIa-R
26	Primer SSIIb-F
27	Primer SSIIb-R
28	Primer ZLhph253
29	Primer ZLhph915
30	PCR primer Bx17-3'
31	PCR primer ZLSSIIa-IR_RM
32	Wildtype Himalaya SSIIa cDNA

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SEQUENCE ID NO:	DESCRIPTION
33	Himalaya292 SSIIa cDNA
34	SSIII conserved amino acid motif
35	SSIII conserved amino acid motif
36	SSIII conserved amino acid motif
37	SSIII conserved amino acid motif
38	SSIII conserved amino acid motif
39	SSIII conserved amino acid motif
40	SSIII conserved amino acid motif
41	SSIII conserved amino acid motif

TABLE 12. Amino acid sub-classification

Sub-class	Amino acids
Acidic	Aspartic acid, Glutamic acid
Basic	Noncyclic: Arginine, Lysine; Cyclic: Histidine
Charged	Aspartic acid, Glutamic acid, Arginine, Lysine, Histidine
Small	Glycine, Serine, Alanine, Threonine, Proline
Polar/neutral	Asparagine, Histidine, Glutamine, Cysteine, Serine, Threonine
Polar/large	Asparagine, Glutamine
Hydrophobic	Tyrosine, Valine, Isoleucine, Leucine, Methionine, Phenylalanine, Tryptophan
Aromatic	Tryptophan, Tyrosine, Phenylalanine
Residues that influence chain orientation	Glycine and Proline

TABLE 13. Exemplary and Preferred Amino Acid Substitutions

Original amino acid	Exemplary amino acid substitution	Preferred amino acid substitution
Ala	Val, Leu, Ile	Val
Arg	Lys, Gln, Asn	Lys
Asn	Gln, His, Lys, Arg	Gln
Asp	Glu	Glu
Cys	Ser	Ser

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Protein	Peptide Sequences	Peptide Sequences
Gln	Asn, His, Lys,	Asn
Glu	Asp, Lys	Asp
Gly	Pro	Pro
His	Asn, Gln, Lys, Arg	Arg
Ile	Leu, Val, Met, Ala, Phe, Norleu	Leu
Leu	Norleu, Ile, Val, Met, Ala, Phe	Ile
Lys	Arg, Gln, Asn	Arg
Met	Leu, Ile, Phe	Leu
Phe	Leu, Val, Ile, Ala	Leu
Pro	Gly	Gly
Ser	Thr	Thr
Thr	Ser	Ser
Trp	Tyr	Tyr
Tyr	Trp, Phe, Thr, Ser	Phe
Val	Ile, Leu, Met, Phe, Ala, Norleu	Leu

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CLAIMS:

1. Barley grain comprising:
 - (a) starch,
 - (b) a first genetic variation which is
 - (i) an induced mutation in an endogenous gene encoding a starch synthase III (SSIII), or
 - (ii) a transgene which encodes an RNA molecule which reduces expression of an endogenous gene encoding an SSIII, and
 - (c) a reduced level or activity of SSIII protein relative to the level or activity in barley grain lacking the first genetic variation.
2. The barley grain of claim 1 which comprises an allele of *SSIIIa* other than an allele comprising the nucleic acid sequence of SEQ ID NO: 3.
3. The barley grain of claim 1 or claim 2 further comprising a second genetic variation in a gene encoding a protein involved in starch synthesis, starch catabolism, starch phosphorylation or non-starch carbohydrate synthesis.
4. The barley grain of claim 3, wherein the second genetic variation is
 - (i) a mutation in a gene encoding a starch synthase II (SSII), preferably a starch synthase IIa (SSIIa), or
 - (ii) a transgene which encodes an RNA molecule which inhibits expression of a gene encoding an SSII, preferably an SSIIa.
5. The barley grain of claim 3 or claim 4 which comprises (i) an allele of *SSIIIa* other than an allele comprising the nucleic acid sequence of SEQ ID NO: 3 and (ii) an allele of *SSIIa* other than an allele comprising the nucleic acid sequence of SEQ ID NO: 33.

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6. The barley grain of claim 5 wherein the grain lacks the allele of *SSIIIa* comprising the nucleic acid sequence of SEQ ID NO: 3 and lacks the allele of *SSIIa* comprising the nucleic acid sequence of SEQ ID NO: 33.
7. Barley grain comprising:
 - (a) starch,
 - (b) a first genetic variation which is
 - (i) a mutation in a gene encoding an *SSIII*, or
 - (ii) a transgene which encodes an RNA molecule which inhibits expression of a gene encoding an *SSIII*, and
 - (c) a reduced level or activity of *SSIII* protein relative to the level or activity in barley grain lacking the first genetic variation,
 - (d) a second genetic variation which is
 - (i) a mutation in a gene encoding an *SSII*, or
 - (ii) a transgene which encodes a nucleic acid molecule which inhibits expression of a gene encoding an *SSII*,
 - (e) a reduced level or activity of *SSII* protein relative to the level or activity in barley grain lacking the second genetic variation, andwherein the grain comprises an allele of *SSIIIa* other than an allele comprising the nucleic acid sequence of SEQ ID NO: 3.
8. The barley grain of claim 7, wherein the *SSIII* is *SSIIIa* and/or the *SSII* is *SSIIa*.
9. The barley grain of any one of claims 1 to 8 whose starch content is at least 43% (w/w), at least 45% (w/w), at least 47% (w/w), at least 50% (w/w), or 41-65% (w/w).

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10. The barley grain of any one of claims 1 to 9, whose amylose content is at least 40%, at least 50% or at least 60% as a proportion of the total starch in the grain.
11. The barley grain of any one of claims 1 to 10, whose β -glucan content is 5-9% (w/w), or greater than 9% (w/w).
12. The barley grain of to any one of claims 1 to 11, whose fructan content is 2-11%, 3-11%, or 4-11% (w/w).
13. The barley grain of claim 12 wherein the fructan comprises a degree of polymerization from about 3 to about 12.
14. The barley grain of any one of claims 1 to 13 comprising an elevated protein content relative to wild-type barley, or relative to HAG.
15. The barley grain of any one of claims 1 to 14 which is homozygous for the *sex6-292* allele of *SSIIa*.
16. The barley grain of any one of claims 1 to 15 wherein the *SSIII* gene encodes an SSIII protein comprising an amino acid sequence having at least 98% sequence identity to SEQ ID NO: 4.
17. The barley grain of any one of claims 1 to 16 which is wholegrain or cracked, ground, polished, milled, kibbled, rolled or pearled grain.
18. A barley plant capable of producing the grain according to any one of claims 1 to 17.
19. Barley wholemeal or flour produced from the grain of any one of claims 1 to 17.

20. A process for producing a food ingredient or beverage ingredient, wherein the process comprises:
- (i) obtaining or producing barley grain according to any one of claims 1 to 17; and
 - (ii) processing the grain to produce the ingredient.
21. The process of claim 20, wherein the ingredient is wholemeal, flour, starch, bran, β -glucan, fructan, non-starch polysaccharides, a baking ingredient, a baking mix, a thickening agent, or cracked, ground, polished, milled, kibbled, rolled or pearled grain, or any combination thereof.
22. A process for producing a food or beverage product, wherein the process comprises:
- (i) obtaining or producing an ingredient produced according to the process of claim 20 or claim 21; and
 - (ii) mixing the ingredient with another food ingredient or beverage ingredient to produce the product.
23. The process of claim 22, wherein the ingredient is used as a bulking agent, a dietary fibre, a texturizing agent, a preservative, a sweetener, a thickening agent, a probiotic agent or any combination of these, in the food or beverage product.
24. The process according to any one of claims 20 to 23 further comprising assessing the level or type of starch or starch synthase activity, starch content, amylose, amylopectin, β -glucan, fructan, non-starch polysaccharides, dietary fibre, resistant starch in the barley grain or the ingredient or the product produced therefrom.

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25. The process according to any one of claims 22 to 24 wherein the food product is a breakfast cereal, biscuit, muffin, muesli bar, noodle, bread, cake, pancake, tortilla, buns, pastry, crackers, pizza, croissants, bagels, pretzels, pasta, soup, sauce, confectionary, and other farinaceous goods,.
26. A process for producing a barley plant capable of producing grain according to any one of claims 1 to 17, wherein the process comprises: (i) introducing into a barley plant a nucleic acid which down-regulates the level or activity of an SSIII in the plant, or introducing into a barley plant a mutation in an endogenous gene encoding SSIII in the plant, and (ii) selecting a barley plant from step (i) which is capable of producing said grain.
27. The process of claim 26 wherein the SSIII gene of the barley plant encodes a protein having at least 98% sequence identity to SEQ ID NO: 4.
28. The process according to claim 26 or claim 27 wherein the process further comprises assessing the level, activity and/or type of starch, starch content, the level or activity of SSIIIa and/or a non-SSIII starch synthase such as SSI and/or GBSSI, amylose, amylopectin, β -glucan, fructan, non-starch polysaccharide, dietary fibre or resistant starch in the barley grain or a product therefrom, or analysing the plant with one or more genetic markers.
29. The process of any one of claims 26 to 28 wherein the level or activity of SSIII protein is less than 25%, less than 10%, less than 5%, or is essentially lacking relative to that of a control plant or the plant prior to the introduction of the nucleic acid or mutation.
30. A process for producing the barley grain of claims 1 to 17, comprising the steps of growing a barley plant and harvesting the grain.

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31. The process of claim 30 comprising processing the grain to produce grain that is cracked, ground, polished, milled, kibbled, rolled or pearled.
32. Barley grain according to any one of claims 1 to 17, or an ingredient produced according to the process of claims 20 or 21, wholemeal or flour therefrom, when used or for use in the production of a product to increase the level of starch, amylose, amylopectin, resistant starch, dietary fibre, protein, lipid, water soluble carbohydrate, β -glucan, fructan or non-starch carbohydrate in said product or to decrease the glycemic index (GI) of said product.
33. Use of a grain according to any one of claims 1 to 17, or an ingredient produced according to the process of claims 20 or 21 such as flour, wholemeal, starch, β -glucan or fructan isolated from a plant, grain, wholemeal or flour in the production of a food product.
34. A food product comprising a food ingredient at a level of at least 10% on a dry weight basis, wherein the food ingredient is a barley grain according to any one of claims 1 to 17 or an ingredient produced according to the process of claims 20 or 21 such as wholemeal or flour.
35. The food product claim 34 wherein the product is selected from the group consisting of bread, buns, breakfast cereal, cake, biscuit, pastry, crackers, muffins, pizza, croissants, bagels, pretzels, pasta, noodles, soup, sauce, confectionary and other farinaceous goods.
36. The product of claim 34 or 35 wherein the barley grain comprises the *sex6-292* allele.

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37. A process for preparing a food or beverage, comprising mixing the barley grain of any one of claims 1 to 17 or the ingredient produced according to the process of claims 20 or 21 such as barley wholemeal or flour of claim 19 with another food or beverage ingredient.
38. A process for providing starch, amylose, amylopectin, β -glucan, fructan, non-starch polysaccharide, dietary fibre or resistant starch to improve one or more indicators of health in a mammal, wherein the process comprises administering, to the mammal, a composition comprising barley grain of any one of claims 1 to 17, or wholemeal or flour therefrom or the food product or ingredient according to any one of claims 34 to 36.
39. The process of claim 38 wherein the grain, flour, starch, amylose, amylopectin, β -glucan, fructan, non-starch polysaccharide, dietary fibre or resistant starch is in the form of a food product, a beverage or a pharmaceutical composition.
40. The process of claim 37 or 38, wherein the one or more indicators of health is an increased number of beneficial intestinal bacteria, reduced number of aberrant crypt foci, increased mineral absorption, reduced level of insulin, reduced glycaemic index, reduced glycaemic load, reduced blood glucose, reduced blood pressure, reduced body weight, reduced blood cholesterol level, increased HDL cholesterol level, increased bone density, increased calcium levels, more frequent bowel movement, or improved blood serum cardiovascular profile.
41. A process for ameliorating one or more symptoms of a condition associated with low levels of dietary starch, amylose, amylopectin, β -glucan, fructan, non-starch polysaccharide, dietary fibre or resistant starch in a subject, said process comprising administering orally to the subject grain according to any one of claims 1 to 17 or a processed product or ingredient comprising starch, starch content, amylose,

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amylopectin, β -glucan, fructan, non-starch polysaccharide, dietary fibre or resistant starch obtained therefrom for a time and under conditions sufficient to ameliorate one or more symptoms.

42. The process of claim 41 wherein the condition is selected from the group consisting of diabetes, obesity, heart disease, hypertension, constipation, osteoporosis and cancer.
43. A process for analyzing a barley plant or a part thereof, comprising the steps of:
 - (i) analysing DNA, RNA, protein, starch granules, starch or grain obtained from the barley plant or part thereof, and
 - (ii) determining from step (i) whether the barley plant or part thereof comprises an allele of *SSIIIa* which comprises the nucleic acid sequence of SEQ ID NO: 3 or which comprises a nucleic acid sequence other than SEQ ID NO: 3.
44. The process of claim 43, wherein the barley plant or part thereof is a member of a population of barley plants or parts thereof.

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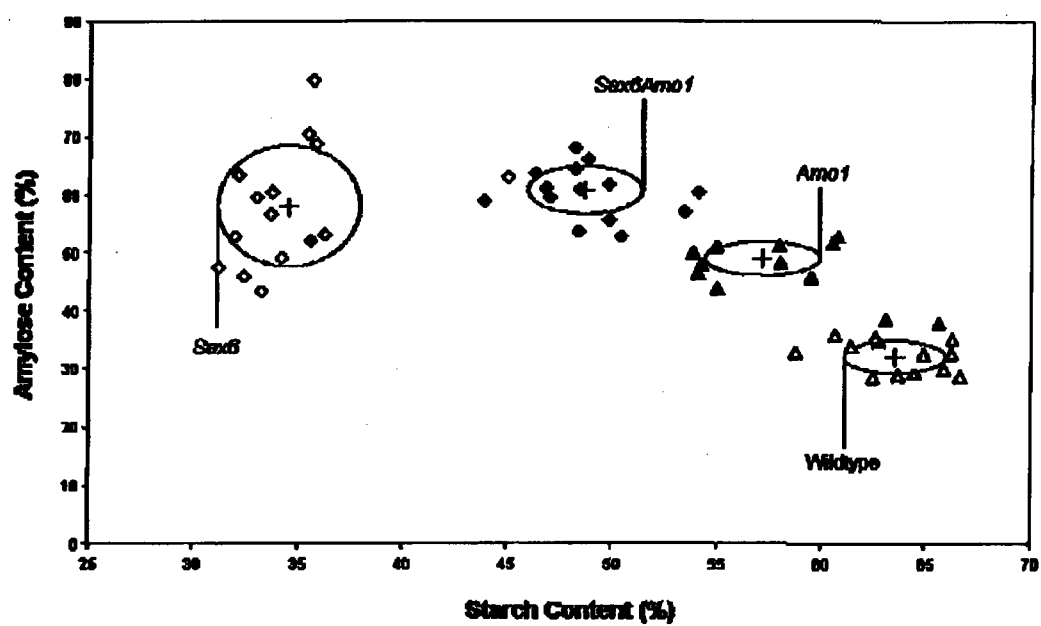
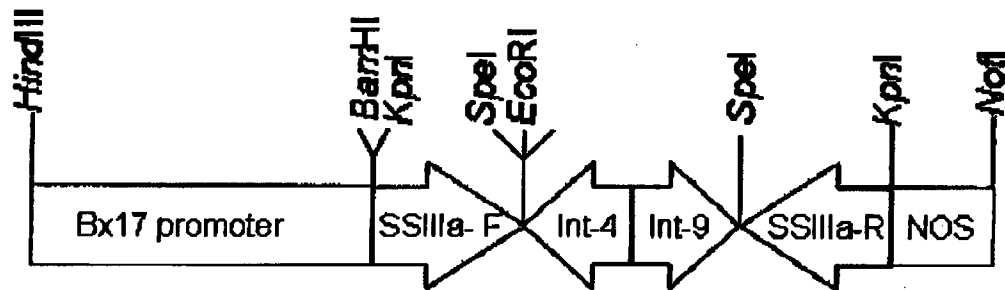


FIGURE 1

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(A)



(B)

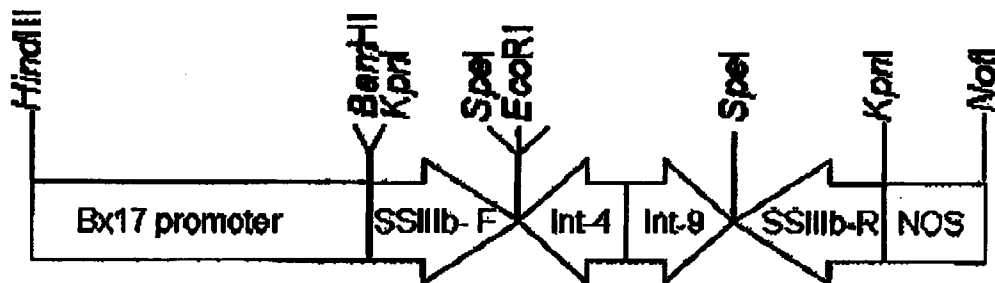


FIGURE 2

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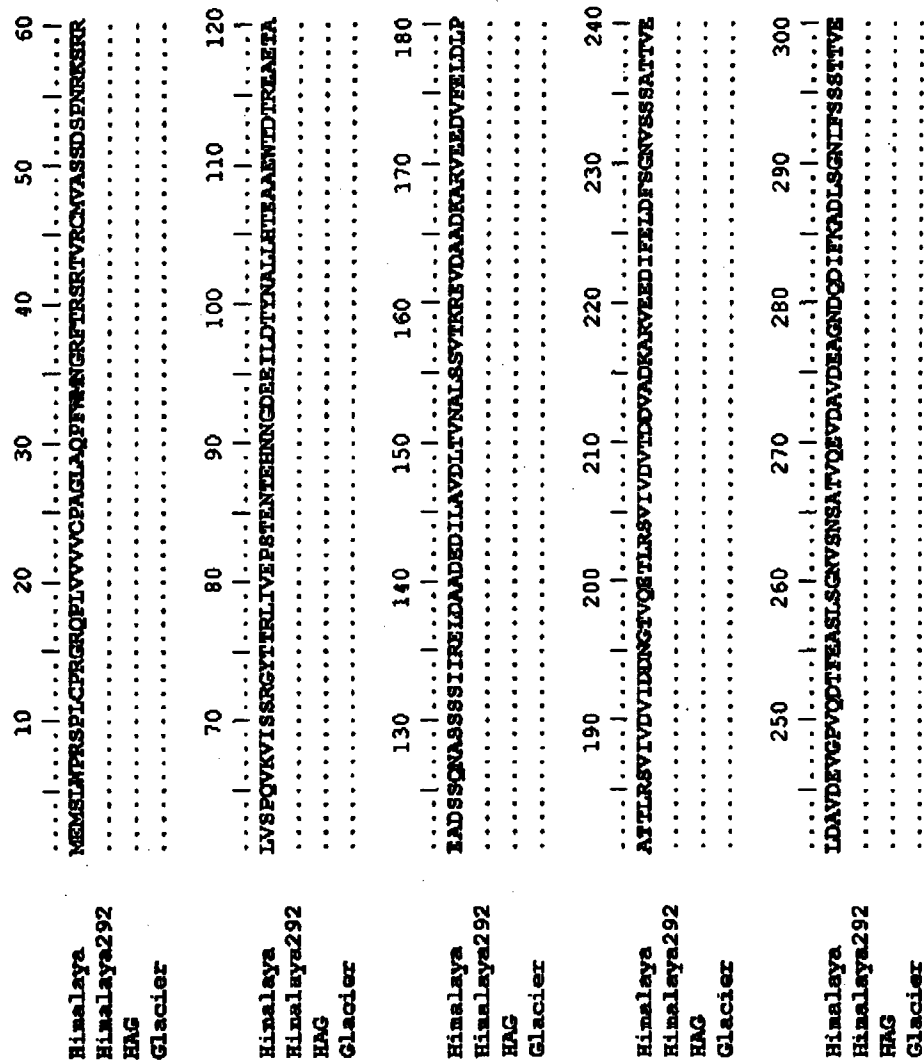


FIGURE 3

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Himalaya	310	320	330	340	350	360
Himalaya292						
HAG	VCWDLAGSTKDRFMDSSGNVTSATWDAIDAVADQDAVADLSGNASSWATYRELD					
Glacier						
Himalaya	370	380	390	400	410	420
Himalaya292						
HAG	DNVDNRSEETFWDLVCEADDEENYQQYFVPSSFSMDKALAKTGVSLNPSPLRITS					
Glacier						
Himalaya	430	440	450	460	470	480
Himalaya292						
HAG	VQEQKYNFTSDKQDLSIADLPQNSIVGSKCKDKSLADVAGPPQSIFGSSQHRPIVAF					
Glacier						
Himalaya	490	500	510	520	530	540
Himalaya292						
HAG	RKQNHSTVEDPQKQSIWGFBSQDLSAVDLFNQNIPIVGTSSQGTQKQPVVDRQDALYV					
Glacier						
Himalaya	550	560	570	580	590	600
Himalaya292						
HAG	NGLEFLKIGDHRSEKTEDEALAVFNIDNVLQKHLADRTQAVETTIWEKVEDEHLINTEHQ					
Glacier						

FIGURE 3 (CONTINUED)

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	610	620	630	640	650	660
Himalaya						
Himalaya292	IGSTECHEMVLNDELSTITEICAGSGDKIQHALSEELSWSEDFVQINKDDGQYEVDEISA					
HAG						
Glacier						
	670	680	690	700	710	720
Himalaya						
Himalaya292	SFTVLEQDIQGPDPDWDVDPQALRAMLOELADKNYSNNKLFVFPFVVKADSVIDLXLNRDL					
HAG						
Glacier						
	730	740	750	760	770	780
Himalaya						
Himalaya292	TALANEFDWVIRKGAENGKMKLFSERLHRSDLGGVWNSCKLHIPKEAYRLDFVFNCRIV					
HAG						
Glacier						
	790	800	810	820	830	840
Himalaya						
Himalaya292	YENNGNDFCIGTGTNEDLFEDFLVKNQRELEKLAMEALERSQTDEQSRKKEARA					
HAG						
Glacier						
	850	860	870	880	890	900
Himalaya						
Himalaya292	DEAVRAQANAEIIEIKKKLHNLSLARTCCDDNLMYIIAASDTNCGDILRLYINENSRLAH					
HAG						
Glacier						

FIGURE 3 (CONTINUED)

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Himalaya	910	920	930	940	950	960
Himalaya292						
HAG	STETMGGCYNNWTDGLSTVSEFVKCDEDCDWWYADVILPEKALVLDWTFADGFPACNAR					
Glacier						
Himalaya	970	980	990	1000	1010	1020
Himalaya292						
HAG	NYDNNSEGOFFHALIPKNNVTKGFWQLEQNIYITLLQERREKEEIMRRKAERSANNKAL					
Glacier						
Himalaya	1030	1040	1050	1060	1070	1080
Himalaya292						
HAG	NEAKDMRRFTLSQKHIVYTFLEVRACIADVLYNPSNTVLNGCTEVWFCSENLWHPHS					
Glacier						
Himalaya	1090	1100	1110	1120	1130	1140
Himalaya292						
HAG	CALPTQKQWKSQSGSLKATVNVFPDAPMDVFFSESDENGIYDNGMDYHIPVSDSIE					
Glacier						
Himalaya	1150	1160	1170	1180	1190	1200
Himalaya292						
HAG	TENTMRILHIAVENAPVAKVGGICGVVTSLSRAVDLGHTEVILLPKYDCLNQSSVVDLE					
Glacier						

FIGURE 3 (CONTINUED)

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	1210	1220	1230	1240	1250	1260
Himalaya						
Himalaya292	LYQSTSWSGTEIKVMVCGVEMLLVYFLFPQNGMFCVCGVGRNDRRFGFCHSALEFIL					
HAG						
Glacier						
	1270	1280	1290	1300	1310	1320
Himalaya						
Himalaya292	QNGLSPHLIHCHEWSSAPVAVWLYKHYHQSRMSATRVVFTIENLEFGAHYIGKANTYCDK					
HAG						
Glacier						
	1330	1340	1350	1360	1370	1380
Himalaya						
Himalaya292	ATTWSPTYSREVAGHGALAPEREKTYCIINGIDPDIDWPYIDNFIIPVPTTENVVEGRSA					
HAG						
Glacier						
	1390	1400	1410	1420	1430	1440
Himalaya						
Himalaya292	ANGALQONFGLOQTDFVFWVGLITRLTAGSGTHLKNATHQTLESNGQVLLGSAFDHRIQ					
HAG						
Glacier						
	1450	1460	1470	1480	1490	1500
Himalaya						
Himalaya292	GDFCLADALHGVTYHGKVKLVLTYTEPLSHLIYAGSDFIIVPSIFEPCGLTQLVANRYGS					
HAG						
Glacier						

FIGURE 3 (CONTINUED)

	1510	1520	1530	1540	1550	1560
Himalaya						
Himalaya292	IFIVRKTGCLLEDTVFVDVNDKRRARSIGLEPNQHSFDCADSNQVDYALNRAIAAMFDARD					
HAG						
Glacier						
	1570	1580	1590			
Himalaya						
Himalaya292	WTHSLCKRYMEQDWSNNRPALDYITLYHAARKF					
HAG						
Glacier						

FIGURE 3 (CONTINUED)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2012/000098

A. CLASSIFICATION OF SUBJECT MATTER		
Int. Cl.		
A01H 5/10 (2006.01)	C12N 15/29 (2006.01)	C12N 15/54 (2006.01)
A01H 1/06 (2006.01)	C12N 5/14 (2006.01)	C12Q 1/25 (2006.01)
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) GENOMEQUEST: SEQ ID NOs 3 and 4; CAPLUS, MEDLINE, BIOSIS, AGRICOLA, WPI, EPODOC: starch synthase III, barley, amol, HAG and like terms		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO2000/066745 A1 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION et al) 9 November 2000 See in particular SEQ ID NOs 7 and 8, p. 16 lines 6-19 and p. 48 lines 3-15	1-44
X	WO1999/066050 A1 (NATIONAL STARCH AND CHEMICAL INVESTMENT HOLDING CORPORATION) 23 December 1999 See in particular pps 9-12, examples, claims	1-44
X	Newman, C.W. et al "Comparative Nutritive Value of Glacier and High Amylose Glacier Barleys" Journal of Animal Science, 1978, vol. 47, pps 448-456 See whole document	1, 9-13, 16-25, 32-35, 37-40-42
Y		3, 4, 14, 15, 36
☒ Further documents are listed in the continuation of Box C ☒ See patent family annex		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 10 May 2012		Date of mailing of the international search report 14 May 2012
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustalia.gov.au Facsimile No. +61 2 6283 7999		Authorized officer FELIX WHITE AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 2 6283 2565

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2012/000098

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Morell, M.K. et al "Barley sex6 mutants lack starch synthase IIa activity and contain a starch with novel properties" The Plant Journal, 2003, vol. 34, pps 173-185 See abstract	3, 4, 14, 15, 36
P,X	WO2011/011833 A1 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION et al) 3 February 2011 See Examples 1, 3, 4	1-44
P,X	Li, Z. et al "The barley amol locus is tightly linked to the starch synthase IIIa gene and negatively regulates expression of granule-bound starch synthetic genes" Journal of Experimental Botany, 3 August 2011, Vol. 62, pps 5217-5231 See whole document	1-44
A	Fujita, N. et al "Characterization of SSIIIa-Deficient Mutants of Rice: The Function of SSIIIa and Pleiotropic Effects by SSIIIa Deficiency in the Rice Endosperm" Plant Physiology, 2007, vol. 144, pps 2009-2023 See whole document	
A	Zhang, X. et al "Overlapping functions of the starch synthases SSII and SSIII in amylopectin biosynthesis in Arabidopsis" BMC Plant Biology, 2008, 8:96 See whole document	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU2012/000098

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
WO	2000/066745	AU	40924/00	CA	2365285	EP	1179074
		US	7001771	US	2006035379	US	7700826
WO	1999/066050	AU	43802/99	CA	2331300	EP	1092033
		JP	2002518015	US	6635756	US	2004025204
		US	7534931				
WO	2011/011833	AU	2010278678	CA	2769311	SG	178164
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.							
END OF ANNEX							