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(54) Title: HIGH AMYLOSE WHEAT - IV

(57) Abstract: The present invention provides wheat grain of the species *Triticumaestivum*, the grain comprising i) mutations in each of its *SSIIa* genes such that the grain is homozygous for a mutation in its *SSIIa-A* gene, homozygous for a mutation in its *SSIIa-B* gene and homozygous for a mutation in its *SSIIa-D* gene, wherein at least two of the mutations in said *SSIIa* genes are null mutations, mutations in each of its *SSIIIa* genes such that the grain is homozygous for a mutation in its *SSIIIa-A* gene, homozygous for a mutation in its *SSIIIa-B* gene and homozygous for a mutation in its *SSIIIa-D* gene, wherein at least two of the mutations in said *SSIIIa* genes are null mutations ii) a total starch content comprising an amylose content and an amylopectin content, iii) a fructan content which is increased relative to wild-type wheat grain on a weight basis, preferably between 3% and 12% of the grain weight, iv) a β-glucan content, v) an arabinoxylan content, vi) a cellulose content. The grain has a weight of between 25mg and 60mg, and the amylose content is between 45% and 70% on a weight basis of the total starch content of the grain as determined by iodine binding assay. The amylopectin content on a weight basis is reduced relative to the wild-type wheat grain, and each of the β-glucan content, arabinoxylan content and cellulose content are increased relative to the wild-type wheat grain on a weight basis, such that the sum of the fructan content, β-glucan content, arabinoxylan content and cellulose content is between 15% and 30% of the grain weight.

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HIGH AMYLOSE WHEAT - IV

RELATED APPLICATION

[0001] This application claims priority to AU Provisional Patent Application No. 2018900073 filed 10 January 2018, the entire disclosure of which is incorporated herein by cross reference.

FIELD

[0002] The specification describes methods of obtaining hexaploid wheat plants having high amylose starch and the use of such plants, and particularly grain or starch therefrom in a range of food and non-food products.

BACKGROUND

[0003] Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country. Throughout this application, various publications are referenced, including referenced in parenthesis. Full citations for publications referenced in parenthesis may be found listed in alphabetical order at the end of the specification immediately preceding the claims. The disclosures of all referenced publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

[0004] Food produced from wheat grain supplies at least 20% of the food kilojoules for the world population and provides a significant portion of the protein and non-starch polysaccharides as well as energy intake to the human diet. Starch is the major component of wheat grain and is used in a vast range of food and non-food products. Starch characteristics vary and they play a key role in determining the suitability of wheat starch for a particular end use. Despite this huge global consumption and despite an increased awareness of the importance of starch functionality on end product quality, research on genetic variation in wheat and its precise impact on starch characteristics lags behind that for other commercially important plant crops.

[0005] Carbohydrate accounts for about 65-75% of the mature wheat grain (Stone and Morell, 2009). The main carbohydrate in wheat grain is starch, which is made up of two polymers of glucose, amylose and amylopectin. Amylose is an essentially linear polymer

of α-1,4 linked glucose units with a few branches, while amylopectin is relatively highly branched with α-1,6 glucosidic unit bonds linking linear chains of α-1,4 linked glucose units. The ratio of amylose to amylopectin appears to be a major determinant in (i) the health benefit of wheat grain and wheat starch and (ii) the end quality of products comprising wheat starch.

[0006] A second important determinant in wheat grain for health is the amount of non-starch polysaccharide in the grain, which forms part of the dietary fibre. Wild-type wheat grain has about 1% by weight of oligosaccharides such as raffinose, about 1% fructans and about 10% cell wall polysaccharides, mainly cellulose, arabinoxylan and β-glucan (Stone and Morell, 2009). These form the major components of dietary fibre which is not digested and absorbed in the small intestine but passes to the colon where it undergoes bacterial degradation. Dietary fibre is important for regulating blood glucose and insulin levels as well as bowel health.

[0007] Cereal grain having starch with increased relative amounts of amylose are of particular interest for their health benefits. Foods comprising increased amylose have been found to be higher in levels of resistant starch (RS), a form of dietary fibre. RS is starch or partially digested starch products that are not digested and absorbed in the small intestine. Resistant starch is increasingly seen to have an important role in promoting intestinal health and in protecting against diseases such as colorectal cancer, type II diabetes, obesity, heart disease and osteoporosis. High amylose starches have been developed in certain cereals such as maize and barley for use in foods as a means of promoting bowel health. The beneficial effects of resistant starch result from the provision of a nutrient to the large bowel wherein the intestinal microflora are given an energy source which is fermented to form short chain fatty acids. These short chain fatty acids provide nutrients for the colonocytes, enhance the uptake of certain nutrients by the large bowel and promote physiological activity of the colon. Generally, if resistant starches or other dietary fibre are not provided to the colon it becomes metabolically relatively inactive. Thus high amylose products have the potential to provide for an increased consumption of fibre. Further potential health benefits of consuming high amylose wheat grains or their products such as starch include an enhanced regulation of sugar, insulin and lipid levels in the blood. Additionally, such foods may promote satiety, improving laxation, promotion of growth of probiotic bacteria and enhancing faecal bile acid excretion.

[0008] Most processed starchy foods contain very little RS. Breads made using wild-type wheat flour and conventional formulation and baking processes contain <1% RS. In comparison, breads baked using the same process and storage conditions but containing high amylose wheat flour by virtue of reduced starch branching enzyme activity in the grain had levels of RS as much as 10-fold higher (WO2006/069422). Legumes, which are one of the few rich sources of RS in the human diet, contain levels of RS that are normally <5%. Therefore, consumption of the high amylose wheat bread in amounts normally consumed by adults (e.g. 200 g/d) would readily supply at least 5-12 g of RS. Thus, incorporation of high amylose wheat grain into food products has the potential to make a considerable contribution to dietary RS intakes for humans.

[0009] Starch is initially synthesized in plants in chloroplasts of photosynthesizing tissues such as leaves in the form of transitory starch. This is mobilized during subsequent dark periods to supply carbon for export to sink organs and energy metabolism or for storage in organs such as seeds or tubers. Synthesis and long-term storage of starch occurs in the amyloplasts of the storage organs, such as the endosperm of cereals, where the starch is deposited as semicrystalline granules up to 100μm in diameter. Granules contain both amylose and amylopectin, the former typically as amorphous material in the native starch granule while the latter is semicrystalline through stacking of the linear glucosidic chains. Granules also contain some of the proteins involved in starch biosynthesis.

[0010] The synthesis of starch in the endosperm involves at least four types of enzymes (Figure 1). First, ADP-glucose pyrophosphorylase (ADGP) catalyses the synthesis of ADP-glucose from glucose-1-phosphate and ATP. Secondly, a diverse set of starch synthases (SS; EC 2.4.1.21) catalyse the transfer of glucose residues from ADP-glucose to the non-reducing end by α-1,4 linkages to elongate an α-glucan chain. Thirdly, starch branching enzymes (SBE) form new α-1,6 linkages in α-polyglucans. Lastly, starch debranching enzymes (DBE) then remove some of the branch linkages through a mechanism that has not been fully resolved.

[0011] While it is clear that at least these four activities are required for normal starch granule synthesis in higher plants, multiple isoforms of each of the enzymes are found in the endosperm of higher plants. Specific roles for some isozymes have been proposed on the basis of mutational analysis or through the modification of gene expression levels using

transgenic approaches (Abel *et al.,* 1996; Jobling *et al.,* 1999; Schwall *et al.,* 2000). However, the contributions of each of the isozymes differs markedly between species and the precise contribution of each isoform to starch biosynthesis are still not known. This is especially true for the hexaploid, bread wheat (*Triticum aestivum*), which has three sets of homologous chromosomes defining genomes A, B and D. Hexaploidy has been considered a significant obstacle in researching and developing useful variants of wheat. In fact, knowledge is limited regarding how homoeologous genes of wheat interact, how their expression is regulated, and how the different proteins produced by homoeologous genes work separately or in concert.

[0012] In maize, rice and wheat, the enzymes starch synthase I (SSI), starch synthase IIa (SSIIa) and starch synthase IIIa (SSIIIa) participate in amylopectin synthesis, perhaps along with other SS. In rice, for example, there are 10 different starch synthases including two granule-bound forms (GBSS). In cereals, GBSS is essential for the biosynthesis of amylose (Nelson & Rines, 1962; Murata *et al.*, 1965; Eriksson, 1969, Delrue *et al.*, 1992, Nakamura *et al.*, 1995) and also contributes to the synthesis of the long chains of amylopectin (Maddelein *et al.*, 1994; Denyer *et al.*, 1996). GBSSI is expressed predominantly in endosperm during grain development, whereas GBSSII is expressed in organs other than the grain, including leaves and stems. Mutational analysis of the roles of SSI, SSIIa and SSIIIa suggests that while each of these enzyme classes is primarily involved in amylopectin synthesis, they are preferentially involved in the extension of specific subsets of chain lengths within the amylopectin molecule. For example, SSI is preferentially involved in synthesis of the shorter outer chains of the amylopectin (DP8 – 12) in the endosperm starch of rice (Fujita *et al.*, 2006). Starch from barley and wheat *ssIIa* mutants showed an increase in the proportion of chains of DP3 – 8, indicating that the SSIIa enzyme plays a role in extending shorter glucan chains of DP3 – 8 to longer glucan chains DP12 – 35 (Morell *et al.*, 2003; Yamamori *et al.*, 2000; Konik-Rose *et al.*, 2007). Loss of SSIIIa in maize, rice and barley confers a reduction in the proportion of longer chains in amylopectin (Jane *et al.*, 1999; Fujita *et al.*, 2007, Li *et al.* 2011). *Arabidopsis* mutants defective for SSIV appear to have fewer, larger starch granules within the plastid. A role in priming starch granule formation has been postulated for the SSIV protein (Roldan *et al.*, 2007).

[0013] Wheat, barley and rice mutants which are defective for SSIIa have been isolated but the three species show different effects on the phenotypes generated by the loss of SSIIa, especially in the extent of the effects. An *ssIIa* mutant wheat plant entirely lacking the SGP-1 (SSIIa) protein was produced by crossing lines which were lacking the A, B and D genome specific forms of SGP-1 protein (Yamamori *et al.,* 2000). The *ssIIa* triple-null grain exhibited deformed starch granules and the starch had altered amylopectin structure. The starch had an amylose content of 30-37% w/w, which was an increase of about 8% over the wild-type level, and a substantial reduction in starch content (Yamamori *et al.*, 2000). Starch from the *ssIIa* triple-null mutant exhibited a decreased gelatinisation temperature compared to starch from corresponding, wild-type grain. The starch content of the *ssIIa* triple-null grain was reduced to less than 50% from at least 60% w/w in the wild-type grain. There is no suggestion in Yamamori *et al.*, (2000) that wheat having greater than 45% amylose content in its starch could be produced by combining *ssIIa* gene mutations, indeed Yamamori *et al.*, teaches to the contrary. This was substantiated by Konik-Rose *et al.*, (2007) who obtained a maximum of 43.98% amylose in the starch of a triple-null *ssIIa* mutant crossed into wheat variety Sunco.

[0014] In barley, a chemically induced null mutation in the *SSIIa* gene greatly reduced the synthesis of amylopectin and thereby raised the proportion of amylose in grain starch to 65-70% w/w (WO02/37955-A1; Morell *et al.,* 2003). Japonica rice comprises an *SSIIa* mutation which reduces the SSIIa enzyme in the endosperm relative to Indica rice, but the level of amylose in Japonica grain starch was not substantially elevated compared to Indica grain starch. In rice, a combination of mutations in the *SBEIIb* and *SSIIIa* genes had a more substantial effect on the relative amount of amylose (Asai *et al.*, 2014).

[0015] The different effects of the *ssIIa* null mutations in wheat, barley and rice were attributed to different extent of pleiotropic effects of the lack of SSIIa protein on the partitioning of starch synthase I (SSI) and starch branching enzyme IIb (SBEIIb) enzymes inside and outside the starch granules in the developing endosperms of these *ssIIa* mutants (Luo *et al.*, 2015). In addition, differential effects on post-translational levels of the SSI and SBEIIb proteins may have affected the remaining amylopectin structure. This was one example where observations in one cereal species could not be simply extrapolated to another cereal species in the area of starch synthesis.

[0016] In maize and rice, high amylose phenotypes have been generated by mutations in the *SBEIIb* gene, encoding starch branching enzyme IIb, also known as the *amylose extender (ae)* gene (Boyer and Preiss, 1981, Mizuno *et al.,* 1993; Nishi *et al.,* 2001) rather than an *SSIIa* gene. In these *sbeIIb* mutants, the amylose content was significantly elevated as a proportion of the starch content, the branch frequency of the residual amylopectin was reduced and the proportion of short chains (<DP17, especially DP8-12) was reduced. Moreover, the gelatinisation temperature of the starch was increased. To obtain further increases in amylose levels in maize, varieties were produced having reduced starch branching enzyme I (SBEI) activity together with an almost complete inactivation of SBEII activity (Sidebottom *et al.,* 1998).

[0017] Wheat having at least 50% amylose as a proportion of the starch content has been generated by reduction in SBEIIa activity alone (Regina *et al.*, 2006) rather than reducing SBEIIb or SSIIa activity. In contrast to maize and rice, wheat having reduced SBEIIb by itself did not yield increased amylose content. International Publication No. WO2005/001098 and International Publication No. WO2006/069422 describe transgenic hexaploid wheat comprising exogenous duplex RNA that reduced expression of one or both of *SBEIIa* and *SBEIIb* genes in the endosperm. Grain from transgenic lines expressed reduced levels of SBEIIa and/or SBEIIb proteins. The reduction of SBEIIa protein in endosperm was associated with increased relative amylose levels of more than 50% whereas the lack of SBEIIb protein by itself did not appear to substantially alter the proportion of amylose in grain starch. International Publication Nos. WO2012/058730 and WO2013/063653 report production of non-transgenic *sbeIIa* and/or *sbeIIb* triple-null mutants substantially lacking expression of SBEIIa and SBEIIb proteins which exhibited increased amylose levels. The grain of *sbeIIa* triple-null genotypes were viable provided that at least one of the *sbeIIa* gene mutations was a point mutation rather than a deletion extending beyond the gene into adjacent regions. Therefore, if it were desired to produce high amylose wheat having at least 50% amylose, the *SBEIIa* gene is the gene that would be targeted in bread wheat. Levels of non-starch polysaccharides such as fructans were not increased in wheat having reduced SBEII activity and increased (>50%) amylose in its starch (e.g. WO2010/006373).

[0018] There is a need in the art for improved high amylose wheat plants and for methods of producing same.

SUMMARY

[0019] The inventors have found, unexpectedly, that hexaploid wheat grain comprising one or more or all of the characteristics selected from the group consisting of an increased total fibre content, an increased total dietary fibre (TDF) content, an increased fructan content, an increased ß-glucan content, an increased amylose content and an increased starch content can be produced by combining null mutations in each of the *SSIIa* and *SSIIIa* genes, each increase being relative to wheat grain comprising the null mutations in the *SSIIa* genes but wild-type for the *SSIIIa* genes. The grain can be produced by combining mutations in the three *SSIIIa* or the *SSIIa* and *SSIIIa* genes on the A, B and D genomes of the wheat by breeding and selection from wheat plants comprising one or two mutations, or by mutagenesis. In a preferred embodiment, the wheat grain comprises an amylose content of at least 45%, as a weight percentage of the total starch content of the grain. Prior publications had indicated that 45% amylose was not achievable through modification of starch synthases (SS), indeed the amylose level in *ssIIa* mutant wheat was generally 30-38% (Yamamori *et al.*, 2000). Since loss of function mutations in each of *SSIIa* and *SSIIIa* are recessive, the phenotypes were seen, in certain lines, when the mutations were in the homozygous state. The inventors also found that the mutant *ssIIa-ssIIIa* wheat grain had significantly increased levels of non-starch polysaccharides, particularly β-glucan and fructan, each as a percentage of the grain weight. This yielded a substantial increase in total fibre content, as well as associated increases in protein content and other favourable phenotypes.

[0020] In a first aspect, the present invention therefore provides a wheat grain of the species *Triticum aestivum*, the grain comprising

i) mutations in each of its *SSIIa* genes and each of its *SSIIIa* genes such that the grain is homozygous for a null mutation in its *SSIIa-A* gene, homozygous for a null mutation in its *SSIIa-B* gene, homozygous for a null mutation in its *SSIIa-D* gene, homozygous for a null mutation in its *SSIIIa-A* gene, homozygous for a null mutation in its *SSIIIa-B* gene and homozygous for a null mutation in its *SSIIIa-D* gene,

ii) a total starch content comprising an amylose content and an amylopectin content,

iii) a fructan content which is increased relative to wild-type wheat grain on a weight basis, preferably between 3% and 12% of the grain weight,

iv) a β-glucan content,

v) an arabinoxylan content,

vi) a cellulose content, and

vii) a total dietary fiber (TDF) content,

the grain having a grain weight of between 25mg and 60mg, wherein the amylose content is between 45% and 70% on a weight basis of the total starch content of the grain as determined by iodine binding assay, wherein the amylopectin content on a weight basis is reduced relative to the wild-type wheat grain, wherein one or more or preferably all of the β-glucan content, TDF content, arabinoxylan content and cellulose content are increased relative to the wild-type wheat grain on a weight basis. In an embodiment, the sum of the fructan content, β-glucan content, arabinoxylan content and cellulose content is between 15% and 30% of the grain weight. In an embodiment, the wheat grain further comprises one or more or all of the characteristics selected from the group consisting of an increased total fibre content, an increased total dietary fibre (TDF) content, the fructan content is increased, the ß-glucan content is increased, the amylose content is increased and an increased starch content, each increase being relative to wheat grain which is homozygous for the null mutation in its *SSIIa-A* gene, homozygous for the null mutation in its *SSIIa-B* gene, homozygous for the null mutation in its *SSIIa-D* gene, homozygous wild-type for the *SSIIIa-A* gene, homozygous wild-type for the *SSIIIa-B* gene and homozygous wild-type for the *SSIIIa-D* gene. In an embodiment, one or more or all of the fructan content, the ß-glucan content, arabinoxylan content, cellulose content and the amylose content of the grain of the invention is about the same (+/- 10%) as a corresponding *ssIIa* mutant-*SSIIIa* wild-type grain i.e. the lack of SSIIIa as well as SSIIa does not significantly reduce these fibre components relative to the grain lacking SSIIa alone. In preferred embodiments, the grain has increased total fibre content and increased TDF content, and preferably increased grain weight relative to the *ssIIa* mutant-*SSIIIa* wild-type grain, or the grain has increased TDF, increased starch content and increased weight relative to the *ssIIa* mutant-*SSIIIa* wild-type grain. The grain has an increased resistant starch (RS) content in the grain starch relative to the wild-type grain, more preferably also has increased RS relative to the *ssIIa* mutant-*SSIIIa* wild-type grain. In embodiments, the RS content is increased at least 2-fold, as much as 10-fold or 12-fold relative to the wild-type grain, and about the same or preferably increased relative to the *ssIIa* mutant-*SSIIIa* wild-type grain.

[0021] The present invention further provides wheat plants which are capable of producing, or are obtained from, this grain and products such as flour, bran, wheat starch granules and wheat starch produced from this grain.

[0022] The present invention also provides food ingredients comprising the grain of the present invention or material produced from this grain. Also provided are food products including these food ingredients and compositions comprising the grain of the present invention or material produced from this grain. The food ingredient may be kibbled, cracked, par-boiled, rolled, pearled, milled or ground grain or any combination of these. A preferred ingredient is flour, most preferably wholemeal or a blend of wholemeal and white flour. These ingredients have an increased level of total fibre relative to a corresponding ingredient from wild-type wheat by virtue of the incorporation of the material from the wheat grain of the invention.

[0023] In another aspect, the present invention provides a process for producing a wheat plant that is capable of producing the grain of the present invention, the process comprising step (i) crossing a first parental wheat plants comprising a null mutation in each of one, two or three *SSIIa* genes and a second parental wheat plant comprising a null mutation in each of one, two or three *SSIIIa* genes, the genes being selected from the group consisting of *SSIIa-A*, *SSIIa-B, SSIIa-D, SSIIIa-A*, *SSIIIa-B,* and *SSIIIa-D*, , or of mutagenising a parental plant, preferably comprising one or two of said null mutations; and step (ii) screening plants or grain obtained from the cross or mutagenesis, or progeny plants or grain obtained therefrom, by analysing DNA, RNA, protein, starch granules or starch from the plants or grain, and step (iii) selecting a fertile wheat plant that has reduced SSIIa activity relative to the second parental plant and reduced SSIIIa activity relative to the first parental wheat plants of step (i).

[0024] In another aspect, the present invention provides a process for improving one or more parameters of metabolic health, bowel health or cardiovascular health in a subject in need thereof, or of preventing or reducing the severity or incidence of a metabolic disease such as diabetes, bowel disease or cardiovascular disease, the method comprising providing to the subject the grain or food product of the present invention.

[0025] The present invention also provides wheat starch granules or wheat starch produced from the wheat grain of the present invention. Also provided are methods of

producing starch comprising extracting the starch from the wheat grain of the present invention.

[0026] In a still further aspect, the present invention provides a process of producing bins of wheat grain comprising:

a) reaping wheat stalks comprising the wheat grain of the present invention;

b) threshing and/or winnowing the stalks to separate the grain from the chaff; and

c) sifting and/or sorting the grain separated in step b), and loading the sifted and/or sorted grain into bins, thereby producing bins of wheat grain.

[0027] In embodiments of each of the above aspects, the wheat grain is further characterised by one or more or all of the features as follows. The amylose content is increased relative to wild-type wheat grain, for example between 48% and 70%, preferably between 50% and 65% of the total starch content of the grain as determined by iodine binding assay. In embodiments, the amylose content is between 50% and 70%, or about 48%, about 50%, about 53%, about 55%, about 60% or about 65%. The starch content of the grain is reduced relative to wild-type wheat grain, for example at least 25%. In embodiments, the starch content of the grain of the invention is between 30% and 70% of the grain weight, between 25% and 65%, between 25% and 60%, between 25% and 55%, between 25% and 50%, between 30% and 70%, between 30% and 65%, between 30% and 60%, between 30% and 55%, or between 30% and 50%. In further embodiments, the starch content is about 35%, about 40%, about 45%, about 50%, about 55%, about 60% or about 65% as a percentage of the grain weight (w/w). In an embodiment, at least 50%, preferably at least 60% or at least 70%, more preferably at least 80% of the starch granules obtained from the grain of the invention show distorted shape and/or surface morphology. The starch of the grain comprises at least 2% resistant starch, preferably at least 3% resistant starch, more preferably between 3% and 15% RS, or between 3% and 10% RS. The starch is characterised by a reduced gelatinisation temperature, which is readily measured by differential scanning calorimetry (DSC), for example the first peak in the DSC scan occurs at a temperature 2-8^{o}C lower than for wild-type starch. In embodiments, the BG content of the grain is the β-glucan content is increased by 1% or by 2% on an absolute basis relative to wild-type grain and/or is increased by between 2-fold and 7-fold

relative to the wild-type wheat grain on a weight basis. In embodiments, the BG level is at least 1% or at least 2%, preferably between 1% and 4% or between 1% and 5% by weight of the grain, preferably about 2%, about 3%, about 4%, more preferably between 2% and 5%. In embodiments, the arabinoxylan content is increased by between 1% and 5% on an absolute basis and/or the cellulose content is increased by between 1% and 5% on an absolute basis. In a preferred embodiment, the grain (prior to any treatment that prevents it germinating) has a germination rate which is between about 70% and about 100% relative to the wild-type wheat grain and the grain, when sown, gives rise to wheat plants which are male and female fertile. Each of these phenotypes are associated with the reduction in SSIIa and SSIIIa activity whilst the grain is developing in the wheat plant, the result of the mutations in the SSIIa and *SSIIIa* genes. All combinations of these features are contemplated in the present invention.

[0028] 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

[0029] Figure 1. Schematic representation of the enzymes involved in starch synthesis in cereal grain, for amylose and amylopectin.

[0030] Figure 2. Schematic gene map of wheat *SSIIa-A* gene mutation in the A genome of wheat line C57. The upper line shows a map of the exons of the *SSIIa-A* gene. Below are the nucleotide sequences of a region of the *SSIIa-A* gene from the wild-type Chinese Spring and mutant C57 showing the deletion of 289 nucleotides in exon 1 including the ATG translation start codon and an insertion of 8 nucleotides, net deletion size of 281 nucleotides. Positions of the primers JKSS2AP1F and JKSS2AP2R are shown.

[0031] Figure 3. Schematic gene map of wheat *SSIIa-B* gene mutation in the B genome of wheat line K79. The upper line shows a map of the exons of the *SSIIa-B* gene. Below are the nucleotide sequences of a region of the *SSIIa-B* gene from the wild-type Chinese Spring (CS) and mutant K79 showing the insertion of 179 nucleotides into exon 8 of *SSIIa-B*. Positions of the primers JKSS2BP7F and JLTSS2BPR1 are shown.

[0032] Figure 4. Schematic gene map of wheat *SSIIa-D* gene mutation in the D genome of wheat line Turkey 116. The upper line shows a map of the exons of the *SSIIa-D* gene. Below are the nucleotide sequences of a region of the *SSIIa-D* gene from the wild-type Chinese Spring (CS) and mutant T116 showing the deletion of 63 nucleotides spanning the junction of exon 5 and intron 5 (intron 5 splice site) of SSIIa-D. Positions of the primers JTSS2D3F and JTSS2D4R are shown.

[0033] Figure 5. Schematic of the crossing and backcrossing program to produce triple null *ssIIa* mutants in the Sunco genetic background.

[0034] Figure 6. Schematic of the crossing and backcrossing program to produce triple null ssIIa mutants in the EGA Hume genetic background.

[0035] Figure 7. Schematic of the crossing and backcrossing program to produce triple null ssIIa mutants in the Westonia genetic background.

[0036] Figure 8. Upper panel shows the average grain weight (mg per grain) and the lower panel shows the total lipid content (% weight of the grain) in the triple null ssIIa grain (abd) and wild-type SSIIa grain (WT) for individual wheat lines in the EGA Hume, Sunco and Westonia genetic backgrounds.

[0037] Figure 9. Means of the data in Figure 8 for the triple null mutant (abd) and WT genotypes in the EGA Hume, Sunco and Westonia genetic backgrounds, showing the standard deviation. Bars indicated with the same letters (a, b, c) are not statistically significantly different, whereas bars with different letters are significantly different.

[0038] Figure 10. Uppermost panel shows the amylopectin content (% of starch content on a weight basis), middle panel shows the amylose content (% of starch content on a weight basis, by iodine binding method) and the lower panel shows the total starch content (% weight of the grain) in the triple null ssIIa grain (abd) and wild-type SSIIa grain (WT) for individual wheat lines in the EGA Hume, Sunco and Westonia genetic backgrounds.

[0039] Figure 11. Means of the data in Figure 10 for the triple null mutant (abd) and WT genotypes in the EGA Hume, Sunco and Westonia genetic backgrounds, showing the standard deviation. Bars indicated with the same letters (a, b, c) are not statistically significantly different, whereas bars with different letters are significantly different.

[0040] Figure 12. Upper panel shows the total fibre content (% of grain on a weight basis) and the lower panel shows the total BG content (% of grain on a weight basis) in the triple null ssIIa grain (abd) and wild-type SSIIa grain (WT) for individual wheat lines in the EGA Hume, Sunco and Westonia genetic backgrounds.

[0041] Figure 13. Means of the data in Figure 12 for the triple null mutant (abd) and WT genotypes in the EGA Hume, Sunco and Westonia genetic backgrounds, showing the standard deviation. Bars indicated with the same letters (a, b, c) are not statistically significantly different, whereas bars with different letters are significantly different.

[0042] Figure 14. Uppermost panel shows the fructan content (% of grain on a weight basis), middle panel shows the arabinoxylan content (% of grain on a weight basis) and the lower panel shows the cellulose content (% of grain on a weight basis) in the triple null ssIIa grain (abd) and wild-type SSIIa grain (WT) for individual wheat lines in the EGA Hume, Sunco and Westonia genetic backgrounds.

[0043] Figure 15. Upper panel shows the average grain weight (mg per grain) and the lower panel shows the total lipid content (mg per grain) in the triple null ssIIa grain (abd) and wild-type SSIIa grain (WT) for individual wheat lines in the EGA Hume, Sunco and Westonia genetic backgrounds.

[0044] **Figure 16.** Means of the data in Figure 15 for the triple null mutant (abd) and WT genotypes in the EGA Hume, Sunco and Westonia genetic backgrounds, showing the standard deviation. Bars indicated with the same letters (a, b, c) are not statistically significantly different, whereas bars with different letters are significantly different.

[0045] **Figure 17.** Uppermost panel shows the amylopectin content (mg per grain), middle panel shows the amylose content (mg per grain) and the lower panel shows the total starch content (mg per grain) in the triple null ssIIa grain (abd) and wild-type SSIIa grain (WT) for individual wheat lines in the EGA Hume, Sunco and Westonia genetic backgrounds.

[0046] **Figure 18.** Means of the data in Figure 17 for the triple null mutant (abd) and WT genotypes in the EGA Hume, Sunco and Westonia genetic backgrounds, showing the standard deviation. Bars indicated with the same letters (a, b, c) are not statistically significantly different, whereas bars with different letters are significantly different.

[0047] **Figure 19.** Upper panel shows the total fibre content (mg per grain) and the lower panel shows the total BG content (mg per grain) in the triple null ssIIa grain (abd) and wild-type SSIIa grain (WT) for individual wheat lines in the EGA Hume, Sunco and Westonia genetic backgrounds.

[0048] **Figure 20.** Means of the data in Figure 19 for the triple null mutant (abd) and WT genotypes in the EGA Hume, Sunco and Westonia genetic backgrounds, showing the standard deviation. Bars indicated with the same letters (a, b, c) are not statistically significantly different, whereas bars with different letters are significantly different.

[0049] **Figure 21.** Uppermost panel shows the fructan content (mg per grain), middle panel shows the arabinoxylan content (mg per grain) and the lower panel shows the cellulose content (mg per grain) in the triple null ssIIa grain (abd) and wild-type SSIIa grain (WT) for individual wheat lines in the EGA Hume, Sunco and Westonia genetic backgrounds.

[0050] **Figure 22.** Means of the data in Figure 21 for the triple null mutant (abd) and WT genotypes in the EGA Hume, Sunco and Westonia genetic backgrounds, showing the standard deviation. Bars indicated with the same letters (a, b, c) are not statistically significantly different, whereas bars with different letters are significantly different.

[0051] Figure 23. Resistant starch content as a percentage of the starch in three selected triple null ssIIa mutants (Sunco-abd) and wild-type (WT) grain in the Sunco genetic background. The three mutants were not significantly different to each other but were significantly different to WT.

[0052] Figure 24. Average mol% differences of chain length distribution (CLD) profiles of debranched starch from 5 triple ssIIa mutant lines compared with the corresponding wild-type starch. The short chains (DP 6-10) were increased in frequency, while the intermediate chains (DP 11-24) were decreased in frequency for the ssIIa mutant starch compared with that of corresponding wild-type.

[0053] Figure 25. Size exclusion chromatography (SEC) profiles of starch from triple null ssIIa mutant grain and corresponding wild-type wheat starch after isoamylase debranching of the starches. The traces show the distribution of normalized refractometer Index (RI) signals of eluted fractions according to their degree of polymerisation (X-axis). The first eluting peak (I) is amylose, the second (II) is long chain amylopectin and peak III is the (debranched) amylopectin. The black trace was for the ssIIa mutant starch, the grey trace was for wild-type starch.

[0054] Figure 26. Immunological characterisation of starch granule bound proteins from mature wheat grains of mutant ssIIa (B22) and wild-type SSIIa (B70) wheat. The top band in the Western blots was identified as SSIIa, the second band from the top was identified as a mixture of SBEIIa and SBEIIb, and the bands of 70 and 60 kDa were SSI and GBSSI, based on their binding with specific antisera. The identity of each band is labelled and indicated by arrows. The identity of the antibodies used for the different blots are labelled on the left or underneath each panel. M: protein molecular weight marker (kDa).

[0055] Figure 27. Alignment of genomic nucleotide sequences of an exon 3-intron 3 portion of mutant ssIIIa genes from the A, B and D genomes, showing the sequences of the SSIIIa mutant gene region corresponding to nucleotide 4473 to 5511 of Genbank Accession No. AF258609. The SSIII-B sequence is for the B genome, SSIII-A for A genome and SSIII-D for D genome. Distinguishing polymorphisms for the wild-type and mutant SSIIIa genes are present in exon 3 at positions 41, 51, 52, 62, 71, 74, 182, 194, 218, 251, 344, 416, 509, 516, 575, 578, 587, 656, 657, 677, 747 and 793 of the sequences shown. Other distinguishing polymorphisms are in the intron 3 portion of the sequence,

which starts at nucleotide 820 and continues beyond the end of the sequence shown. The positions of the SSIIIa-B-W758stop, SSIIIa-A-Q865stop, SSIIIa-A-W931stop and SSIIIa-D-W923stop mutations are indicated by arrows. The ssIIIa-B-W758stop mutation was a G to A mutation at nucleotide position 227, the ssIIIa-A-Q865stop mutation was a C to T mutation at nucleotide position 546, the ssIIIa-A-W931stop mutation was a G to A mutation at nucleotide position 747 and the ssIIIa-D-W923 stop mutation was a G to A mutation at nucleotide position 722, each in the sequences shown in this Figure. The positions of oligonucleotide primers used in this study are indicated by the underlined nucleotides.

[0056] **Figure 28.** Genome specific amplification of portions of the SSIIIa genes in wheat. Lanes labelled as "CS" were for Chinese Spring null-tetrasomic lines lacking chromosome 1 of the A, B or D genomes.

LISTING OF THE SEQUENCES

[0057] SEQ ID NO:1 Amino acid sequence of SSIIa-A polypeptide, encoded by the wheat A genome; Accession number: AAD53263, 799aa.

[0058] SEQ ID NO:2 Amino acid sequence of wheat SSIIa-B polypeptide, encoded by the B genome of wheat; Accession number: CAB96627, 798aa.

[0059] SEQ ID NO:3 Amino acid sequence of SSIIa-D polypeptide, encoded by the wheat D genome; Accession number: BAE48800, 799aa; Shimbata *et al.*, (2005).

[0060] SEQ ID NO:4 Nucleotide sequence of full length cDNA of wheat SSIIa-A gene; 2821 nucleotides; Accession number: AF155217; Li *et al.*, (1999); translation start codon nucleotides 89-91, stop codon 2486-2488.

[0061] SEQ ID NO:5 Nucleotide sequence of full length cDNA of wheat SSIIa-B gene; 2793 nucleotides; Accession number: AJ269504; Gao and Chibbar, (2000); translation start codon nucleotides 135-137, stop codon 2529-2531.

[0062] SEQ ID NO:6 Nucleotide sequence of full length cDNA of wheat SSIIa-D gene; 2846 nucleotides; Accession number: AJ269502; Gao and Chibbar, (2000); translation start codon nucleotides 210-212, stop codon 2607-2609. Transit peptide encoded by nucleotides 201-384, mature peptide 385-2606.

[0063] SEQ ID NO:7 The nucleotide sequence of the SSIIa-A gene of wheat; Accession number: AB201445; 6898nt (IWGSC: Chromosome 7AS, Traes_7AS_53CAFB43A, 52346437 bp to 52346905 bp, 52351676 to 52351931 bp reverse strand).

[0064] SEQ ID NO:8 The nucleotide sequence of the SSIIa-B gene of wheat (Accession number: AB201446) (IWGSC: Chromosome 7DS, Traes_7DS_E6C8AF743, 3877787: 1 to 396 bp, 5137 to 5419 bp forward strand), 6811nt.

[0065] SEQ ID NO:9 The nucleotide sequence of the SSIIa-D gene of wheat (Accession number: AB201447) (IWGSC: Chromosome 7DS, Traes_7DS_E6C8AF743, 3877787: 1 to 396 bp, 5137 to 5419 bp forward strand); 6950nt.

[0066] SEQ ID NO:10 Amino acid sequence of wheat SSIIb-A encoded in the A genome, 676aa, deduced from the nucleotide sequence of Accession number AK332724.

[0067] SEQ ID NO:11 Amino acid sequence of wheat SSIIb-D encoded in the D genome, 674aa, Accession number ABY56824 (which has 100% identity with EU333947).

[0068] SEQ ID NO:12 Nucleotide sequence of full length cDNA of wheat SSIIb-A gene on the A genome, Accession number: AK332724. 2727nt (IWGSC: Chromosome 6AL, Traes_6AL_AE01DC0EA, 187,500,495 bp to 187,505,249 bp forward strand).

[0069] SEQ ID NO:13 The nucleotide sequence of partial length cDNA of wheat SSIIb-B on the B genome, 1282nt, IWGSC: Chromosome 6DL, gene: Traes_6BL_61D83E262, 162,113,784 bp to 162,116,959 bp reverse strand).

[0070] SEQ ID NO:14 The nucleotide sequence of full length cDNA of wheat SSIIb-D on the D genome, 2025nt (Accession number: EU333947) (IWGSC: Chromosome 6DL, gene: Traes_6DL_19F1042C7, 147,049,693 bp to 147,051,708 bp reverse strand).

[0071] SEQ ID NOs:15-49 Oligonucleotide primers.

[0072] SEQ ID NOs:50-51 Amino acid sequences of peptides

[0073] SEQ ID NO:52 Amino acid sequence of a representative SSIIIa-A polypeptide; 1629 amino acids.

[0074] SEQ ID NO:53 Nucleotide sequence of wheat SSIIIa A genome cDNAs, deduced from genomic DNA sequences identified by BLAST using Genbank Accession No:

AF258608 as a query to search the EnsemblPlants databases; 5343 nucleotides. Translation start codon ATG is at nucleotides 28-30.

[0075] SEQ ID NO:54 Nucleotide sequence of four contigs from representative wheat SSIIIa-A genes. The sequences of the four contigs were assembled from sequences identified in EnsemblPlants database by BLAST using Genbank Accession No: AF258608 as the query sequence. The four contig sequences in the sequence below are separated by nnnnnn at nucleotides 708 to 713, 6019 to 6025 and 7410 to 7415.

[0076] SEQ ID NO:55 Amino acid sequence of a representative SSIIIa-B polypeptide; 1612 amino acids.

[0077] SEQ ID NO:56 Nucleotide sequence of wheat SSIIIa B genome cDNA, deduced from genomic DNA sequences identified by BLAST using Genbank Accession No: AF258608 as a query to search the EnsemblPlants databases; 5279 nucleotides.

[0078] SEQ ID NO:57 Nucleotide sequence of three contigs from representative wheat SSIIIa-B genes. The sequences of the three contigs were assembled from sequences identified in EnsemblPlants database by BLAST using Genbank Accession No: AF258608 as the query sequence. The three contig sequences in the sequence below are separated by nnnnnn at nucleotides 673 to 5678 and nucleotides 7074 to 7079.

[0079] SEQ ID NO:58 Amino acid sequence of a representative SSIIIa-D polypeptide, from Aegilops tauschii, the presumed D genome progenitor of hexaploid wheat; 1611 amino acids.

[0080] SEQ ID NO:59 Nucleotide sequence of 5' part of wheat SSIIIa D genome cDNA, deduced from genomic DNA sequences identified by BLAST using Genbank Accession No: AF258608 as a query to search the EnsemblPlants databases. 660 nucleotides. The sequence corresponds to the first 211 amino acids of SSIIIa-D. A full length cDNA sequence for SSIIIa-D from Aegilops tauschii is provided in Accession No. AF258609.

[0081] SEQ ID NO:60 Nucleotide sequence of three contigs from representative wheat SSIIIa-D genes. The sequences of the four contigs were assembled from sequences identified in EnsemblPlants database by BLAST using Genbank Accession No: AF258608 as the query sequence. The four contig sequences in the sequence below are separated by nnnnnn at nucleotides 964 to 969 and nucleotides 2347 to 2352.

[0082] SEQ ID NO:61 Partial length amino acid sequence of a representative SSIIIb-A polypeptide; 1002 amino acids.

[0083] SEQ ID NO:62 Nucleotide sequence of 5' part of wheat SSIIIb A genome cDNA, deduced from genomic DNA sequences identified by BLAST using Genbank Accession No: EU333946 as a query to search the EnsemblPlants databases; 3108 nucleotides. The partial sequence is missing about 600nt from the 3' end of the cDNA.

[0084] SEQ ID NO:63 Nucleotide sequence of three contigs from representative wheat SSIIIb-A genes. The sequences of the three contigs were assembled from sequences identified in EnsemblPlants database by BLAST using Genbank Accession No: EU333946 as the query sequence. The three contig sequences in the sequence below are separated by nnnnnn at nucleotides 3843 to 3848 and nucleotides 3843 to 3849 and nucleotides 5915 to 5920.

[0085] SEQ ID NO:64 Amino acid sequence of a representative SSIIIb-B polypeptide; 1276 amino acids.

[0086] SEQ ID NO: 65 Nucleotide sequence of wheat SSIIIb B genome cDNA, deduced from genomic DNA sequences identified by BLAST using Genbank Accession No: EU333946 as a query to search the EnsemblPlants databases; 3866 nucleotides. The translation start codon ATG is at nucleotides 26-28.

[0087] SEQ ID NO:66 Nucleotide sequence of three contigs from representative wheat SSIIIb-B genes. The sequences of the three contigs were assembled from sequences identified in EnsemblPlants database by BLAST using Genbank Accession No: EU333946 as the query sequence. The three contig sequences in the sequence below are separated by nnnnnn at nucleotides 3843 to 3848 and nucleotides 5449 to 5454. Exons correspond to nucleotides: exon 1, 289-415; exon 2, 807-870; exon 3, 959-2656; exon 4, 2922-3136; exon 5, 3272-3542; exon 6, 4152-4327; exon 7, 4403-4510; exon 8, 5038-5147; exon 9, 5756-5858; exon 10, 6149-6219; exon 11, 6325-6453; exon 12, 6578-6760; exon 13, 6888-7019; exon 14, 7111-7222; exon 15, 7311-7434; exon 16, 7552-7695.

[0088] SEQ ID NO:67 Amino acid sequence of a representative SSIIIb-D polypeptide; 1195 amino acids.

[0089] SEQ ID NO: 68 Nucleotide sequence of wheat SSIIIb D genome cDNA, deduced from genomic DNA sequences identified by BLAST using Genbank Accession No: EU333946 as a query to search the EnsemblPlants databases; 4110 nucleotides. The translation start codon ATG is at nucleotides 97-99.

[0090] SEQ ID NO:69 Nucleotide sequence of two contigs from representative wheat SSIIIb-D genes. The sequences of the two contigs were assembled from sequences identified in EnsemblPlants database by BLAST using Genbank Accession No: EU333946 as the query sequence. The three contig sequences in the sequence below are separated by nnnnnn at nucleotides 5450 to 5455; 8011 nucleotides.

[0091] SEQ ID NOs:70-74 Oligonucleotide primers.

DETAILED DESCRIPTION

[0092] 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. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that no other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

[0093] As used herein the singular forms "a", "an" and "the" include plural aspects, and vice versa, 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 "a plant" includes one plant, as well as two or more plants; and so forth.

[0094] As used herein the term "about" in relation to a numerical value or range is intended to cover numbers falling within ± 10% of the specified numerical value or range.

[0095] Each embodiment in this specification is to be applied mutatis mutandis to every other embodiment unless expressly stated otherwise.

[0096] Genes and other genetic material (e.g. mRNA, constructs etc) are represented in italics and their proteinaceous expression products are represented in non-italicised form. Thus, for example, SSIIa is an expression product of SSIIa.

[0097] 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 sequence listing is provided after the claims. A list describing the SEQ ID NOs in the sequence listing is provided after the Figure Legends.

[0098] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the invention pertains. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, preferred methods and materials are described.

[0099] The present invention is based in part on the surprising observations made in the experiments described herein that hexaploid wheat grain comprising one or more or all of the characteristics selected from the group consisting of an increased total fibre content, an increased total dietary fibre (TDF) content, an increased fructan content, an increased ß-glucan content, an increased amylose content and an increased starch content can be produced by combining null mutations in each of the SSIIIa genes, preferably in each of the SSIIa and SSIIIa genes. Each increase is relative to the content of the component in wild-type wheat grain in the case of triple null ssIIIa mutants, or for wheat grain comprising the null mutations in the ssIIa genes but wild-type for the SSIIIa genes in the case of the double triple null ssIIa-ssIIIa mutant grain. The grain can be produced by combining mutations in the three SSIIIa genes on the A, B and D genomes of the wheat by breeding and selection, or by mutagenesis, or by combining such mutations with ssIIa mutations. In a preferred embodiment, hexaploid wheat grain comprising null mutations in each of its three SSIIa genes and each of its three SSIIIa genes can be produced which have an amylose content of at least 45% (w/w). These surprising observations were unexpected on the basis of reports made by others that the level of amylose in triple null

ssIIa hexaploid wheat grain was less than 45% (Yamamori *et al.*, 2000; Konik-Rose *et al.*, 2007). In this content, the amylose content is defined as a percentage of the total starch content of the grain on a weight/weight basis. Furthermore, the wheat grain has other desirable properties including increased total fibre content, based on increases in the fructan, β-glucan, arabinoxylan and cellulose contents, which provides for health benefits when the grain or products produced from the grain are used as food or feed.

[0100] Accordingly, in a first aspect the present invention provides a wheat grain of the species Triticum aestivum, the grain comprising

i) mutations in each of its SSIIIa genes such that the grain is homozygous for a mutation in its SSIIIa-A gene, homozygous for a mutation in its SSIIIa-B gene and homozygous for a mutation in its SSIIIa-D gene, wherein each of the mutations in said SSIIIa genes are null mutations,

ii) a total starch content comprising an amylose content and an amylopectin content,

iii) a fructan content which is increased relative to wild-type wheat grain on a weight basis, preferably between 3% and 12% of the grain weight,

iv) a β-glucan content,

v) an arabinoxylan content,

vi) a cellulose content, and

vii) a total dietary fiber (TDF) content,

the grain having a grain weight of between 25mg and 60mg, wherein one or more or all of the β-glucan content, TDF content, arabinoxylan content and cellulose content are increased relative to the wild-type wheat grain on a weight basis, and/or preferably increased relative to a homozygous, triple null ssIIa wheat grain on a weight basis. In a preferred embodiment, the sum of the fructan content, β-glucan content, arabinoxylan content and cellulose content is between 15% and 30% of the grain weight. In an embodiment, the amylose content is between 45% and 70% on a weight basis of the total starch content of the grain as determined by iodine binding assay, and/or the amylopectin content on a weight basis is reduced relative to the wild-type wheat grain, preferably

wherein the grain further comprises homozygous, null mutations in each of the three SSIIa genes.

[0101] The present invention further provides wheat plants which produced or are obtained from this grain and flour and/or wheat starch granules produced from this grain.

[0102] The present invention also provides food ingredients comprising the grain of the present invention or material produced from this grain. Also provided are food products including these food ingredients and compositions comprising the grain of the present invention or material produced from this grain. The food ingredient may be kibbled, cracked, par-boiled, rolled, pearled, milled or ground grain or any combination of these.

[0103] In another aspect the present invention provides a process for producing a wheat plant that produces the grain of the present invention, the process comprising step (i) crossing two parental wheat plants each comprising a null mutation in each of one, two or three *SSIIIa* genes selected from the group consisting of *SSIIIa*-A, *SSIIIa*-B and *SSIIIa*-D, or of mutagenising a parental plant comprising said null mutations; and step (ii) screening plants or grain obtained from the cross or mutagenesis, or progeny plants or grain obtained therefrom, by analysing DNA, RNA, protein, starch granules or starch from the plants or grain, and step (iii) selecting a fertile wheat plant that has reduced SSIIIa activity relative to at least one of the parental wheat plants of step (i), or grain therefrom. Preferably, one or both parental plants comprise null mutations in one, two or three *ssIIa* genes, whereby the selected wheat plant has a reduced SSIIa polypeptide amount or activity relative to wild-type grain. More preferably, the selected wheat plant or grain therefrom is double triple null mutant for *ssIIa-ssIIIa*.

[0104] In another aspect the present invention provides a process for improving one or more parameters of metabolic health, bowel health or cardiovascular health in a subject in need thereof, or of preventing or reducing the severity or incidence of a metabolic disease such as diabetes, bowel disease or cardiovascular disease, the method comprising providing to the subject the grain or food product of the present invention.

[0105] As used herein "improving one or more parameters of metabolic health" is a relative term and means an improvement in comparison to the consumption of an equivalent amount of food or drink produced with wild-type wheat.

[0106] In a still further aspect the present invention provides a process of producing bins of wheat grain comprising:

a) reaping wheat stalks comprising the wheat grain of the present invention;

b) threshing and/or winnowing the stalks to separate the grain from the chaff; and

c) sifting and/or sorting the grain separated in step b), and loading the sifted and/or sorted grain into bins, thereby producing bins of wheat grain.

[0107] In certain embodiments the wheat grain of the present invention is further characterised by one or more or all of the following features:

i) a starch content of between 30% and 70% or between 32% and 65%, of the grain weight,

ii) the amylose content is between 45% and 65% of the total starch content of the grain as determined by iodine binding assay,

iii) the starch content has a chain length distribution as determined by fluorescence-activated capillary electrophoresis (FACE) after debranching of the starch samples which is increased in the proportion of chain lengths of DP 7-10 and decreased in the proportion of chain lengths DP 11-24, relative to wild-type wheat starch,

iv) the fructan content comprises fructan of DP 3-12 such that at least 50% of the fructan content is of DP 3-12,

v) the fructan content is increased by between 2-fold and 10-fold relative to the wild-type wheat grain on a weight basis,

vi) the β-glucan content is increased by 1% or by 2% on an absolute basis, and/or is increased by between 2-fold and 7-fold relative to the wild-type wheat grain on a weight basis,

vii) the β-glucan content is between 1% and 4% of the grain weight,

viii) the arabinoxylan content is increased by between 1% and 5% on an absolute basis,

ix) the cellulose content is increased by between 1% and 5% on an absolute basis,

x) the grain has a germination rate which is between about 70% and about 100% relative to the wild-type wheat grain, and

xi) the grain, when sown, gives rise to wheat plants which are male and female fertile.

[0108] The grain may also comprise a level and/or activity of an SSIIa or SSIIIa protein which is less than 5% of the level or activity of a SSIIa or SSIIIa protein in the wild-type wheat grain, or which lacks one or more or all of SSIIa-A protein, SSIIa-B protein, SSIIa-D protein, SSIIIa-A protein, SSIIIa-B protein and SSIIIa-D protein. The grain may also be homozygous for a null mutation in its *SSIIa-A* gene, homozygous for a null mutation in its *SSIIa-B* gene and homozygous for a null mutation in its *SSIIa-D* gene. The grain is preferably homozygous for a null mutation in its *SSIIIa-A* gene, homozygous for a null mutation in its *SSIIIa-B* gene and homozygous for a null mutation in its *SSIIIa-D* gene. Each null mutation may be selected, independently, from the group consisting of a deletion mutation, an insertion mutation, a premature translation stop codon, a splice site mutation and a non-conservative amino acid substitution mutation, preferably wherein the grain comprises deletion mutations in each of one, two or three *SSIIa* genes, one, two or three *SSIIIa* genes, or deletions of the genes entirely. In a preferred embodiment, the grain comprises stop codons in each of one, two or three *SSIIa* genes and/or one, two or three *SSIIIa* genes, most preferably in all three *SSIIIa* genes.

[0109] In certain embodiments the grain further comprises a loss of function mutation in an endogenous gene which encodes a starch synthesis polypeptide, or a chimeric polynucleotide which encodes an RNA which reduces the expression of the endogenous gene which encodes the starch synthesis polypeptide, said starch synthesis polypeptide being selected from the group consisting of SSI and SSIV, wherein said mutation is selected from the group consisting of a deletion mutation, an insertion mutation, a premature translation stop codon, a splice site mutation and a non-conservative amino acid substitution mutation. It is preferred that at least one, more than one, or all of the mutations are i) introduced mutations, ii) were induced in a parental wheat plant or seed by mutagenesis with a mutagenic agent such as a chemical agent, biological agent or irradiation, or iii) were introduced in order to modify the plant genome.

[0110] It is preferred that the grain has an amylose content of about 45%, about 50%, about 55% or about 60% on a weight basis of the total starch content of the grain and/or

that the grain is non-transgenic or is free of any exogenous nucleic acid that encodes an RNA which reduces expression of a *SSIIa* gene, and/or an *SSIIIa* gene, and/or a *SBEIIa* gene.

[0111] The SSIIa level and/or activity is determined by assaying the SSIIa level and/or activity in developing endosperm, or by assaying the amount of SSIIa protein in harvested grain by immunological or other means. Likewise, the SSIIIa level and/or activity is determined by assaying the SSIIIa level and/or activity in developing endosperm, or by assaying the amount of SSIIIa protein in harvested grain by immunological or other means. The endosperm may be either from the plant from which the grain was obtained or a progeny plant.

[0112] The starch granules of the grain and/or the starch of the grain of the present invention may also be characterised by one or more of the properties selected from the group consisting of:

i) comprising at least 2% resistant starch;

ii) the starch characterised by a reduced glycaemic index (GI);

iii) the starch granules being distorted in shape;

iv) the starch granules having reduced birefringence when observed under polarized light;

v) the starch characterized by a reduced swelling volume;

vi) modified chain length distribution and/or branching frequency in the starch;

vii) the starch characterized by a reduced peak temperature of gelatinisation;

viii) the starch characterized by a reduced peak viscosity;

ix) reduced starch pasting temperature;

x) reduced peak molecular weight of amylose as determined by size exclusion chromatography;

xi) reduced starch crystallinity; and

xii) reduced proportion of A-type and/or B-type starch, and/or increased proportion of V-type crystalline starch;

wherein each property is relative to wild-type wheat starch granules or wild-type wheat starch.

[0113] In certain embodiments of the present invention the grain is processed so that it is no longer capable of germinating. Examples of such processed grain include heat-treated grain, and kibbled, cracked, par-boiled, rolled, pearled, milled or ground grain. Alternatively, the grain is capable of germinating at a rate between 70% and 100% relative to the wild-type. In an embodiment, the grain of the invention is capable of giving rise to a wheat plant of the invention, i.e. the grain is viable.

[0114] The present invention clearly extends to the grain as described above when comprised in a wheat plant. The present invention also extends to wheat plants which produce, or are obtained from, the grain of the present invention. such wheat plants may be characterised by a level and/or activity of SSIIIa protein in its endosperm which is less than 5% of the level or activity of SSIIIa protein in the wild-type wheat grain, or which preferably lacks one or more or all of SSIIIa-A protein, SSIIIa-B protein and SSIIIa-D protein. In a more preferred embodiment, the grain does not have detectable levels of both SSIIa protein and SSIIIa protein. Preferably, the wheat plant is male and female fertile.

[0115] Flour produced from the grain of the present invention is also encompassed. In an embodiment, the flour is white flour. The flour is preferably wholemeal, or a blend of white flour and wholemeal, for example in a ration from 1:2 to 2:1. The invention also provides wheat bran from the grain of the invention. Each of these products comprise wheat cells having the genetic composition of the wheat grain (i.e the DNA of the wheat grain).

[0116] Wheat starch granules or wheat starch produced from the grain is also part of the present invention. The wheat starch granules or wheat starch will typically comprise 45%, preferably about 50%, about 55% or about 60% amylose, or between 45% and 70% amylose, each on a weight basis as a proportion of the total starch content of the starch granules or starch, the starch granules preferably comprising wheat GBSSI polypeptide. The starch granules and/or starch is will also be characterised by one or more of:

a) having no detectable SSIIIa polypeptide as determined by an immunological means;

b) comprising at least 2% resistant starch on a weight basis;

c) the starch characterised by a reduced glycaemic index (GI);

d) the starch granules being distorted in shape;

e) the starch granules having reduced birefringence when observed under polarized light;

f) the starch characterized by a reduced swelling volume;

g) modified chain length distribution and/or branching frequency in the starch;

h) the starch characterized by a reduced peak temperature of gelatinisation;

i) the starch characterized by a reduced peak viscosity;

j) reduced starch pasting temperature;

k) reduced peak molecular weight of amylose as determined by size exclusion chromatography;

l) reduced starch crystallinity; and

m) reduced proportion of A-type and/or B-type starch, and/or increased proportion of V-type crystalline starch;

wherein each property is relative to wild-type wheat starch granules or starch.

[0117] The present invention also provides a food ingredient that comprises the grain, the flour, preferably the wholemeal, or wheat bran, or the wheat starch granules or wheat starch of present invention, preferably at a level of at least 10%, preferably about 20% to about 80%, more preferably 25% to 70%, on a dry weight basis. The food ingredient may be kibbled, cracked, par-boiled, rolled, pearled, milled or ground grain or any combination of these. The food ingredient may also be incorporated in a food product, preferably at a level of at least 10% or at least 15% on a dry weight basis.

[0118] The present invention also provides a composition comprising the wheat grain, the flour, preferably the wholemeal, or wheat bran, or the wheat starch granules or wheat starch of the present invention, at a level of at least 10% or at least 15% by weight, or wheat grain having a level of amylose lower than 45% (w/w) or flour, wholemeal, starch granules or starch obtained therefrom. The composition may comprise a blend of flours.

[0119] The present invention also provides a process for producing a food comprising steps of (i) adding a food ingredient of the present invention to another food ingredient, and (ii) mixing the food ingredients, thereby producing the food. The process may also

involve processing the grain to produce the food ingredient, prior to step (i), or a step of heating the mixed food ingredients from step (ii) at a temperature of at least 100°C or at least 110°C for at least 10 minutes.

[0120] As used herein, the term "by weight" or "on a weight basis" refers to the weight of a substance as a percentage of the weight of the material or item comprising the substance. This is abbreviated herein as "w/w". For example the amylose content is defined as the weight of amylose as a percentage of the weight of the total starch content.

[0121] The synthesis of starch in the endosperm of higher plants including wheat is carried out by a suite of enzymes that catalyse four key steps, shown schematically in Figure 1. Firstly, ADP-glucose pyrophosphorylase (EC 2.7.7.27) activates the monomer precursor of starch through the synthesis of ADP-glucose from G-1-P and ATP. Secondly, the activated glucosyl donor, ADP-glucose, is transferred to the non-reducing end of a pre-existing α-1,4 linkage by starch synthases (EC 2.4.1.24). Thirdly, starch branching enzymes introduce branch points through the cleavage of a region of α-1,4 linked glucan followed by transfer of the cleaved chain to an acceptor chain, forming a new α-1,6 linkage. Starch branching enzymes are the only enzymes that can introduce the α-1,6 linkages into α-polyglucans and therefore play an essential role in the formation of amylopectin. Fourthly, starch debranching enzymes (EC 2.4.4.18) remove some of the branch linkages.

[0122] In the cereal endosperm, two isoforms of ADP-glucose pyrophosphorylase (ADGP) are present, one form within the amyloplast, and one form in the cytoplasm. Each form is composed of two subunit types. The shrunken (*sh2*) and brittle (*bt2*) mutants in maize represent lesions in large and small subunits respectively.

[0123] As used herein, the term "starch synthase" (SS) refers to an enzyme that transfers a glucosyl residue from the activated glucosyl donor, ADP-glucose, to the non-reducing end of a pre-existing glucan chain by a α-1,4 linkage (EC 2.4.1.24). SS enzyme activity may be assayed as described by Guan and Keeling (1998). At least five classes of starch synthase are found in the cereal endosperm including in hexaploid wheat *T. aestivum*, namely an isoform exclusively localised within or bound to the starch granule, granule-bound starch synthase (GBSS), two forms that are partitioned between the granule and the soluble fraction (SSI and SSII), a form that is entirely located in the soluble fraction

(SSIII) and more recently a fifth form, SSIV (Figure 1). Each of these are included in term "starch synthase". They are active during endosperm development during growth of the wheat plant, when storage starch is being synthesized and deposited, but may be present in an inactive state in mature (dormant) wheat grain. GBSS has been shown to be essential for amylose synthesis. Each of SSI-IV are involved primarily in amylopectin synthesis on the basis of biochemical and genetic evidence. For example, mutations in *SSII* and *SSIII* genes have been shown to alter amylopectin structure (Schondelmaier *et al.*, 1992; Yamamori *et al.*, 2000). Starch synthases are classified according to their amino acid sequence as belonging to one of these five groups based on the extent of homology to known members of these classes.

[0124] Within cereals, at least two sub-classes of SSII have been identified, SSIIa and SSIIb, although a third subclass SSIIc has been identified in rice (Ohdan *et al.*, 2005) and genes which appear to encode a corresponding SSIIc enzyme in wheat are identified as described in Example 2. Starch synthase IIa (SSIIa) primarily catalyses the polymerisation of intermediate length glucan chains (DP 12-24) of amylopectin in the endosperm of cereals by transferring a glucosyl moiety from ADP-Glucose to the non-reducing end of a pre-existing α-1,4-linked glucan chains (Fontaine *et al.*, 1993). SSIIa thereby elongates short chains (DP<10) of amylopectin. In a wheat *ssIIa* mutant, glucan chains of DP 6-11 were increased in frequency and chains of DP 11-25 were decreased in frequency (Yamamori *et al*, 2000). The different classes of SSII and SSIIIa are distinguished by their homology to amino acid sequences of type members of each class i.e. by phylogenetic analysis. Different SSII and SSIIIa enzymes and in some cases different wheat SSIIa isoenzymes can be distinguished by the number of amino acids in the polypeptides, as described below.

[0125] The level of expression of genes encoding SSII, SSIIa or SSIIIa may be assessed by assessing transcript levels such as by Northern blot hybridisation analysis or by RT-PCR analysis. In a preferred method, the amount of SSIIa or SSIIIa protein in grain or developing endosperm is measured by separating the proteins in extracts of the grain/endosperm on gels by electrophoresis, then transferring the proteins to a membrane by Western blotting, followed by quantitative detection of the protein on the membrane using specific antibodies ("Western blot analysis"). Exemplary methods for gel electrophoresis and immunoblotting are described in Example 1.

[0126] Starch synthase I (SSI) appears to exist as a single isoform in cereals. In rice, SSI accounts for about 70% of the total soluble SS activity in the endosperm (Fujita *et al*, 2006). SSI preferentially synthesizes short glucan chains of DP6-15, preferring the shortest amylopectin chains as substrates. Despite its important role, the complete absence of SSI enzyme in the rice endosperm does not affect the size and shape of the seed or starch granules, suggesting that other SS enzymes are capable of compensating for absent SSI function.

[0127] In contrast, SSIIIa produces the relatively longer chains of amylopectin, particularly of DP>30, and extends intermediate length glucan chains. *ssIIIa* mutants exhibit an increase in intermediate length chains in the amylopectin. There are two forms of SSIII, SSIIIa being the main form expressed in endosperm, and SSIIIb being a minor form. Little is known about the contribution of SSIV isoforms to glucan chain length in cereal grain, but it appears to function mostly in leaves (Leterrier *et al*., 2008). Two SSIV genes, SSIVa and SSIVb, are expressed in rice throughout the plant and at relatively constant levels during grain filling, so they appear to have a function throughout the plant. *ssIV* mutants in *Arabidopsis* had decreased levels of leaf starch.

[0128] Each of the starch synthases are expressed as polypeptides with N-terminal signal peptides that are cleaved off during translocation into the amyloplasts.

[0129] As used herein, "starch branching enzyme" (SBE) means an enzyme that introduces α-1,6 glycosidic bonds between chains of glucose residues (EC 2.4.1.18), thereby introducing the α-1,6 branch points in amylopectin. Two main classes of SBEs are known in plants, SBEI and SBEII. SBEII can be further categorized into two types in cereals, SBEIIa and SBEIIb (Hedman and Boyer, 1982; Boyer and Preiss, 1978; Mizuno *et al*., 1992, Sun *et al.,* 1997). Additional forms of SBEs are also reported in some cereals, a putative 149 kDa SBEI from wheat and a 50/51 kDa SBE from barley. Sequence alignment revealed a high degree of sequence similarity at both the nucleotide and amino acid levels and allows the grouping into the SBEI, SBEIIa and SBEIIb classes. The amino acid sequences of SBEIIa and SBEIIb generally exhibit around 80% identity to each other, mostly concentrated in the central regions of the polypeptides.

[0130] SBEI, SBEIIa and SBEIIb may also be distinguished by their expression patterns, but this differs in different species. In wheat endosperm, SBEI (Morell *et al*, 1997) is

found exclusively in the soluble fraction, while SBEIIa and SBEIIb are found in both soluble and starch-granule associated fractions (Rahman *et al.,* 1995). In maize, SBEIIb is the predominant form in the endosperm whereas SBEIIa is expressed relatively more strongly in the leaf and appears to be expressed throughout the plant (Gao *et al.*, 1997). In rice, SBEIIa and SBEIIb are found in the endosperm in approximately equal amounts. However, there are also differences in timing of gene expression. SBEIIa is expressed at an earlier stage of seed development, being detected at 3 days after flowering, and was expressed in leaves, while SBEIIb was not detectable at 3 days after flowering and was most abundant in developing seeds at 7-10 days after flowering and was not expressed in leaves. In wheat endosperm, SBEIIa is expressed about 3-4-fold more highly than SBEIIb. Different cereal species show significant differences in SBEIIa and SBEIIb expression, and conclusions drawn in one species cannot readily be applied to another species. Specific antibodies may also be used to distinguish the enzymes.

[0131] Genomic and cDNA sequences for each of the SBE genes have been characterized, including for wheat. Sequence alignment reveals a high degree of sequence similarity at both the nucleotide and amino acid levels, but also the sequence differences and allows the grouping into the SBEI, SBEIIa and SBEIIb classes. In wheat, apparent gene duplication events have increased the number of SBEI genes in each genome (Rahman *et al.*, 1999). The elimination of greater than 97% of the SBEI activity in wheat endosperm by combining mutations in the highest expressing forms of the SBEI genes from the A, B and D genomes had no measurable impact on starch structure or functionality (Regina *et al.*, 2004). In contrast, reduction of SBEIIa expression by a gene silencing construct in hexaploid wheat resulted in high amylose levels (>70%), while a corresponding construct that reduced SBEIIb expression but not SBEIIa had minimal effect (Regina *et al.*, 2006). In barley, a gene silencing construct which reduced both *SBEIIa* and *SBEIIb* expression in endosperm was used to generate high amylose barley grain (Regina *et al.,* 2010). In maize, *SBEIIb* mutants known as *amylose extender* (*ae*) produced high amylose phenotypes.

[0132] Enzyme activity assays of branching enzymes to detect the activity of all three isoforms, SBEI, SBEIIa and SBEIIb are based on the method of Nishi *et al.*, 2001 with minor modification as follows. After electrophoresis, the gel is washed twice in 50 mM HEPES, pH 7.0 containing 10% glycerol and incubated at room temperature in a reaction

mixture consisting of 50 mM HEPES, pH 7.4, 50 mM glucose-1-phosphate, 2.5 mM AMP, 10% glycerol, 50 U phosphorylase a, 1mM DTT and 0.08% maltotriose for 16 h. The bands are visualised with a solution of 0.2% (w/v) I_2 and 2% KI. The SBEI, SBEIIa and SBEIIb isoform-specific activities are separated under these conditions of electrophoresis. This is confirmed by immunoblotting using anti-SBEI, anti-SBEIIa and anti-SBEIIb antibodies. Densitometric analysis of immunoblots which measures the intensity of each band is conducted to determine the level of enzyme activity of each isoform.

[0133] Starch branching enzyme (SBE) activity may be measured by enzyme assay, for example by the phosphorylase stimulation assay (Boyer and Preiss, 1978). This assay measures the stimulation by SBE of the incorporation of glucose 1-phosphate into methanol-insoluble polymer (α-D-glucan) by phosphorylase A. Isoforms of SBE show different substrate specificities, for example SBEI exhibits higher activity in branching amylose, while SBEIIa and SBEIIb show higher rates of branching with an amylopectin substrate. SBEI preferentially produces longer chains with DP>16 by branching less branched polyglucans, whereas SBEII isoenzymes generates shorter chains with DP<12. The isoforms may also be distinguished on the basis of the length of the glucan chain that is transferred.

[0134] Two classes of debranching enzyme (DBE) are known in cereals, namely isoamylase (ISA) and pullulanase (PUL). ISA mainly debranches phytoglycogen and amylopectin, whereas PUL acts upon pullulan and amylopectin but not phytoglycogen (Nakamura *et al.*, 1996). Mutants for ISA have been termed *sugary* and produce more short chains of amylopectin, and ISA therefore appears to act in the editing of excessively branched chains or improper branches in amylopectin. In contrast, PUL is believed to function in starch degradation in germinating grain as well as starch synthesis.

[0135] Developing hexaploid wheat endosperm expresses SSIIa from the *SSIIa* genes on each of the A, B and D genomes. As used herein, "SSIIa expressed from the A genome" or "SSIIa-A" means a polypeptide whose amino acid sequence is set forth in SEQ ID NO:1 or which is at least 99% identical to the amino acid sequence set forth in SEQ ID NO: 1 or comprising such a sequence. The amino acid sequence provided as SEQ ID NO:1 (Li *et al.*, 1999; Genbank Accession No. AAD53263) is used herein as the reference sequence for a wild-type SSIIa-A polypeptide. The polypeptide of SEQ ID NO:1 is 799 amino acid residues long, as would amino acid substitution mutants of SEQ ID NO:1. Enzymatically

active variants of this enzyme exist in wheat, for example in cultivar Fielder, see Accession No. CAB96626.1 (Gao and Chibbar, 2000) whose amino acid sequence is 99.5% (795/799) identical to SEQ ID NO.1, and in diploid relatives of *Triticum aestivum* such as from *Triticum urartu*, provided as Accession Nos. CUS28065.1 and CDI68213.1. Such variants are included in "SSIIa-A". SSIIa-A does not include the homoeologous polypeptides, SSIIa-B and SSIIa-D, because those polypeptides are about 96% identical to SEQ ID NO:1.

[0136] As used herein, "SSIIa expressed from the B genome" or "SSIIa-B" means a polypeptide whose amino acid sequence is set forth in SEQ ID NO:2 or which is at least 99% identical to the amino acid sequence set forth in SEQ ID NO:2 or comprising such a sequence. The amino acid sequence provided as SEQ ID NO:2 (Genbank Accession No. CAB99627.1) corresponds to the starch synthase IIa expressed from the B genome of wheat variety Fielder, which is used herein as the reference sequence for a wild-type SSIIa-B polypeptide. The polypeptide of SEQ ID NO:2 is 798 amino acids long, as would amino acid substitution mutants of SEQ ID NO:2. Enzymatically active variants of this enzyme exist in wheat, such variants are included in "SSIIa-B". SSIIa-B does not include the homoeologous polypeptides, SSIIa-A and SSIIa-D, because those polypeptides are about 96% identical to SEQ ID NO:2 (Li *et al.*, 1999).

[0137] As used herein, "SSIIa expressed from the D genome" or "SSIIa-D" means a polypeptide whose amino acid sequence is set forth in SEQ ID NO:3 or which is at least 99% identical to the amino acid sequence set forth in SEQ ID NO:3 or comprising such a sequence. The amino acid sequence of SEQ ID NO:3 (Genbank Accession No. BAE48800; Shimbata *et al.*, 2005) corresponds to the SSIIa expressed from the D genome in wheat cultivar Chinese Spring, which is used herein as the reference sequence for wild-type SSIIa-D. The protein of SEQ ID NO:3 is 799 amino acids long. Enzymatically active variants of this enzyme exist in wheat, for example in cultivar Fielder, see Accession No. CAB86618 (Gao and Chibbar, 2000) whose amino acid sequence is 99.9% (798/799) identical to SEQ ID NO.3, and in diploid relatives of *Triticum aestivum* such as *Aegilops tauschii*, a likely progenitor of the D genome of hexaploid wheat, provided as Accession No CAB86618. Such variants are included in "SSIIa-D". SSIIa-D does not include the homoeologous polypeptides, SSIIa-A and SSIIa-B, because those polypeptides are about 96% identical to SEQ ID NO:3. The amino acid sequence provided as SEQ ID NO: 3 is

95.9% identical to each of SEQ ID NO:1 and SEQ ID NO:2. Alignment of the three amino acid sequences shows amino acid differences which may be used to distinguish the proteins or to classify variants as SSIIa-A, SSIIa-B or SSIIa-D.

[0138] When comparing amino acid sequences to determine the percentage identity in this context, for example by Blastp, the full length sequences should be compared, and gaps in a sequence counted as amino acid differences.

[0139] As used herein, an "SSIIa polypeptide" means an SSIIa-A polypeptide, SSIIa-B polypeptide or SSIIa-D polypeptide.

[0140] As used herein, each of the SSIIa-A, SSIIa-B and SSIIa-D polypeptides include polypeptide variants which have reduced or no starch synthase enzyme activity, as well as polypeptides having wild-type or essentially wild-type enzyme activity. Comparison of the amino acid sequence of a mutant form of an SSIIa polypeptide with SEQ ID NOs:1, 2 and 3 is used to determine which of the SSIIa-A, -B or -D polypeptides it is derived from and so to classify the mutant form. For example, a mutant SSIIa polypeptide is considered to be a mutant SSIIa-A polypeptide if its amino acid sequence is more closely related, i.e. having a higher degree of sequence identity, to SEQ ID NO:1 than to SEQ ID NOs:2 and 3. Analogously, a mutant SSIIa polypeptide is a mutant SSIIa-B polypeptide if it is more closely related to SEQ ID NO:2 than SEQ ID NOs:1 or 3, and a mutant SSIIa polypeptide is a mutant SSIIa-D polypeptide if it is more closely related to SEQ ID NO:3 than SEQ ID NOs:1 and 2. Those skilled in the art are thereby able to classify a mutant SSIIa polypeptide.

[0141] A mutant SSIIa polypeptide may have reduced starch synthase enzyme activity (a partial mutant) or lacks starch synthase activity (null mutant polypeptide). A mutant *SSIIa* gene may be expressed to produce an SSIIa polypeptide in wheat endosperm, for example a truncated polypeptide, or it may not be expressed, and produce no polypeptide at all. It may also be expressed to produce a transcript but no translation product.

[0142] It is also understood that SSIIa proteins may be present in grain, particularly mature grain as commonly harvested commercially, but in an inactive or dormant state because of the physiological conditions in the grain. Such polypeptides are included in "SSIIa polypeptides" as used herein. The SSIIa polypeptides may be enzymatically active during only part of grain development, in particular in developing endosperm when storage

starch is typically deposited, but in an inactive state otherwise. Such SSIIa polypeptides may be detected and quantitated readily using immunological methods such as Western blot analysis.

[0143] Thus, "wild-type" as used herein when referring to an SSIIa-A polypeptide means a polypeptide whose amino acid sequence is set forth in SEQ ID NO: 1 or enzymatically active variants at least 99% identical in amino acid sequence which are found in nature and which have essentially the same activity as SEQ ID NO:1; "wild-type" as used herein when referring to SSIIa-B means a polypeptide whose amino acid sequence is set forth in SEQ ID NO: 2 or enzymatically active variants at least 99% identical in amino acid sequence which are found in nature and which have essentially the same activity as the polypeptide whose sequence is provided as SEQ ID NO:2; "wild-type" as used herein when referring to SSIIa-D means a polypeptide whose amino acid sequence is set forth in SEQ ID NO: 3 or enzymatically active variants at least 99% identical in amino acid sequence which are found in nature and which have essentially the same activity as the polypeptide whose sequence is provided as SEQ ID NO:3. In each case, the wild-type polypeptide has starch synthase II activity and has not been modified by the present invention.

[0144] Wild-type wheat produces two other classes of SSII polypeptides, namely SSIIb and SSIIc polypeptides. As used herein, "SSIIb expressed from the A genome" or "SSIIb-A" means a polypeptide whose amino acid sequence is set forth in SEQ ID NO:10 or which is at least 99% identical to the amino acid sequence set forth in SEQ ID NO:10 or comprising such a sequence. The amino acid sequence provided as SEQ ID NO:10 (deduced from the nucleotide sequence of Genbank Accession No. AK332724) is used herein as the reference sequence for a wild-type SSIIb-A polypeptide. The polypeptide of SEQ ID NO:10 is 676 amino acid residues long. Enzymatically active variants of this enzyme are included in "SSIIb-A". SSIIb-A does not include the homoeologous polypeptide SSIIb-D which is about 90% identical to SEQ ID NO:8.

[0145] As used herein, "SSIIb expressed from the D genome" or "SSIIb-D" means a polypeptide whose amino acid sequence is set forth in SEQ ID NO:11 or which is at least 99% identical to the amino acid sequence set forth in SEQ ID NO:11 or comprising such a sequence. The amino acid sequence provided as SEQ ID NO:11 (Genbank Accession No. ABY56824) corresponds to the starch synthase IIb expressed from the D genome of bread

wheat, which is used herein as the reference sequence for a wild-type SSIIb-D polypeptide. The polypeptide of SEQ ID NO:11 is 674 amino acids long. Enzymatically active variants of this enzyme are included in "SSIIb-D". SSIIb-D does not include the homoeologous polypeptide SSIIb-A which is about 90% identical to SEQ ID NO:11.

[0146] The amino acid sequences of the SSIIa homoeologs and the SSIIb homoeologs are 71-79% identical, and therefore the polypeptides can be readily distinguished even though they have similar enzyme activities.

[0147] As described in Example 2 herein, wheat SSIIc sequences were also identified and could be readily distinguished from the SSIIa sequences.

[0148] As used herein, the terms "*SSIIa* gene" and "wheat *SSIIa* gene" and the like refer to the genes that encode a SSIIa polypeptide, including wild-type SSIIa polypeptides such as homologous polypeptides present in other wheat varieties as well as mutant forms of the genes which either encode SSIIa polypeptides with reduced activity or undetectable activity, or genes which were derived therefrom by mutation. *SSIIa* genes include, but are not limited to, the wheat *SSIIa* genes which have been cloned, including the genomic and cDNA sequences listed in Table 1 which are annotated as *SSIIa* genes. The term *SSIIa* gene includes, collectively, each of the more specific terms "*SSIIa-A* gene", "*SSIIa-B* gene" and "*SSIIa-D* gene", encoding an SSIIa-A, SSIIa-B and SSIIa-D polypeptide, respectively, or mutant forms derived from such genes. The *SSIIa* genes as used herein encompasses mutant forms which do not encode any polypeptide at all, or polypeptides which have no starch synthase activity, in which cases the mutant forms represent null alleles of the genes. Alleles of the genes include mutant alleles where at least part of the gene is deleted, including where the entire gene is deleted, which alleles also represent null alleles of the genes.

[0149] An "endogenous *SSIIa* gene" refers to an *SSIIa* gene which is in its native location in the wheat genome, including wild-type and mutant forms. As is understood in the art, hexaploid wheats such as bread wheat comprise three genomes which are commonly designated the A, B and D genomes, while tetraploid wheats such as durum wheat comprise two genomes commonly designated the A and B genomes. Each genome comprises 7 pairs of chromosomes which may be observed by cytological methods during meiosis and thus identified, as is well known in the art. Endogenous *SSIIa-A* genes, *SSIIa-*

B genes and *SSIIa-D* genes are located on the short arm of chromosomes 7A, 7B and 7D, respectively, in hexaploid wheat. In contrast, the terms "isolated *SSIIa* gene" and "exogenous *SSIIa* gene" refer to an *SSIIa* gene which is not in its native location, for example having been removed from a wheat plant, cloned, synthesized, comprised in a vector or in the form of a transgene in a cell, such as transgene in a transgenic wheat plant. The *SSIIa* gene in this context may be any of the specific forms as described as follows.

Table 1. Starch synthase enzyme genes characterized from cereals

Species	SS isoform	Type of clone	Accession No.	Reference
Wheat	SSI	cDNA and genomic	AF091803 (cDNA) AF091802 (genomic)	Li *et al.*, 1999 Li *et al.*, 1999
	SSIIa-A	cDNA and genomic	AF155217 (cDNA) AB201445 (genomic)	Li *et al.*, 1999 Shimbata *et al.*, 2005
	SSIIa-B	cDNA and genomic	AJ269504 (cDNA) AB201446 (genomic)	Gao and Chibbar, 2000 Shimbata *et al.*, 2005
	SSIIa-D	cDNA and genomic	AJ269502 (cDNA) AB201447 (genomic)	Gao and Chibbar, 2000 Shimbata *et al.*, 2005
	SSIIb-A	cDNA and genomic	AK332724 (cDNA) Traes_6AL_AE01DC0EA, 6A:187503905 bp to 187505233 bp (genomic)	Kawaura *et al.*, 2009 The IWGSC databases
	SSIIb-B	cDNA and genomic	Traes_6BL_61D83E262, 6B:162116364 - 162116691 bp (genomic)	The IWGSC databases
	SSIIb-D	cDNA and genomic	EU333947 (cDNA) Traes_6DL_19F1042C7, 6D: 147050072 - 147051031 bp (genomic)	NCBI The IWGSC databases
	SSIIc-A	cDNA and genomic	Traes_1AL_729BF3204, 1A: 68687585 – 68688377,	The IWGSC databases
	SSIIc-B	cDNA and genomic	Traes_1BL_447468BDE, 1B: 31475067-314776087 bp	The IWGSC databases
	SSIIc-D	cDNA and genomic	EU307274 (cDNA) Traes_1DL_F667ED844, IWGSC_CSS_1DL_scaff_2205619:1950-3041 bp	NCBI The IWGSC databases
	SSIIIa	cDNA	AF258608 (cDNA) AF258609 (genomic)	Li *et al.*, 2000 Li *et al.*, 2000
	SSIIIb	cDNA and genomic	EU333946 (cDNA)	NCBI
	SSIVa	cDNA	AY044844 (cDNA) DQ400416 (genomic)	NCBI Leterrier *et al.*, 2008
Rice	SSIIa	cDNA	AF419099 (cDNA)	NCBI
	SSIIb	cDNA	AF395537 (cDNA)	NCBI
	SSIIc	cDNA	AF383878 (cDNA)	NCBI
Barley	SSIIa	cDNA	AY133249 (cDNA)	Li *et al.*, 2003
	SSIIb	cDNA	AK372518 (cDNA)	Matsumoto *et al.*, 2011
	SSIIc	cDNA	AK372414 (cDNA)	Matsumoto *et al.*, 2011
Maize	SSIIa	cDNA	AF019296 (cDNA)	Harn *et al.*, 1998
	SSIIb	cDNA	NM_001112544 (cDNA)	Schnable *et al.*, 2009

Species	SS isoform	Type of clone	Accession No.	Reference
	SSIIc	cDNA	EU284113 (cDNA)	Yan *et al.*, 2008
Arabidopsis	SSII	cDNA	NM_110984 (cDNA)	Salanoubat *et al.*, 2000

[0150] As used herein, "a *SSIIa* gene on the A genome of wheat" or "*SSIIa*-A gene" means any polynucleotide which encodes an SSIIa-A polypeptide as defined herein or which is derived from a polynucleotide which encodes SSIIa-A in a wheat plant, including naturally occurring polynucleotides, sequence variants or synthetic polynucleotides, including "wild-type *SSIIa-A* gene(s)" which encode an SSIIa-A polypeptide with essentially wild-type SSIIa activity, and "mutant *SSIIa-A* gene(s)" which do not encode an SSIIa-A polypeptide with essentially wild-type activity but which are recognizably derived from a wild-type *SSIIa-A* gene. Comparison of the nucleotide sequence of a mutant form of an *SSIIa* gene with a suite of wild-type *SSIIa* genes is used to determine which of the *SSIIa* genes it is derived from and so to classify it. For example, a mutant *SSIIa* gene is considered to be a mutant *SSIIa-A* gene if its nucleotide sequence is more closely related, i.e. having a higher degree of sequence identity, to a wild-type *SSIIa-A* gene than to any other *SSIIa* gene. A mutant *SSIIa-A* gene encodes an SSIIa polypeptide with reduced starch synthase enzyme activity (partial mutant), or a polypeptide which lacks starch synthase activity or no protein at all (null mutant gene). An exemplary nucleotide sequence of a cDNA corresponding to an *SSIIa-A* gene is given in SEQ ID NO:4 (Genbank Accession No. AF155217; Li *et al.*, 1999). Other exemplary nucleotide sequences are provided in Accession Nos. AK330838 (cDNA from *SSIIa-A* gene from cultivar Chinese Spring, Kawaura *et al.*, 2009), and Accession No. AJ269503 which provides a cDNA from an *SSIIa-A* gene from cultivar Fielder (Gao and Chibbar, 2000).

[0151] As used herein, the terms "*SSIIa* gene on the B genome" or "*SSIIa-B* gene", and "*SSIIa* gene on the D genome" or "*SSIIa-D* gene" have corresponding meanings to that for *SSIIa-A* in the previous paragraph. An exemplary nucleotide sequence of a cDNA corresponding to an *SSIIa-B* gene is given in SEQ ID NO:5 (Genbank Accession No. AJ269504; Gao and Chibbar 2000) and of an *SSIIa-D* gene is given in SEQ ID NO:6 (Genbank Accession No. AJ269502; from cultivar Fielder, Gao and Chibbar, 2000). Sequences of parts of *SSIIa* genes are also given herein as referred to in Figures 2-4.

[0152] As used herein, "a *SSIIb* gene on the A genome of wheat" or "*SSIIb*-A gene" means any polynucleotide which encodes an SSIIb-A polypeptide as defined herein or which is derived from a polynucleotide which encodes SSIIb-A in a wheat plant, including naturally occurring polynucleotides, sequence variants or synthetic polynucleotides, including "wild-type *SSIIb-A* gene(s)" which encode an SSIIb-A polypeptide with essentially wild-type SSIIb activity, and "mutant *SSIIb-A* gene(s)" which do not encode an SSIIb-A polypeptide with essentially wild-type activity but which are recognizably derived from a wild-type *SSIIb-A* gene. Comparison of the nucleotide sequence of a mutant form of an *SSIIb* gene with a suite of wild-type *SSIIb* genes is used to determine which of the *SSIIb* genes it is derived from and so to classify it. For example, a mutant *SSIIb* gene is considered to be a mutant *SSIIb-A* gene if its nucleotide sequence is more closely related, i.e. having a higher degree of sequence identity, to a wild-type *SSIIb-A* gene than to any other *SSIIb* gene. A mutant *SSIIb-A* gene encodes an SSIIb polypeptide with reduced starch synthase enzyme activity (partial mutant), or a polypeptide which lacks starch synthase activity or no protein at all (null mutant gene). An exemplary nucleotide sequence of a cDNA corresponding to an *SSIIb-A* gene is given in SEQ ID NO:12 (Genbank Accession No. AK332724).

[0153] As used herein, the terms "*SSIIa* gene on the B genome" or "*SSIIa-B* gene", and "*SSIIa* gene on the D genome" or "*SSIIa-D* gene" have corresponding meanings to that for *SSIIa-A* in the previous paragraph. An exemplary nucleotide sequence of a cDNA corresponding to an *SSIIa-B* gene is given in SEQ ID NO:5 (Genbank Accession No. AJ269504; Gao and Chibbar 2000) and of an *SSIIa-D* gene is given in SEQ ID NO:6 (Genbank Accession No. AJ269502; from cultivar Fielder, Gao and Chibbar, 2000).

[0154] The *SSIIa* genes as defined above include any regulatory sequences that are 5' or 3' of the transcribed region, including the promoter region, that regulate the expression of the associated transcribed region, and introns within the transcribed regions. An exemplary nucleotide sequence of an SSIIa gene is provided as SEQ ID NO:7, which provides the nucleotide sequence of an *SSIIa-A* gene from the A genome of wheat; Accession No. AB201445. In analogous fashion, the nucleotide sequence of a wild-type *SSIIa-B* gene of wheat is provided as Accession number AB201446 (IWGSC: Chromosome 7DS, Traes_7DS_E6C8AF743, 3877787: 1 to 396 bp, 5137 to 5419 bp forward strand) and of an *SSIIa-D* gene of wheat is provided as Accession number AB201447 (IWGSC:

Chromosome 7DS, Traes_7DS_E6C8AF743, 3877787: 1 to 396 bp, 5137 to 5419 bp forward strand). Each of these wild-type genes were from wheat cultivar Chinese Spring (Shimbata *et al.*, 2005).

[0155] It would be understood that there is natural variation in the sequences of *SSIIa* genes from different wheat varieties. The homoeologous genes are readily recognizable by the skilled artisan on the basis of sequence identity. The degree of sequence identity between the nucleotide sequences of the homoeologous wild-type *SSIIa* genes and the amino acid sequences of the wild-type polypeptides is 95-96%.

[0156] Developing hexaploid wheat endosperm also expresses SSIIIa from the *SSIIIa* genes on each of the A, B and D genomes. As used herein, "SSIIIa expressed from the A genome" or "SSIIIa-A" means a polypeptide whose amino acid sequence is set forth in SEQ ID NO:52 or which is at least 98% identical to the amino acid sequence set forth in SEQ ID NO:52 or comprising such a sequence. The amino acid sequence provided as SEQ ID NO:52 is used herein as the reference sequence for a wild-type SSIIIa-A polypeptide. The polypeptide of SEQ ID NO:52 is 1629 amino acid residues long, as would amino acid substitution mutants of SEQ ID NO:52. Enzymatically active variants of this enzyme exist in wheat and are included in "SSIIIa-A". SSIIIa-A does not include the homoeologous polypeptides, SSIIIa-B and SSIIIa-D, because those polypeptides are about 94-96% identical to SEQ ID NO:52.

[0157] As used herein, "SSIIIa expressed from the B genome" or "SSIIIa-B" means a polypeptide whose amino acid sequence is set forth in SEQ ID NO:55 or which is at least 98% identical to the amino acid sequence set forth in SEQ ID NO:55 or comprising such a sequence. The amino acid sequence provided as SEQ ID NO:55 is used herein as the reference sequence for a wild-type SSIIa-B polypeptide. The polypeptide of SEQ ID NO:55 is 1612 amino acids long, as would amino acid substitution mutants of SEQ ID NO:55. Enzymatically active variants of this enzyme exist in wheat, such variants are included in "SSIIIa-B". SSIIIa-B does not include the homoeologous polypeptides, SSIIIa-A and SSIIIa-D, because those polypeptides are about 94-96% identical to SEQ ID NO:55.

[0158] As used herein, "SSIIIa expressed from the D genome" or "SSIIIa-D" means a polypeptide whose amino acid sequence is set forth in SEQ ID NO:58 or which is at least 98% identical to the amino acid sequence set forth in SEQ ID NO:58 or comprising such a

sequence. The amino acid sequence of SEQ ID NO:58 (Genbank Accession No. AAF88000; Li *et al.*, 2000) corresponds to the SSIIIa expressed from the D genome in *Aegilops tauschii*, a progenitor of hexaploid wheat, and is used herein as the reference sequence for wild-type SSIIIa-D. The protein of SEQ ID NO:58 is 1611 amino acids long. Enzymatically active variants of this enzyme exist in wheat, which variants are included in "SSIIIa-D". SSIIIa-D does not include the homoeologous polypeptides, SSIIIa-A and SSIIIa-B, because those polypeptides are about 94-96% identical to SEQ ID NO:58. Alignment of the three amino acid sequences shows amino acid differences which may be used to distinguish the proteins or to classify variants as SSIIIa-A, SSIIIa-B or SSIIIa-D, see for example Figure 27.

[0159] When comparing amino acid sequences to determine the percentage identity in this context, for example by Blastp, the full length sequences should be compared, and gaps in a sequence counted as amino acid differences.

[0160] As used herein, an “SSIIIa polypeptide” means an SSIIIa-A polypeptide, SSIIIa-B polypeptide or SSIIIa-D polypeptide.

[0161] As used herein, each of the SSIIIa-A, SSIIIa-B and SSIIIa-D polypeptides include polypeptide variants which have reduced or no starch synthase enzyme activity, as well as polypeptides having wild-type or essentially wild-type enzyme activity. Comparison of the amino acid sequence of a mutant form of an SSIIIa polypeptide with SEQ ID NOs:52, 55 and 58 is used to determine which of the SSIIIa-A, -B or -D polypeptides it is derived from and so to classify the mutant form. For example, a mutant SSIIIa polypeptide is considered to be a mutant SSIIIa-A polypeptide if its amino acid sequence is more closely related, i.e. having a higher degree of sequence identity, to SEQ ID NO:52 than to SEQ ID NOs:55 and 58. Analogously, a mutant SSIIIa polypeptide is a mutant SSIIIa-B polypeptide if it is more closely related to SEQ ID NO:55 than SEQ ID NOs:52 and 58, and a mutant SSIIIa polypeptide is a mutant SSIIIa-D polypeptide if it is more closely related to SEQ ID NO:58 than SEQ ID NOs:52 and 55. Those skilled in the art are thereby able to classify a mutant SSIIIa polypeptide.

[0162] A mutant SSIIIa polypeptide may have reduced starch synthase enzyme activity (a partial mutant) or lacks starch synthase activity (null mutant polypeptide). A mutant *SSIIIa* gene may be expressed to produce an SSIIIa polypeptide in wheat endosperm, for example

a truncated polypeptide, or it may not be expressed, and produce no polypeptide at all. It may also be expressed to produce a transcript but no translation product.

[0163] It is also understood that SSIIIa proteins may be present in grain, particularly mature grain as commonly harvested commercially, but in an inactive or dormant state because of the physiological conditions in the grain. Such polypeptides are included in "SSIIIa polypeptides" as used herein. The SSIIIa polypeptides may be enzymatically active during only part of grain development, in particular in developing endosperm when storage starch is typically deposited, but in an inactive state otherwise. Such SSIIIa polypeptides may be detected and quantitated readily using immunological methods such as Western blot analysis.

[0164] Thus, "wild-type" as used herein when referring to an SSIIIa-A polypeptide means a polypeptide whose amino acid sequence is set forth in SEQ ID NO:52 or enzymatically active variants at least 98% identical in amino acid sequence which are found in nature and which have essentially the same activity as SEQ ID NO:52; "wild-type" as used herein when referring to SSIIIa-B means a polypeptide whose amino acid sequence is set forth in SEQ ID NO:55 or enzymatically active variants at least 98% identical in amino acid sequence which are found in nature and which have essentially the same activity as the polypeptide whose sequence is provided as SEQ ID NO:55; "wild-type" as used herein when referring to SSIIIa-D means a polypeptide whose amino acid sequence is set forth in SEQ ID NO:58 or enzymatically active variants at least 98% identical in amino acid sequence which are found in nature and which have essentially the same activity as the polypeptide whose sequence is provided as SEQ ID NO:58. In each case, the wild-type polypeptide has starch synthase III activity and has not been modified by the present invention.

[0165] Wild-type wheat produces another class of SSIII polypeptide, namely SSIIIb polypeptide. As used herein, "SSIIIb expressed from the A genome" or "SSIIIb-A" means a polypeptide whose amino acid sequence comprises the sequence set forth in SEQ ID NO:61 or which is at least 98% identical to the amino acid sequence set forth in SEQ ID NO:61 or comprising such a sequence. The amino acid sequence provided as SEQ ID NO:61 is used herein as the reference sequence for a wild-type SSIIIb-A polypeptide. The polypeptide of SEQ ID NO:61 is 1002 amino acid residues long, which is about 200 amino

acids short of full length. Enzymatically active variants of this enzyme are included in "SSIIIb-A".

[0166] As used herein, "SSIIIb expressed from the B genome" or "SSIIIb-B" means a polypeptide whose amino acid sequence comprises the sequence set forth in SEQ ID NO:64 or which is at least 98% identical to the amino acid sequence set forth in SEQ ID NO:64 or comprising such a sequence. The amino acid sequence provided as SEQ ID NO:64 is used herein as the reference sequence for a wild-type SSIIIb-A polypeptide. The polypeptide of SEQ ID NO:64 is 1276 amino acid residues long, believed to be full length. Enzymatically active variants of this enzyme are included in "SSIIIb-B".

[0167] As used herein, "SSIIIb expressed from the D genome" or "SSIIIb-D" means a polypeptide whose amino acid sequence is set forth in SEQ ID NO:67 or which is at least 98% identical to the amino acid sequence set forth in SEQ ID NO:67 or comprising such a sequence. The amino acid sequence provided as SEQ ID NO:67 corresponds to the starch synthase IIIb expressed from the D genome of bread wheat, which is used herein as the reference sequence for a wild-type SSIIIb-D polypeptide. The polypeptide of SEQ ID NO:67 is 1195 amino acids long. Enzymatically active variants of this enzyme are included in "SSIIIb-D". SSIIb-D does not include the homoeologous polypeptides SSIIIb-A and SSIIIb-B.

[0168] The amino acid sequences of the SSIIIa homoeologs and the SSIIIb homoeologs are about 80% identical, and therefore the polypeptides can be readily distinguished even though they have similar enzyme activities.

[0169] As used herein, the terms "*SSIIIa* gene" and "wheat *SSIIIa* gene" and the like refer to the genes that encode a SSIIIa polypeptide, including wild-type SSIIIa polypeptides such as homologous polypeptides present in other wheat varieties as well as mutant forms of the genes which either encode SSIIIa polypeptides with reduced activity or undetectable activity, or genes which were derived therefrom by mutation. *SSIIIa* genes include, but are not limited to, the wheat *SSIIIa* genes which have been cloned, including the genomic and cDNA sequences listed in Table 1 which are annotated as *SSIIIa* genes. The term *SSIIIa* gene includes, collectively, each of the more specific terms "*SSIIIa-A* gene", "*SSIIIa-B* gene" and "*SSIIIa-D* gene", encoding an SSIIIa-A, SSIIIa-B and SSIIIa-D polypeptide, respectively, or mutant forms derived from such genes. The *SSIIIa* genes as used herein

encompasses mutant forms which do not encode any polypeptide at all, or polypeptides which have no starch synthase activity, in which cases the mutant forms represent null alleles of the genes. Alleles of the genes include mutant alleles where at least part of the gene is deleted, including where the entire gene is deleted, or alleles comprising an inactivating nucleotide substitution such as a premature translation termination codon (stop codon), which alleles also represent null alleles of the genes.

[0170] An "endogenous *SSIIIa* gene" refers to an *SSIIIa* gene which is in its native location in the wheat genome, including wild-type and mutant forms. Endogenous *SSIIIa-A* genes, *SSIIIa-B* genes and *SSIIIa-D* genes are located on chromosomes 1A, 1B and 1D, respectively, in hexaploid wheat. In contrast, the terms "isolated *SSIIIa* gene" and "exogenous *SSIIIa* gene" refer to an *SSIIIa* gene which is not in its native location, for example having been removed from a wheat plant, cloned, synthesized, comprised in a vector or in the form of a transgene in a cell, such as transgene in a transgenic wheat plant. The *SSIIIa* gene in this context may be any of the specific forms as described as follows.

[0171] As used herein, "a *SSIIIa* gene on the A genome of wheat" or "*SSIIIa*-A gene" means any polynucleotide which encodes an SSIIIa-A polypeptide as defined herein or which is derived from a polynucleotide which encodes SSIIIa-A in a wheat plant, including naturally occurring polynucleotides, sequence variants or synthetic polynucleotides, including "wild-type *SSIIIa-A* gene(s)" which encode an SSIIIa-A polypeptide with essentially wild-type SSIIIa activity, and "mutant *SSIIIa-A* gene(s)" which do not encode an SSIIIa-A polypeptide with essentially wild-type activity but which are recognizably derived from a wild-type *SSIIIa-A* gene. Comparison of the nucleotide sequence of a mutant form of an *SSIIIa* gene with a suite of wild-type *SSIIIa* genes is used to determine which of the *SSIIIa* genes it is derived from and so to classify it. For example, a mutant *SSIIIa* gene is considered to be a mutant *SSIIIa-A* gene if its nucleotide sequence is more closely related, i.e. having a higher degree of sequence identity, to a wild-type *SSIIIa-A* gene than to any other *SSIIIa* gene. A mutant *SSIIIa-A* gene encodes an SSIIIa polypeptide with reduced starch synthase enzyme activity (partial mutant), or a polypeptide which lacks starch synthase activity or no protein at all (null mutant gene). An exemplary nucleotide sequence of a cDNA corresponding to an *SSIIIa-A* gene is given in SEQ ID NO:53.

[0172] As used herein, the terms "*SSIIIa* gene on the B genome" or "*SSIIIa-B* gene", and "*SSIIIa* gene on the D genome" or "*SSIIIa-D* gene" have corresponding meanings to that for *SSIIIa-A* in the previous paragraph. An exemplary nucleotide sequence of a cDNA corresponding to an *SSIIIa-B* gene is given in SEQ ID NO:56 and of an *SSIIIa-D* gene is given in SEQ ID NO:59. Genomic gene sequences of parts of *SSIIIa* genes are also given herein as SEQ ID NOs:54, 57 and 60.

[0173] As used herein, "a *SSIIIb* gene on the A genome of wheat" or "*SSIIIb*-A gene" means any polynucleotide which encodes an SSIIIb-A polypeptide as defined herein or which is derived from a polynucleotide which encodes SSIIIb-A in a wheat plant, including naturally occurring polynucleotides, sequence variants or synthetic polynucleotides, including "wild-type *SSIIIb-A* gene(s)" which encode an SSIIIb-A polypeptide with essentially wild-type SSIIIb activity, and "mutant *SSIIIb-A* gene(s)" which do not encode an SSIIIb-A polypeptide with essentially wild-type activity but which are recognizably derived from a wild-type *SSIIIb-A* gene. Comparison of the nucleotide sequence of a mutant form of an *SSIIIb* gene with a suite of wild-type *SSIIIb* genes is used to determine which of the *SSIIIb* genes it is derived from and so to classify it. For example, a mutant *SSIIIb* gene is considered to be a mutant *SSIIIb-A* gene if its nucleotide sequence is more closely related, i.e. having a higher degree of sequence identity, to a wild-type *SSIIIb-A* gene than to any other *SSIIIb* gene. A mutant *SSIIIb-A* gene encodes an SSIIIb polypeptide with reduced starch synthase enzyme activity (partial mutant), or a polypeptide which lacks starch synthase activity or no protein at all (null mutant gene). An exemplary nucleotide sequence of a cDNA corresponding to an *SSIIIb-A* gene is given in SEQ ID NO:62.

[0174] As used herein, the terms "*SSIIIa* gene on the B genome" or "*SSIIIa-B* gene", and "*SSIIIa* gene on the D genome" or "*SSIIIa-D* gene" have corresponding meanings to that for *SSIIIa-A* in the previous paragraph. An exemplary nucleotide sequence of a cDNA corresponding to an *SSIIIa-B* gene is given in SEQ ID NO:65 and of an *SSIIIa-D* gene is given in SEQ ID NO:68.

[0175] The *SSIIIa* and *SSIIIb* genes as defined above include any regulatory sequences that are 5' or 3' of the transcribed region, including the promoter region, that regulate the expression of the associated transcribed region, and introns within the transcribed regions. Exemplary nucleotide sequences of parts of an *SSIIIa* gene is provided as SEQ ID NO:54,

which provides parts of the nucleotide sequence of an *SSIIIa-A* gene from the A genome of wheat. In analogous fashion, the nucleotide sequence of parts of a wild-type *SSIIIa-B* gene of wheat is provided as SEQ ID NO:57 and of an *SSIIIa-D* gene of wheat is provided as SEQ ID NO:60.

[0176] An allele is a variant of a gene at a single genetic locus. A diploid organism has two sets of chromosomes. Hexaploid wheat was six sets of chromosomes, 7 chromosomes in each set and is thought to have arisen by hybridisation of three progenitor diploid plants contributing the A-, B- and D-genomes. Each chromosome of a pair of chromosomes has one copy (i.e. one allele) of each gene. If both alleles of a gene are the same, the organism is homozygous with respect to that allele or gene. If the two alleles of a gene are different, the organism is heterozygous with respect to that gene. The interaction between alleles at a locus is generally described as dominant or recessive. The two alleles of a gene in the wheat plant or grain may have the same mutation as each other, so are said to be homozygous for that mutation, or the two alleles may comprise different mutations to each other and are said to be heterozygous for those mutations. Different alleles of a gene, or for multiple genes, may be combined using methods known in the art. For example, two parental wheat plants which have different alleles for a gene may be crossed to produce progeny (F1) which contain both alleles, in the heterozygous state, and the progeny plants then self-fertilised to produce a further generation of plants (F2) which comprise one or the other of the alleles in the homozygous state or both alleles in the heterozygous state, according to Mendelian genetics.

[0177] Alleles that do not encode or are not capable of leading to the production of any active enzyme are null alleles. Such null alleles may or may not encode a polypeptide, for example encoding a truncated polypeptide or a polypeptide having an inactivating change in amino acid sequence relative to a corresponding wild-type SSIIa or SSIIIa polypeptide.

[0178] Reference to a null mutation(s) includes a null mutation independently selected from the group consisting of a deletion mutation, an insertion mutation, a splice-site mutation, a premature translation termination mutation, and a frameshift mutation, or any combination thereof, preferably a premature translation termination mutation or a small deletion (1-20 basepairs). In an embodiment, one or more of the null mutations are non-conservative amino acid substitution mutations or a null mutation has a combination of two

or more non-conservative amino acid substitutions. In this context, non-conservative amino acid substitutions are as defined herein.

[0179] A loss of function mutation, which includes a partial loss of function mutation in an allele of a gene as well as complete loss of function mutation (null mutation), means a mutation in the allele leading to a reduced level or activity of the enzyme, such as an SSIIa or SSIIIa enzyme in the grain. The mutation in the allele may mean, for example, that less protein having wild-type or reduced activity is translated or that wild-type or reduced levels of transcription are followed by translation of an enzyme with reduced enzyme activity, or preferably the mutant allele is both transcribed at a reduced rate compared to wild-type and any translation product has less activity than the corresponding wild-type polypeptide. The mutation may result in, for example, that no or less RNA is transcribed from the gene comprising the mutation or that the polypeptide that is produced has no or reduced activity relative to wild-type, preferably both. If there is no transcript detected from an allele, for example by RT-PCR assay, that result indicates that the allele is a null allele.

[0180] A "point mutation" refers to a single nucleotide base change which includes a deletion, substitution or insertion of a single nucleotide. The point mutation may further be a splice-site mutation, a premature translation termination mutation, a frame shift mutation or other loss of function mutation wherein the mutation results in no SSIIa and/or SSIIIa protein being produced or the protein is produced in lower amounts or the protein produced has lower or preferably no SSII activity. A frame shift mutation in a protein coding region of a gene is considered a null mutation because of its effect on the structure of the encoded polypeptide. Likewise, a premature translation termination mutation is considered a null mutation unless it occurs very close to the C-terminus of the protein coding region of the gene, in which case an enzyme assay can be used to determine whether the polypeptide has enzyme activity. In some embodiments, the point mutation results in a conservative or preferably a non-conservative amino acid substitution.

[0181] A "reduced" or "lower" amount or level of polypeptide or enzyme activity means a reduced or lower amount or level relative to the amount or level produced by the corresponding wild-type allele or gene. Typically, the reduction is by at least 40%, preferably at least 50% or at least 60%, more preferably at least 80% or 90% relative to the wild-type, most preferably at least 98% or even 100%. In a most preferred embodiment,

the protein is not detected, such as for example in a Western blot assay as described herein, preferably for one or more or all of SSIIa-A, SSIIa-B, SSIIa-D, SSIIIa-A, SSIIIa-B and SSIIIa-D.

[0182] A "reduced" activity means reduced relative to the corresponding wild-type enzyme, such as an SSIIa, SSIIIa or other enzyme.

[0183] Protein "activity" refers to SS activity which may be measured directly or indirectly by various means known in the art and as described herein.

[0184] In some embodiments, the amount by weight of an SSIIa, SSIIIa or other polypeptide is reduced even though the number of polypeptide molecules in the grain is the same as in the wild-type, but each molecule having less, preferably no, activity than the wild-type. For example, the polypeptides produced are shorter than wild-type SSIIa or SSIIIa protein or other protein, such as occurs if the mutant SSIIa or SSIIIa protein or other protein is truncated due to a premature translation termination signal. Such truncated polypeptides may or may not be detectable, depending on their stability.

[0185] As used herein, “two identical alleles of an *SSIIa-A* gene”, means that the two alleles of the *SSIIa-A* gene are identical to each other i.e the plant or grain is homozygous for those alleles or that gene; “two identical alleles of an *SSIIa-B* gene”, means that the two alleles of the *SSIIa-B* gene are identical to each other; “two identical alleles of an *SSIIa-D* gene”, means that the two alleles of the *SSIIa-D* gene are identical to each other. Analogous terms apply for *SSIIIa* genes.

[0186] The wheat plants of the invention can be produced and identified after mutagenesis. In some embodiments, the wheat plant is non-transgenic, which is desirable in some markets, or which is free of any exogenous nucleic acid molecule which reduces expression of an *SSIIa* gene or an *SSIIIa* gene. In another embodiment, the wheat plant is transgenic, for example it comprises an exogenous nucleic acid molecule other than one which reduces expression of an *SSIIa* gene and/or an *SBEIIa* gene, such as for example, an exogenous nucleic acid molecule which encodes a polypeptide that confers herbicide tolerance to the plant.

[0187] Mutant wheat plants having a mutation in a single *SSIIIa* gene which can be combined by crossing of plants and selection of progeny having other *SSIIIa* gene mutations to generate the wheat plants of the invention can be either synthetic, for

example, by performing site-directed mutagenesis on the nucleic acid, or induced by mutagenic treatment, or may be naturally occurring, i.e. isolated from a natural source. In some embodiments, a progenitor plant cell, tissue, seed or plant may be subjected to mutagenesis to produce single or multiple mutations, such as nucleotide substitutions, deletions, insertions and/or codon modification. Preferred wheat plants and grain of the invention comprise at least one introduced *SSIIIa* gene mutation, more preferably two or more introduced *SSIIIa* gene mutations, and may comprise no mutations from a natural source i.e. all of the mutant *SSIIIa* alleles in the plant were obtained by synthetic means or by mutagenic treatment. As used herein, an "induced mutation" or "introduced 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, or an endonuclease induced mutation.

[0188] Mutagenesis can be achieved by chemical or radiation means, for example EMS or sodium azide (Zwar and Chandler, 1995) treatment of seed, or gamma irradiation, well know in the art. 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*, 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 specific *SSIIa* or *SSIIIa* genes. Mutants which are identified may be backcrossed with non-mutated wheat plants as recurrent parents in order to remove and therefore reduce the effect of unlinked mutations in the mutagenised genome.

[0189] Isolation of mutants may be achieved by screening mutagenised plants or seed. For example, a mutagenized population of wheat may be screened directly for the *SSIIa* or *SSIIIa* genotype or indirectly by screening for a phenotype that results from mutations in the *SSIIa* or *SSIIIa* genes. Screening directly for the genotype preferably includes assaying for the presence of mutations in the *SSIIa* or *SSIIIa* genes, which may be observed in PCR assays by the absence of specific *SSIIa* or *SSIIIa* markers as expected when some of the genes are deleted, or heteroduplex based assays as in TILLING. Screening is preferably

based on nucleotide sequencing which is often based on pools of candidate mutants. Screening for the phenotype may comprise screening for a loss or reduction in amount of one or more SSIIa or SSIIIa polypeptides by ELISA or affinity chromatography, or altered starch phenotypes in the grain starch. In hexaploid wheat, screening is preferably done in a genotype that already lacks one or two of the SSIIa or SSIIIa activities, for example in a wheat plant already mutant in the *SSIIa* or *SSIIIa* genes of two of the three genomes, so that a mutant further lacking the functional activity is sought. Affinity chromatography may be carried out to distinguish the SSIIIa-A, SSIIIa-B and SSIIIa-D polypeptides. Large populations of mutagenised seeds (thousands or tens of thousands of seeds) may be screened for high amylose phenotypes using near infra-red spectroscopy (NIR). By these means, high throughput screening is readily achievable and allows the isolation of mutants at a frequency of approximately one per several hundred seeds.

[0190] Plants and seeds of the invention can be produced using the process known as TILLING (Targeting Induced Local Lesions IN Genomes), in that one or more of the mutations in the wheat plants or grain may be produced by this method. In a first step, introduced mutations such as novel single base pair changes are induced in a population of plants by treating seeds or pollen with a chemical or radiation mutagen, and then advancing plants to a generation where mutations will be stably inherited, typically an M2 generation where homozygous mutants may be identified. DNA is extracted, and seeds are stored from all members of the population to create a resource that can be accessed repeatedly over time. For a TILLING assay, PCR primers are designed to specifically amplify a single gene target of interest. Next, dye-labelled 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 Cel I, which recognizes and cleaves mismatched DNA, or the use of High Resolution Melting, is used to discovering novel SNPs within a TILLING population. For example, see Botticella *et al.*, 2011, or Example 14 herein.

[0191] Using this approach, many thousands of plants can be screened to identify any individual with a single base change or 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 and amplifying 1.4 kb fragments with 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, and Henikoff *et al.,* 2004.

[0192] 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 some repeat number polymorphisms. This has been called Ecotilling (Comai *et al.,* 2004). 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 mutations in a single step. In this way, sequencing of the mutant gene is simple and efficient.

[0193] As used herein the term "biological agents" means agent useful in producing site-specific mutants and includes enzymes that induce double stranded breaks in DNA that stimulate endogenous repair mechanisms. These include endonucleases, zinc finger nucleases, TAL effector proteins, transposases, site-specific recombinases and are preferably CRISPR endonucleases. Zinc finger nucleases (ZFNs), for example, facilitate site-specific cleavage within a selected gene 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.,* 2009, See also Durai *et al.,* 2005 and Liu *et al.,* 2010.

[0194] Clustered regularly interspaced short palindromic repeats (CRISPR) are segments of prokaryotic DNA containing short repetitions of base sequences. Each repetition is followed by short segments of "spacer DNA" from previous exposures to a bacteriophage virus or plasmid. The CRISPR/Cas system is a prokaryotic immune system that confers resistance to foreign genetic elements such as those present within plasmids and phages, and provides a form of acquired immunity. CRISPR spacers recognize and cut these

exogenous genetic elements in a manner analogous to RNA interference in eukaryotic organisms. CRISPRs are found in approximately 40% of sequenced bacterial genomes and 90% of sequenced archaea.

[0195] By delivering the Cas9 nuclease and appropriate guide RNAs into a cell, the cell's genome can be cut at a desired location, allowing existing genes to be removed and/or new ones added. CRISPRs have been used in concert with specific endonuclease enzymes for genome editing and gene regulation in various species. Further information regarding CRISPR can be found in WO 2013/188638, WO 2014/093622 and Doudna *et al.*, (2014).

[0196] Transcription activator-like effector nucleases (TALEN) are restriction enzymes that can be engineered to cut specific sequences of DNA. They are made by fusing a TAL effector DNA-binding domain to a DNA cleavage domain (a nuclease which cuts DNA strands). Transcription activator-like effectors (TALEs) can be engineered to bind practically any desired DNA sequence, so when combined with a nuclease, DNA can be cut at specific locations. The restriction enzymes can be introduced into cells, for use in gene editing or for genome editing in situ, a technique known as genome editing with engineered nucleases. Alongside zinc finger nucleases and CRISPR/Cas9, TALEN is a prominent tool in the field of genome editing. Further information regarding TALEN can be found in Boch (2011); Juong *et al.*, (2013) and Sune *et al.*, (2013).

[0197] Identified 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. See, for example, Example 3 herein.

[0198] In some embodiments, mutations are null mutations such as nonsense mutations, frameshift mutations, deletions, 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.

[0199] Insertional nucleotide sequence variants are those in which one or more nucleotides are introduced into a site in the nucleotide sequence, either at a predetermined site as is possible with zinc finger nucleases (ZFN), CRISPR nucleases or other homologous recombination methods, or by random insertion with suitable screening of the resulting product.

[0200] Deletional variants are characterised by the removal of one or more nucleotides from the sequence. In an embodiment, a mutant gene has only a single insertion or deletion of a sequence of nucleotides relative to the wild-type gene. The deletion may be extensive enough to include one or more exons or introns, both exons and introns, an intron-exon boundary, a part of the promoter, the translational start site, or even the entire gene. Deletions may extend far enough to include at least part of, or the whole of, an *SSIIa* or *SSIIIa* gene and one or more adjacent genes on the A, B or D genome. Insertions or deletions within the exons of the protein coding region of a gene which insert or delete a number of nucleotides which is not an exact multiple of three, thereby causing a change in the reading frame during translation, almost always abolish activity of the mutant gene comprising such insertion or deletion; such mutations are null mutations. Insertions or deletions within the exons of the protein coding region of a gene which insert or delete a number of nucleotides which is an exact multiple of three may or may not abolish activity of the gene comprising such insertion or deletion. In the case of a deletion of an exact multiple of three nucleotides, the deletion would be expected to inactivate the encoded polypeptide if the deleted nucleotides encode a highly conserved amino acid. Enzyme assays or phenotypic assays can be used to determine if insertion or deletion mutations are null mutations.

[0201] 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. In some embodiments, the 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 most preferably only one nucleotide. Substitutions may be "silent" in that the nucleotide substitution does not change the amino acid defined by the codon. Nucleotide substitutions may reduce the translation efficiency and thereby reduce the expression level of the affected *SSIIa* or *SSIIIa* gene, for example by reducing the mRNA stability or, if near an exon-intron splice boundary, alter the splicing efficiency. Silent substitutions that do not alter the translation efficiency of an *SSIIa* or *SSIIa* gene are not expected to alter the activity of the gene and are therefore regarded herein as non-mutant, i.e. such genes are active variants and not encompassed in "mutant gene". Alternatively, the nucleotide substitution(s) may change the encoded amino acid sequence and thereby alter the activity of the encoded enzyme, particularly if conserved amino acids are

substituted for another amino acid which is quite different i.e. a non-conservative substitution. For conservative substitutions, see Table 3. Conserved amino acids in wheat SSIIa polypeptides may be identified by aligning SSIIa or SSIIIa amino acid sequences from different species, for example aligning SEQ ID NO:1 with an *Arabidopsis thaliana* SSII and determining which amino acids are in common.

[0202] 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 of the gene including such silent nucleotide substitutions. Screening methods may first involve screening for polymorphisms and secondly for mutations within a group of polymorphic variants. Mutations include deletions of all or part of a gene, insertions such as an insertion into an exon of a gene, and nucleotide substitutions, and any combination thereof.

[0203] The terms "plant(s)" and "wheat plant(s)" as used herein as a noun generally refer to whole plants, but when "plant" or "wheat" is used as an adjective, the terms refer to any substance which is present in, obtained from, derived from, or related to a plant or a wheat plant, such as for example, plant organs (e.g. leaves, stems, roots, flowers), single cells (e.g. pollen), seeds or grains, plant cells including for example tissue cultured cells, products produced from the plant such as "wheat flour", "wheat grain", "wheat starch", "wheat starch granules" and the like. Plantlets and germinated grain 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 whole plant, preferably a wheat plant. Plant parts as used herein comprise plant cells. Plant parts include vegetative structures (for example, leaves including the leaf sheath and leaf blade, stems including the internodes), roots, tillers, floral organs/structures such as the spike (also called the ear or head), pollen, ovules, seed (including embryo, endosperm, and seed coat), plant tissue (for example, vascular tissue, ground tissue, 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, preferably a wheat 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, and various forms of aggregations of plant cells in culture, such as calli. Plant tissues in or from seeds such as wheat grain are the seed coat, endosperm, scutellum, aleurone layer and embryo. Each of the wheat plant tissues or organs and wheat cells comprise genetic material (nucleic acid) of the wheat plant from which is obtained.

[0204] Cereals as used herein means plants or grain of the monocotyledonous families Poaceae or Graminae which are cultivated for the edible components of their grain, and includes wheat, barley, maize, oats, rye, rice, sorghum, triticale, millet, buckwheat. Preferably, the cereal plant or grain is a wheat plant or grain. In a further preferred embodiment, the wheat cell, plant or grain, or products derived therefrom, of the invention is of the species *Triticum aestivum*.

[0205] As used herein, the term "wheat" refers to any species of the Genus *Triticum,* including progenitors thereof, as well as progeny thereof produced by crosses with other species. Wheat includes "hexaploid wheat" which has genome organization of AABBDD, comprised of 42 chromosomes, and "tetraploid wheat" which has genome organization of AABB, comprised of 28 chromosomes. The plants and grain of the invention are of the hexaploid species *T. aestivum*, and do not include the tetraploid species *T. durum*, also referred to as durum wheat or *Triticum turgidum* ssp. *durum*. Diploid progenitors of *T. aestivum* are thought to be *T. uartu*, *T. monococcum* or *T. boeoticum* for the A genome, *Aegilops speltoides* for the B genome, and *T. tauschii* (also known as *Aegilops squarrosa* or *Aegilops tauschii*) for the D genome. Preferably the *T. aestivum* plant of the invention is suitable for commercial production of grain, having suitable agronomic characteristics which are known to those skilled in the art. Most preferably the wheat is *Triticum aestivum* ssp. *aestivum*, herein also referred to as "breadwheat".

[0206] Aspects of the invention provide methods of planting and harvesting wheat grain of the invention, and methods of producing bins of wheat grain of the invention. For instance, after the ground is prepared by ploughing and/or certain other methods, the seeds are typically planted by sowing by drilling furrows and planting the seeds in rows. To prevent scattering of the grain produced upon plant maturity, wheat may be harvested before it is fully ripe, but is typically harvested when the plants show complete loss of green colour. There are several steps in harvesting: cutting, or reaping, the stalks; threshing and winnowing, to separate the grain from the spikes, glumes, and other chaff; sifting and

sorting the grain; typically done by a combine harvester, then loading the grain into trucks. In some embodiments harvested wheat grain may be stored in dry, well-ventilated buildings that keep out insect pests. In some embodiments, harvested wheat grain may be stored for a short time in bins or granaries. The wheat grain may then by hauled to country elevators, tall structures where the grain is dried and stored until it is sold or shipped to terminal elevators. Therefore, embodiments of the invention provide a process of producing bins of wheat grain comprising: a) reaping wheat stalks comprising wheat grain as defined herein; b) threshing and/or winnowing the stalks to separate the grain from the chaff; and c) sifting and/or sorting the grain separated in step b), and loading the sifted and/or sorted grain into bins, thereby producing bins of wheat grain.

[0207] The wheat plants and grain of the invention have uses other than uses for food or animal feed, for example uses in research or breeding. In seed propagated crops such as wheat, the plants can be self-crossed to produce a plant which is homozygous for the desired genes, or haploid tissues such as developing germ cells can be induced to double the chromosome complement to produce a homozygous plant. The inbred wheat plant of the invention thereby produces grain containing the combination of mutant *SSIIa* and *SSIIIa* alleles which are homozygous. The grain can be grown to produce plants that would have the selected phenotype such as, for example, high amylose content in its starch.

[0208] The wheat plants of the invention may be crossed with plants containing a more desirable genetic background, and therefore the invention includes the transfer of the reduced SSIIa trait to other genetic backgrounds. As used herein, "crossing" or "cross" refers to the process by which the pollen of a flower on one plant is applied (artificially or naturally) to the stigma of a flower of another plant. After the initial crossing, a suitable number of backcrosses may be carried out to remove a less desirable background. *SSIIa* or *SSIIIa* allele-specific PCR-based markers such as those described herein may be used to screen for or identify progeny plants or grain with the desired combination of alleles, thereby tracking the presence of the alleles in the breeding program. The desired genetic background may include a suitable combination of genes providing commercial yield and other characteristics such as agronomic performance or abiotic stress resistance. The genetic background might also include other altered starch biosynthesis or modification genes, for example null alleles of *SSI* genes or favourable alleles of *GBSS* genes. The

genetic background may comprise one or more transgenes such as, for example, a gene that confers tolerance to an herbicide such as glyphosate.

[0209] The desired genetic background of the wheat 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, disease resistance and abiotic stress resistance. For Australian use, one might want to cross the altered starch trait of the wheat plant of the invention into wheat cultivars such as Baxter, Kennedy, Janz, Frame, Rosella, Cadoux, Diamondbird or other commonly grown varieties. Other varieties will be suited for other growing regions. It is preferred that the wheat plant of the invention provide a grain yield (tonnes/hectare) of at least 50% relative to the yield of the corresponding wild-type variety in at least some growing regions, more preferably at least 60% or at least 70%, or at least 80% or at least 90%, relative to a wild-type variety having about the same genetic background, grown under the same conditions. In an embodiment, the yield of grain is less than 90% relative to a wild-type variety having about the same genetic background, grown under the same conditions. The yield can readily be measured in controlled field trials, or in simulated field trials in the greenhouse, preferably in the field.

[0210] Marker assisted selection is a well recognised method of selecting for heterozygous plants obtained 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(s) of interest normally present in a 1:1 ratio in a backcross population, and a molecular marker linked to a gene can be used to distinguish the two alleles of the gene. The presence of two markers can be assayed, one for a mutant allele and the other for the wild-type allele. 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. Procedures such as crossing wheat plants, self-fertilising wheat plants or marker-assisted selection are standard procedures and well known in the art. Transferring alleles from tetraploid wheat such as durum wheat to a hexaploid, or other forms of hybridisation, is more difficult but is also known in the art.

[0211] To identify the desired phenotypic characteristic, wheat plants that contain a combination of mutant *ssIIa*, *SSIIIa* or other desired genes are typically compared to a

control plant. When evaluating a phenotypic characteristic associated with mutant *ssIIa* or *SSIIIa* genes such as amylose content in the grain starch, or total fibre content or grain weight or yield, the plants to be tested and control plants are grown under growth chamber, glasshouse or preferably field conditions, under the same conditions (temperature, soil, moisture supply, fertiliser supply, season etc). Identification of a particular phenotypic trait and comparison to controls is based on routine statistical analysis and scoring. Expression of genes or enzyme activity are compared to growth, development and yield parameters which include one or more of germination rate, seedling vigour including seedling emergence, plant morphology, colour, number, size, dimensions, dry and wet weight, ripening, above- and below-ground biomass ratios, and timing, rates and duration of various stages of growth through senescence, including vegetative growth, fruiting, flowering, grain yield and dormancy, harvest index, and soluble carbohydrate content including sucrose, glucose, fructose and starch levels as well as endogenous starch levels. In some embodiments, the wheat plants of the invention differ from wild-type plants in one or more of these parameters by less than 50%, more preferably less than 40%, less than 30%, less than 20%, less than 15%, less than 10%, less than 5%, less than 2% or less than 1% when grown under the same conditions. Preferably, the plant or grain of the invention is about the same as the wild-type plant or grain for one or more of these parameters.

[0212] As used herein, the term "linked" or "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 which case the polymorphism will be 100% linked to the phenotype. Thus, the percent of recombination observed between the loci per generation (calculated as 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 2cM apart and most preferably essentially 0 cM apart.

[0213] As used herein, the "other genetic markers" may be any molecules which are linked to a desired trait in the wheat plants of the invention. Such markers are well known to those skilled in the art and include molecular markers linked to genes determining traits

such disease resistance, 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. Examples of such genes are stem-rust resistance genes *Sr2* or *Sr38*, the stripe rust resistance genes *Yr10* or *Yr17*, the nematode resistance genes such as *Cre1* and *Cre3*, alleles at glutenin loci that determine dough strength such as *Ax*, *Bx*, *Dx*, *Ay*, *By* and *Dy* alleles, the *Rht* genes that determine a semi-dwarf growth habit and therefore lodging resistance (Eagles *et al.,* 2001; Langridge *et al.*, 2001; Sharp *et al.,* 2001).

[0214] The wheat plants, wheat plant parts and products therefrom of the invention are preferably non-transgenic for genes that inhibit expression of an *SSIIIa*, *SSIIa* gene and/or an *SBEIIa* gene, i.e. they do not comprise a transgene encoding an RNA molecule that reduces expression of an endogenous *SSIIIa*, *SSIIa* gene, although in this embodiment they may comprise other transgenes, eg. herbicide tolerance genes such as glyphosate tolerance. More preferably, the wheat plant, grain and products therefrom are non-transgenic, i.e. they do not contain any transgene, which is preferred in some markets. Such products are also described herein as "non-transformed" products. Such non-transgenic plants and grain comprise the multiple mutant *SSIIa* and *SSIIIa* alleles as described herein, such as those produced after mutagenesis.

[0215] The terms "transgenic plant" and "transgenic wheat plant" as used herein refer to a plant that contains a genetic construct ("transgene") not found in a wild-type plant of the same species, variety or cultivar. That is, transgenic plants (transformed plants) contain genetic material that they did not contain prior to the transformation. A "transgene" or "genetic construct" as referred to herein has the normal meaning in the art of biotechnology and refers to a genetic sequence which has been produced or altered by recombinant DNA or RNA technology. If present in a plant cell, the transgene had been introduced into the plant cell or a progenitor cell by a human. 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 typically 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 or a sequence

expressing an inhibitory double-stranded RNA. Plants containing such sequences are included herein in "transgenic plants". Transgenic plants as defined herein include all progeny of an initial transformed and regenerated plant (T0 plant) which has been genetically modified using recombinant techniques, where the progeny comprise the transgene. Such progeny may be obtained by self-fertilisation of the primary transgenic plant or by crossing such plants with another plant of the same species. In an 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. Transgenic plant parts include all parts and cells of said plants which comprise the transgene such as, for example, seeds, cultured tissues, callus and protoplasts.

[0216] A "non-transgenic plant", preferably a non-transgenic wheat plant, is one which has not been genetically modified by the introduction of genetic material by recombinant DNA techniques. The presence in a plant or grain of deletions of part of a gene as generated by site-specific endonucleases such as ZFN, TAL effectors of CRISPR type nucleases, followed by non-homologous end-joining repair in the plant cell, and progeny thereof are included herein as “non-transgenic”. As used herein, “progeny” includes all offspring from a wheat plant, both the immediate and subsequent generations, and both plants and seed (grain). Progeny include the seeds and plants obtained after self-fertilisation (“selfing”) and the grain and plants resulting from a cross between two parental plants, such as the F1 offspring (first generation), F2, F3, F4 etc being the offspring from the second etc generations after selfing of the F1 plants.

[0217] As used herein, the term "corresponding non-transgenic plant" refers to a plant which is the same or similar in most characteristics, preferably isogenic or near-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.

[0218] "Wild-type", as used herein, refers to a cell, tissue, plant or plant part, preferably a *Triticum aestivum* plant, plant part or grain, which has not been modified according to the invention. Such a wild-type plant or grain is at least wild-type for its *SSIIa* and/or *SSIIIa* genes, according to the context. “Corresponding wild-type” refers to a wild-type cell,

tissue, plant, plant part or plant product which is suitable as a comparison to the cell, tissue, plant, plant part or plant product of the invention, as readily understood in the art. Generally, the corresponding wild-type is from a genetically similar, preferably isogenic, wheat plant or plant part to the plant or plant part of the invention but not having the *ssIIa* and/or *ssIIIa* gene mutations. Wild-type cells, tissue or plants are known in the art and may be used as controls to compare gene or polypeptide sequences, in particular *SSIIa* gene sequences, levels of expression of an *SSIIa* or *SSIIIa* gene or the extent and nature of trait modification with cells, tissue or plants modified as described herein. As used herein, "wild-type wheat grain" means a corresponding non-mutagenized, non-transgenic wheat grain. Specific wild-type wheat grains as used herein include but are not limited to Sunstate, Chara and Cadoux. The Sunstate wheat cultivar is described in Ellison *et al.*, (1994).

[0219] 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 that will distinguish the transformed and non-transformed plants. An alternative method to confirm a positive transformant is by Southern blot hybridization, well known in the art. Wheat plants which are transformed may also be identified i.e. distinguished from non-transformed or wild-type wheat plants by their phenotype, for example conferred by the presence of a selectable marker gene, or by immunoassays that detect or quantify the expression of an enzyme encoded by the transgene, or any other phenotype conferred by the transgene.

[0220] The wheat plants of the species *Triticum aestivum*, of the present invention may be grown or harvested for grain, primarily for use as food for human consumption or as animal feed, or for fermentation or industrial feedstock production such as ethanol production, among other uses. Alternatively, the green, aerial parts of wheat plants may be used directly as feed for animals, either directly by grazing in the field or after harvesting. The straw and chaff can also be used as feed or for non-food use. The plant of the present invention is preferably useful for food production and in particular for commercial food production for human consumption. Such food production might include the making of

flour, dough, semolina or other products from the grain such as starch granules or isolated starch that might be an ingredient in commercial food production.

[0221] As used herein, the term "grain" generally refers to mature, harvested seed (also called the kernel) of a plant but can also refer to grain after imbibition or germination, according to the context. Grain includes the mature kernels produced by growers for purposes other than growing further plants. Mature cereal grain such as wheat commonly has a moisture content of less than about 18-20%, typically about 8-10% moisture. As used herein, the term "seed" includes harvested seed but also includes seed which is developing in the plant post anthesis and mature seed comprised in the plant prior to harvest. The parts of the grain include the testa (seedcoat), the pericarp (fruit coat), the aleurone layer, the starchy endosperm and the embryo (germ) which is made up of the scutellum, the plumule (shoot) and radicle (primary root). The combined testa, pericarp and aleurone layer are commonly referred to as the “bran”, which can be removed from the grain by milling, which may also comprise the germ. The scutellum is the region that secretes some of the enzymes involved in germination and absorbs the soluble sugars from the breakdown of starch in the endosperm for growth of the seedling after germination. The aleurone which surrounds the starchy endosperm also secretes enzymes during germination.

[0222] As used herein, "germination" refers to the emergence of the root tip (radicle) from the seed coat after imbibition. The radicle usually emerges first and then the plumule. "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. Germination rates can be calculated using techniques known in the art. For example, a population of seeds, typically at least 100 grains, can be assessed daily over several days to determine the germination percentage over time. With regard to grain of the present invention, as used herein the term "germination rate which is substantially the same" means that the germination rate of the grain is at least 90%, that of corresponding wild-type grain. In an embodiment, the grain of the invention has a germination rate of between about 70% and about 100% relative to wild-type grain, preferably between about 90% and about 100% relative to wild-type grain. When measuring germination, the wheat grain of the invention and the wild-type grain used as a control should have been grown under the same conditions and stored under the same conditions for about the same length of time.

[0223] The invention also provides food ingredients such as flour, preferably wholemeal, bran and other products produced from the grain. These may be unprocessed or processed, for example by heat treatment, fractionation or bleaching.

[0224] The grain of the invention can be processed to produce a food ingredient or a food or non-food product using any technique known in the art. In one embodiment, the food ingredient is flour such as, for example, wholemeal (wholemeal flour) or white flour. As used herein, "flour" is a milled product from the grain which has been milled to a powder. The powder is commonly fractionated by sieving, sifting, centrifugation or other methods known in the art, and may be further refined, heat treated and/or bleached. Refined flour, or "white flour" as referred to herein refers to flour which has been enriched for the endosperm-derived part of the milled powder relative to wholemeal, performed by removing at least some of the bran and germ components of the milled powder. The Food and Drug Administration (FDA) requires flour to meet certain particle size standards in order to be included in the category of refined flour. According to the FDA, the particle size of refined flour is described as flour in which not less than 98% passes through a cloth having openings not larger than those of woven wire cloth designated "212 micrometers (U.S. Wire 70)".

[0225] As used herein, the term "wholemeal", also called wholemeal flour or whole grain flour, is a milled flour which was made from essentially 100% of the grain and which includes a refined flour constituent (refined flour or refined flour) and a coarse fraction (an ultrafine-milled coarse fraction). The coarse fraction includes at least one of bran and germ, typically both. The germ is an embryonic plant found within the grain kernel, comprising the embryo and scutellum. The germ includes lipids, fibre, vitamins, protein, minerals and phytonutrients, such as flavonoids, at levels higher than in the mature endosperm of the grain. The bran includes several cell layers including the pericarp (fruit coat) and testa (seed coat) and also has a significant amount of lipids, fibre, vitamins, protein, minerals and phytonutrients, such as flavonoids. The aleurone layer, while technically considered part of the endosperm of the mature grain, exhibits many of the same characteristics as the bran and therefore is typically removed with the bran and germ during the milling and/or sieving process. The aleurone layer also includes lipids, fibre, vitamins, protein, minerals and phytonutrients, such as flavonoids and ferulic acid.

[0226] Further, the coarse fraction may be blended with the refined flour constituent. Preferably, the coarse fraction is homogenously blended with the refined flour constituent. The coarse fraction may be mixed with the refined flour constituent to form the wholemeal, thus providing a wholemeal with increased nutritional value, fibre content, and antioxidant capacity as compared to refined flour. For example, the coarse fraction or wholemeal may be used in various amounts to replace refined flour in baked goods, snack products, and food products. The wholemeal of the present invention (i.e. ultrafine-milled whole grain flour) may also be marketed directly to consumers for use in their homemade baked products. In an exemplary embodiment, a granulation profile of the wholemeal is such that 98% of particles by weight of the wholemeal are less than 212 micrometers in diameter.

[0227] In further embodiments, enzymes found within the bran and germ of the wholemeal and/or coarse fraction are inactivated in order to stabilize the wholemeal and/or coarse fraction. It is contemplated by the present invention that "inactivated" may also mean inhibited, denatured, or the like. Stabilization is a process that uses steam, heat, radiation, or other treatments to inactivate the enzymes found in the bran and germ layer. In the absence of stabilization, naturally occurring enzymes in the bran and germ catalyze changes to compounds in the flour, which may adversely affecting the cooking characteristics of the flour and the shelf life. Inactivated enzymes do not catalyze changes to compounds found in the flour, therefore, flour that has been stabilized retains its cooking characteristics and has a longer shelf life. For example, the present invention may implement a two-stream milling technique to grind the coarse fraction. Once the coarse fraction is separated and stabilized, the coarse fraction is then ground through a grinder, preferably a gap mill, to form a coarse fraction having a particle size distribution less than or equal to about 500 micrometers. After sifting, any ground coarse fraction having a particle size greater than 500 micrometers may be returned to the process for further milling.

[0228] In additional embodiments, the flour, wholemeal or the coarse fraction may be a component of a food product, for example, may be used as an ingredient in food production. The food product may be, for example, a bagel, a biscuit, a bread, a bun, a croissant, a dumpling, a muffin such as an English muffin, a pita bread, a quickbread, a refrigerated or frozen dough product, dough, baked beans, a burrito, chili, a taco, a tamale,

a tortilla, a pot pie, a ready to eat cereal, a ready to eat meal, stuffing, a microwaveable meal, a brownie, a cake, a cheesecake, a coffee cake, a cookie, a dessert, a pastry, a sweet roll, a candy bar, a pie crust, pie filling, baby food, a baking mix, a batter, a breading, a gravy mix, a meat extender, a meat substitute, a seasoning mix, a soup mix, a gravy, a roux, a salad dressing, a soup, sour cream, a noodle, a pasta, ramen noodles, chow mein noodles, lo mein noodles, an ice cream inclusion, an ice cream bar, an ice cream cone, an ice cream sandwich, a cracker, a crouton, a doughnut, an egg roll, an extruded snack, a fruit and grain bar, a microwaveable snack product, a nutritional bar, a pancake, a par-baked bakery product, a pretzel, a pudding, a granola-based product, a snack chip, a snack food, a snack mix, a waffle, a pizza crust, animal food or pet food.

[0229] In other embodiments, the flour, wholemeal or coarse fraction may be a component of a nutritional supplement. For instance, the nutritional supplement may be a product that is added to the diet containing one or more ingredients, typically including: vitamins, minerals, lipids such as omega-3 fatty acids, amino acids, enzymes, antioxidants such as lutein, herbs, spices, probiotics, extracts, prebiotics and fibre. The flour, wholemeal or coarse fraction of the present invention includes vitamins, minerals, amino acids, enzymes, and fibre. For instance, the coarse fraction contains a concentrated amount of dietary fibre as well as other essential nutrients, such as B-vitamins, selenium, chromium, manganese, magnesium, and antioxidants, which are essential for a healthy diet. For example, 15 grams of the coarse fraction of the present invention delivers 33% of an individual's daily recommend consumption of fibre. Further, 9 grams is all that is needed to deliver 20% of an individual's daily recommend consumption of fibre. Thus, the coarse fraction is an excellent supplemental source for consumption of an individual's fibre requirement.

[0230] In additional embodiments, the wholemeal or coarse fraction may be a fibre supplement or a component thereof. Many current fibre supplements such as psyllium husks, cellulose derivatives and hydrolyzed guar gum have limited nutritional value beyond their fibre content. Additionally, many fibre supplements have an undesirable texture and poor taste. Therefore, in an embodiment, the food ingredients of the invention lack fibre supplements derived from sources other than wheat grain. Supplements made from the wholemeal or coarse fraction of the wheat grain thereby deliver fibre as well as protein, and antioxidants. The fibre supplement may be delivered in, but is not limited to

the following forms: instant beverage mixes, ready-to-drink beverages, nutritional bars, wafers, cookies, crackers, gel shots, capsules, chews, chewable tablets, and pills. One embodiment delivers the fibre supplement in the form of a flavored shake or malt type beverage, this embodiment may be particularly attractive as a fibre supplement for children.

[0231] In an additional embodiment, a milling and blending process may be used to make a multi-grain flour or a multi-grain coarse fraction. For example, bran and germ from one type of grain such as grain of the invention may be ground and blended with ground endosperm or whole grain flour of another type of wheat or other cereal. It is contemplated that the present invention encompasses mixing any combination of one or more of bran, germ, endosperm, and whole grain flour of one or more grains. This multi-grain approach may be used to make custom flour and capitalize on the qualities and nutritional contents of multiple types of grains such as wheat to make one flour.

[0232] The flour or wholemeal of the present invention may be produced by any milling process known in the art. An exemplary embodiment involves grinding grain in a single stream without separating endosperm, bran, and germ of the grain into separate streams. Clean and tempered grain is conveyed to a first passage grinder, such as a hammermill, roller mill, pin mill, impact mill, disc mill, air attrition mill, gap mill, or the like. After grinding, the grain is discharged and conveyed to a sifter. Any sifter known in the art for sifting a ground particle may be used. Material passing through the screen of the sifter is the whole grain flour of the present invention and requires no further processing. Material that remains on the screen is referred to as a second fraction. The second fraction requires additional particle reduction. Thus, this second fraction may be conveyed to a second passage grinder. After grinding, the second fraction may be conveyed to a second sifter.

[0233] It is contemplated that the flour, wholemeal, coarse fraction and/or grain products of the present invention may be modified or enhanced by way of numerous other processes such as: fermentation, instantizing, extrusion, encapsulation, toasting, roasting, or the like. The flour and wholemeal of the invention comprise the genetic material, including the DNA, of the wheat grain from which they were derived, as do the food products produced therefrom. See for example, Tilley (2004) and Bryan *et al.*, (1998).

[0234] A malt-based beverage provided by the present invention involves alcohol beverages (including distilled beverages) and non-alcohol beverages that are produced by using malt as a part or whole of their starting material. Examples include beer, happoshu (low-malt beer beverage), whisky, low-alcohol malt-based beverages (e.g., malt-based beverages containing less than 1% of alcohols), and non-alcohol beverages.

[0235] Malting is a process of controlled steeping and germination followed by drying of the grain. This sequence of events is important for the synthesis of numerous enzymes that cause grain modification, a process that principally depolymerizes the dead endosperm cell walls and mobilizes the grain nutrients. In the subsequent drying process, flavour and colour are produced due to chemical browning reactions. Although the primary use of malt is for beverage production, it can also be utilized in other industrial processes, for example as an enzyme source in the baking industry, or as a flavouring and colouring agent in the food industry, for example as malt or as a malt flour, or indirectly as a malt syrup, etc.

[0236] In one embodiment, the present invention relates to methods of producing a malt composition. The method preferably comprises the steps of:

(i) providing wheat grain of the invention,

(ii) steeping said grain,

(iii) germinating the steeped grains under predetermined conditions and

(iv) drying said germinated grains.

[0237] Malt may be prepared using only grain of the invention or in mixtures comprising other grains. Malt is mainly used for brewing beer, but also for the production of distilled spirits. Brewing comprises wort production, main and secondary fermentations and post-treatment. First the malt is milled, stirred into water and heated. During this "mashing", the enzymes activated in the malting degrade the starch of the kernel into fermentable sugars. The produced wort is clarified, yeast is added, the mixture is fermented and a post-treatment is performed.

[0238] In general, the first step in the wort production process is the milling of malt in order that water may gain access to grain particles in the mashing phase, which is fundamentally an extension of the malting process with enzymatic depolymerization of substrates. During mashing, milled malt is incubated with a liquid fraction such as water.

The temperature is either kept constant (isothermal mashing) or gradually increased. In either case, soluble substances produced in malting and mashing are extracted into said liquid fraction before it is separated by filtration into wort and residual solid particles denoted spent grains. The wort composition may also be prepared by incubating wheat grain of the invention or parts thereof with one or more suitable enzyme, such as enzyme compositions or enzyme mixture compositions, for example Ultraflo or Cereflo (Novozymes). The wort composition may also be prepared using a mixture of malt and unmalted plants or parts thereof, optionally adding one or more suitable enzymes during said preparation.

[0239] The starch of the flour or wholemeal when incorporated in food products provides modified digestive properties, for example the food ingredient comprises increased resistant starch relative to a corresponding food ingredient produced from wild-type wheat grain, such as including between 1% to 20%, 2% to 18%, 3% to 18% or 5% to 15% resistant starch, and a decreased Glycaemic Index (GI), such as a reduction by at least 5 units, preferably between 5 and 25 units. This may be in combination with an increased total fibre content, for example, of 15% to 30% by weight in the food ingredient.

[0240] Carbohydrates, compounds comprising one or more saccharide units comprised of carbon, hydrogen and oxygen, can be classified according to the number and composition of the monosaccharide units making up the carbohydrate. These include polysaccharides (>10 monosaccharide units), oligosaccharides (3-10 monosaccharide units), disaccharides and monosaccharides including glucose, fructose, xylulose and arabinose. Carbohydrates make up more than 65% by weight of the mature wild-type wheat grain, including 65-75% starch and about 10% cell wall polysaccharides such as cellulose, arabinoxylan and BG.

[0241] Starch is the major storage carbohydrate in most plants, including cereals such as wheat. "Starch" is defined herein as polysaccharide composed of glucopyranose units polymerized through α-1,4 linkages and either no or some α-1,6 linkages. Starch is synthesized in the amyloplasts and formed and stored in granules in the developing storage organ such as grain; it is referred to herein as "storage starch" or "grain starch" or “starch of the grain”. In cereal grains including *Triticum aestivum*, the great majority of the storage starch is deposited in the endosperm as starch granules. Starch is synthesized and deposited within amyloplasts during grain development, in particular the grain filling phase of plant

growth, and forms discrete crystalline structures termed starch granules. In wild-type *Triticum aestivum*, the starch granules are of two size classes, namely larger, ellipsoidal granules ranging from 10-40 μm in diameter (A-type granules) and smaller, spherical granules from 1-10 μm in diameter (B-type granules).

[0242] The molecules of starch are classified as belonging to two component fractions, known as amylose and amylopectin, which are distinguished on the basis of their degree of polymerization (DP) and the ratio of α-1,6 to α-1,4 linkages in the polymers. Amylose comprises almost entirely linear α-1,4 linked glucosyl chains which may have either no or a few glucan chains joined by an α-1,6 bond to other α-1,4 linked chains and has a molecular weight of 10^4 to 10^5 daltons. The term "amylose" is defined herein as including essentially linear molecules of α-1,4 linked glucosidic (glucopyranose) units, sometimes referred to as "true amylose", and amylose-like long-chain starch which is sometimes referred to as "intermediate material" or "amylose-like amylopectin" which appears as iodine-binding material in an iodometric assay along with true amylose (Takeda *et al.*, 1993; Fergason, 1994). The linear molecules in true amylose typically have a DP of between 500 and 5000 and contain less than 1% α-1,6 linkages. Recent studies have shown that about 0.1% of α-1,6-glycosidic branching sites may occur in amylose, therefore it is described as "essentially linear". In this context, the percentage (%) refers to the number of α-1,6-glycosidic bonds relative to the total number of glycosidic bonds, being the sum of α-1,4-glycosidic bonds and α-1,6-glycosidic bonds. Granule bound starch synthase (GBSS) is the main enzyme involved in synthesis of amylose.

[0243] Amylopectin is a relatively highly branched glucan polymer in which α-1,4 linked glucosyl chains with 3 to 60 glucosyl units are connected by α-1,6 linkages, so that approximately 4-6% of the total number of glucosyl linkages are α-1,6 linkages. Therefore, amylopectin is a much larger molecule with a DP ranging from 5000 to 500,000 and is more highly branched than amylose. Amylose has a helical conformation with a molecular weight of about 10^4 to about 10^6 Daltons while amylopectin has a molecular weight of about 10^7 to about 10^8 Daltons. These two types of starch can readily be distinguished or separated by methods well known in the art, for example by size exclusion chromatography or by their different binding affinity for iodine. Amylose is digested more

slowly by α-amylases in the small intestine than amylopectin, the latter having multiple sites for enzymatic hydrolysis because of its highly branched structure.

[0244] Starch from wild-type *Triticum aestivum* grain typically comprises 20%-30% of amylose and about 70%-80% of amylopectin as measured by an iodometric method, whereas the starch of the grain of the invention has an amylose content of about 45% to about 70% on a weight basis. The amylose content may be between 45% and 70%, in some embodiments between 45% and 65%, or about 50%, about 55%, about 60% or about 65%. The higher the level, the more preferred is the grain. The proportion of amylose in the starch as defined herein is on a weight/weight (w/w) basis, i.e. the weight of amylose as a percentage of the weight of total starch extractable from the grain, with respect to the starch prior to any fractionation into amylose and amylopectin fractions. The terms "proportion of amylose in the starch" and "amylose content" when used herein in the context of the grain, flour or other product of the invention are essentially interchangeable terms. Amylose content may be determined by any of the methods known in the art including size exclusion high-performance liquid chromatography (HPLC), for example in 90% (w/v) DMSO, concanavalin A methods (Megazyme Int, Ireland), or preferably by an iodometric method, for example as described in Example 1. The HPLC method may involve debranching of the starch (Batey and Curtin, 1996) or not involve debranching. It will be appreciated that methods such as the HPLC method of Batey and Curtin, 1996 which assay only the "true amylose" may underestimate the amylose content as defined herein. Methods such as HPLC or gel permeation chromatography depend on fractionation of the starch into the amylose and amylopectin fractions, while iodometric methods depend on differential iodine binding and therefore do not require fractionation.

[0245] From the grain weight and amylose content, the amount of amylose deposited per grain can be calculated and compared for test and control lines.

[0246] Starch is readily isolated from wheat grain using standard methods, for example the method of Schulman and Kammiovirta, 1991. On an industrial scale, wet or dry milling can be used. Starch granule size is important in the starch processing industry where there may be separation of the larger A granules from the smaller B granules.

[0247] Wild-type wheat grown commercially has a starch content in the grain which is usually in the range 55-75%, depending somewhat on the cultivar grown. In comparison,

the grain of the invention has a starch content of about 25% to about 70%, so in most embodiments its starch content is reduced relative to corresponding wild-type grain. In embodiments, the starch content of the grain of the invention is between 25% and 65%, between 25% and 60%, between 25% and 55%, between 25% and 50%, between 30% and 70%, between 30% and 65%, between 30% and 60%, between 30% and 55%, or between 30% and 50%. In further embodiments, the starch content is about 35%, about 40%, about 45%, about 50%, about 55%, about 60% or about 65% as a percentage of the grain weight (w/w). The starch content of the grain of the invention may also be defined on a relative basis, i.e. relative to the starch content of corresponding wild-type grain. In embodiments, the starch content is between about 50% and about 90%, or between 50% and 80%, between 50% and 75%, between 50% and 70%, between 60% and 90% or between 60% and 80%, each being relative to that of wild-type grain. The starch content may also be between 90% and 100% relative to the starch content of wild-type grain. In each case, the comparison should be made by growing the plants under the same conditions, for example in field trials.

[0248] Flour, starch granules and bran of the invention may be obtained from grain by a milling process, optionally followed by a sifting or sieving process. Purified starch may also be obtained from grain by a milling process, for example a wet milling process, followed by further separation of the starch from protein, oil and fibre of the grain. As used herein, the term "milled product" refers to a product produced from grinding grain, preferably wheat grain of the invention, and includes flour (for example, wholemeal), middlings (also known as wheat midds), bran (including the germ) and starch granules. The middlings are usually in the form of granular particles and include bran and germ from the grain, and typically comprise about 18% protein, 20-30% starch, and about 5-6% lipid. This fraction from the milling process is relatively nutrient dense compared to white flour. Grain shape is a feature that can impact on the commercial usefulness of a plant, since grain shape can have an impact on the ease or otherwise with which the grain can be milled. The milling yield is an important parameter for commercial usefulness of the grain. The milling yield of the grain of the invention may be reduced by at least 10% relative to wild-type grain, but alternatively may be about the same as wild-type grain.

[0249] In another aspect, the invention provides starch granules or starch obtained from the grain of the plant of the invention. The starch of the granules has an increased

proportion of amylose and a reduced proportion of amylopectin relative to wild-type wheat starch granules. The initial product of the milling process is a mixture or composition which includes starch granules, such as for example, white flour or wholemeal, and the invention therefore encompasses such granules. The starch granules from wild-type wheat comprise starch granule-bound proteins including GBSS, SSI, SBEIIa and SBEIIb amongst other proteins and therefore the presence of these proteins distinguish wheat starch granules from starch granules of other cereals. In contrast, the starch granules from wheat grain of the invention comprise wheat GBSS, including the GBSS polypeptides encoded by each of the A, B and D genomes of hexaploid wheat, but are reduced for SSIIa and/or SSIIIa polypeptides, indeed some embodiments lack both SSIIa and SSIIIa polypeptides. These starch granules may also comprise reduced levels of one or more or all of the wheat SSI, SBEIIa and SBEIIb polypeptides, even if the wheat grain is wild-type for the genes encoding these enzymes. The starch granules from the wheat grain of the invention are typically distorted in shape and surface morphology when observed under light microscopy, see for Example 7, particularly for wheat grain having an amylose content of at least 45% or at least 50% as a percentage of the total starch of the grain. In an embodiment, at least 50%, preferably at least 60% or at least 70%, more preferably at least 80% of the starch granules obtained from the grain of the invention show distorted shape and/or surface morphology. The starch granules also show a loss of birefringence when observed under polarised light. See, for instance, Example 7 herein for determining the incidence of birefringence. For example, less than 50% or less than 25% of the starch granules show the "maltese cross" that is observed when wild-type starch granules are observed under polarised light.

[0250] The starch from starch granules may be purified by removal of the proteins after disruption and dispersal of the starch granules by heat and/or chemical treatment. The starch of the grain, the starch of the starch granules, and the purified starch of the invention may be further characterized by one or more or all of the following properties:

i) its amylose content is at least 45% (w/w), preferably between 45% and 70% on a weight basis, or at least 50% (w/w), or about 60% (w/w) amylose as a proportion of the total starch;

ii) comprising at least 2% resistant starch, preferably at least 3% resistant starch;

iii) the starch is characterised by a reduced glycaemic index (GI);

iv) the starch granules being distorted in shape;

v) the starch granules having reduced birefringence when observed under polarized light;

vi) the starch characterized by a reduced swelling volume;

vii) modified chain length distribution and/or branching frequency in the starch;

viii) the starch characterized by a reduced peak temperature of gelatinisation;

ix) the starch characterized by a reduced peak viscosity;

x) reduced starch pasting temperature;

xi) reduced peak molecular weight of amylose as determined by size exclusion chromatography;

xii) reduced starch crystallinity; and

xiii) reduced proportion of A-type and/or B-type starch, and/or increased proportion of V-type crystalline starch;

each property being relative to wild-type wheat starch granules or starch.

[0251] The flour or starch of the invention may also be characterized by its swelling volume in heated excess water compared to wild-type flour or starch. Swelling volume is typically measured by mixing either a starch or flour with excess water and heating to elevated temperatures, typically greater than 90°C. The sample is then collected by centrifugation and the swelling volume is expressed as the mass of the sedimented material divided by the dry weight of the sample. A low swelling characteristic is useful where it is desired to increase the starch content of a food preparation, in particular a hydrated food preparation. The flour and starch of the invention preferably has a decreased swelling volume, such as for example decreased by 30-70% relative to a wild-type wheat flour or starch.

[0252] One measure of an altered amylopectin structure is the distribution of chain lengths, or the degree of polymerization, of the starch. The chain length distribution may be determined by using fluorophore-assisted carbohydrate electrophoresis (FACE) following isoamylase de-branching. The amylopectin of the starch of the invention may have a distribution of chain lengths in the range from 5 to 60, or in a subrange such as DP 7-11, that is greater than the corresponding chain length distribution of starch from wild-type plants, and/or reduced in frequency in other subranges, for example DP 12-24. See for instance, Example 7 herein. Starch with longer chain lengths will also have a

commensurate decrease in frequency of branching. The starch of the grain of the invention is distinctively different to the starch of wheat grain having reduced SBEIIa activity (Regina *et al.*, 2006) where the amylopectin of the grain comprises an increased proportion of the DP 4 - 12 chain length fraction relative to the amylopectin of wild-type grain, as measured after isoamylase debranching of the amylopectin. The difference can be readily determined by FACE.

[0253] In another aspect of the invention, the wheat starch may have an altered gelatinisation temperature, preferably a reduced gelatinisation temperature, which is readily measured by differential scanning calorimetry (DSC). Gelatinisation is the heat-driven collapse (disruption) of molecular order within the starch granule in excess water, with concomitant and irreversible changes in properties such as granular swelling, crystallite melting, loss of birefringence, viscosity development and starch solubilisation. The gelatinisation temperature may be either increased or decreased compared to starch from wild-type plants, depending on the chain length of the remaining amylopectin. High amylose starch from amylose extender (ae) mutants of maize showed a higher gelatinisation temperature than normal maize (Fuwa *et al.*, 1999; Krueger *et al.,* 1987).

[0254] The gelatinisation temperature, in particular the temperature of onset of the first peak or the temperature for the apex of the first peak, may be reduced by at least 3°C, preferably at least 5°C or more preferably at least 7°C as measured by DSC compared to starch extracted from a similar, but unaltered grain. The starch may comprise an elevated level of resistant starch, with an altered structure indicated by specific physical characteristics including one or more of the group consisting of physical inaccessibility to digestive enzymes which may be by reason of having altered starch granule morphology, the presence of appreciable starch associated lipid, altered crystallinity, and altered amylopectin chain length distribution. The high proportion of amylose also contributes to the level of resistant starch when the starch has been heated and then cooled.

[0255] The starch structure of the wheat of the present invention may also differ in that the degree of crystallinity is reduced and/or the type of crystallinity is modified compared to starch isolated from wild-type wheat grain. Crystallinity is typically investigated by X-ray crystallography. The reduced crystallinity of a starch is also thought to be associated with enhance organoleptic properties and contributes to a smoother mouth feel.

[0256] The invention also provides wheat grain, flour, wholemeal, starch granules and starch from the grain comprising increased amounts of dietary fibre, by way of at least an elevated level of RS, but also by way of increased levels of other dietary fibre components such as arabinoxylans, β-glucan and fructans. As used herein, "dietary fibre" (DF) or "total dietary fibre" (TDF) means the sum of carbohydrate polymers in food that are not digested in the small intestine of a healthy human subject and that have physiological benefit in the large intestine. As used herein, DF is different to "total fiber content" (below). DF is not digested and absorbed in the small intestine but passes to the colon where it may be degraded by bacteria. DF includes RS, non-α-glucan oligosaccharides and non-starch polysaccharides (NSP) such as arabinoxylans, β-glucan and fructans. DF is usually divided into forms which are soluble in water (soluble fibre) or not (insoluble fibre), and RS. The most-health promoting fraction of dietary fibre in wild-type wheat grain is the soluble fibre which mostly comprises the AX component, since wild-type starch is very low in RS. In contrast, in oats and barley the soluble fibre is mostly BG. The National Heart Foundation of Australia has recommended a daily intake of 30-35 g of DF for cardiovascular and colonic health. A variety of candidate genes have been identified in wheat which affect dietary fibre content (Quraishi *et al.*, 2011), but none to the level as provided by the grain of the invention. DF may be measured by the method AOAC 991.43 (Megazyme).

[0257] As used herein, a "prebiotic" is a non-digestible (by human digestive enzymes) food ingredient that beneficially affects a subject by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon after passing through the small intestine. For example, fructans and BG cannot be digested except through bacterial activity, but can alter the composition of human gut microbes by specific fermentation, producing short chain carboxylic acids including acetate, propionate and butyrate.

[0258] In embodiments, the wheat grain, flour, starch granules and starch of the invention provide modified digestive properties such as increased resistant starch. As used herein, "resistant starch" (RS) refers to the starch and products of starch digestion that are not absorbed in the small intestine of healthy individuals but enter into the large bowel. This is defined in terms of a percentage of the total starch of the grain, or a percentage of the total starch content in the food, according to the context. Therefore, resistant starch excludes products digested and absorbed in the small intestine. RS is therefore part of the dietary fibre content of the food ingredient (flour etc) or food product of the invention. RS is

divided into five categories: physically inaccessible starch (RSI) such as, for example, in incompletely milled grain, resistant starch granules (RSII) such as, for example, found to a small extent in potatoes and green bananas, retrograded starch (RSIII) which is formed when gelatinised starch is cooled for an extended period of time, chemically modified starch (RSIV) such as formed by etherifying or esterifying free hydroxyl groups on the glycosyl residues, and starch capable of forming complexes between amylose or long branch chains of amylopectin with lipids (RSV) (Birt *et al.*, 2013). RSIII is especially formed from longer chains of amylose which tend to recrystallize and form retrograded starch after gelatinisation. The RS in products based on starch with an elevated amylose content is mainly retrograded amylose (Hung *et al.*, 2006). The increased RS of the starch of the grain, starch granules, starch and products therefrom of the invention is thought to be due to an increase in the RSII and RSV contents and to RSIII after retrogradation if the starch has been heated and then cooled, relative to the corresponding wild-type product. Starch-lipid association as measured by V-complex crystallinity is also likely to contribute to the level of resistant starch, increasing the RSV component by virtue of increased lipid content in the grain of the invention. Some of the starch may also be in an RSI form, being somewhat inaccessible to digestion.

[0259] Several methods are available to measure RS levels in food ingredients or food, all relying on an initial removal of digestible starch using starch hydrolysing enzymes (Dupuis *et al.*, 2014). RS estimation by the Prosky method (AOAC 985.29) uses gravimetric determination of dietary fibre after α-amylase, glucoamylase and protease digestion (Prosky *et al.*, 1985). The McCleary method (AOAC 2009.01) is the official method of the AOAC and is commercially available (Megazyme International, Ireland). It is the preferred method of measuring RS, see Example 1 herein. In this assay, non-resistant starch is solubilized by treatment with pancreatic α-amylase, the RS recovered and dissolved in 2 M KOH, and then hydrolysed to glucose with amyloglucosidase and measured.

[0260] In embodiments, the starch has between 2% and 20%, between 2% and 18%, between 3% and 18%, between 3% and 15%, or between 5% and 15% resistant starch on a weight basis, as a percentage of the total starch content. In embodiments, the RS content is increased by between 2- and 10-fold relative to a corresponding food ingredient or food product made with an equivalent amount of wild-type wheat starch. In embodiments, the

RS content is increased by about 3-fold, about 4-fold, about 5-fold, about 6-fold, about 7-fold, about 8-fold, or about 9-fold relative to the wild-type. The extent of increased RS can be adjusted by blending the products of the invention with a corresponding wild-type product. The altered starch structure and in particular the high amylose levels of the starch of the invention give rise to an increase in RS when consumed in food. RS has beneficial physiological effects associated with metabolic products released during its fermentation in the bowel (Topping and Clifton, 2001), in particular the SCFA butyrate, propionate and acetate, and is effective in reducing postprandial blood glucose levels.

[0261] The grain, food ingredients and food products produced therefrom of the invention can be used advantageously for the provision in the diet of, or production of, compositions enriched for β-glucan, cellulose, fructan or arabinoxylan, based on the increased levels of these components in the grain of the invention. The cell walls of cereal grain are complex and dynamic structures composed of a variety of polysaccharides such as cellulose, xyloglucans, pectin (rich in galacturonic acid residues), callose (1,3-β-D-glucan), arabinoxylans (arabino-1,4-β-D-xylan, hereinafter AX) and BG, as well as polyphenolics such as lignin. In cell walls of the grasses and some other monocot plants, glucuronoarabinoxylans and BG predominate and the levels of pectic polysaccharides, glucomannans and xyloglucans are relatively low (Carpita *et al.*, 1993). These polysaccharides are synthesized by a large number of diverse polysaccharide synthases and glycosyltranferases, with at least 70 gene families present in plants and in many cases, multiple members of gene families.

[0262] As used herein, the term "(1,3;1,4)-β-D-glucan", also referred to as "β-glucan" and abbreviated herein as "BG", refers to an essentially linear polymer of unsubstituted and essentially unbranched β-glucopyranosyl monomers covalently linked mostly through 1,4-linkages with some 1,3-linkages. The glucopyranosyl residues, joined by 1,4- and 1,3-linkages, are arranged in a non-repeating but non-random fashion, i.e. the 1,4- and 1,3-linkages are not arranged randomly, but equally they are not arranged in regular, repeating sequences (Fincher, 2009a, 2009b). Most (about 90%) of the 1,3-linked residues follow 2 or 3 1,4-linked residues in wheat BG, as in oat and barley BG. BG can therefore be considered to be a chain of mainly β-1,4 linked cellotriosyl (each with 3 glucopyranosyl residues) and cellotetrosyl (each with 4 glucopyranosyl residues) units linked together by single β-1,3 linkages with approximately 10% longer β-1,4 linked cellodextrin units of four

to about ten 1,4-linked glucopyranosyl residues, up to about 28 glucopyranosyl residues (Fincher and Stone, 2004). Typically, the BG polymers have at least 1000 glycosyl residues and adopt an extended conformation in aqueous media. The ratio of tri- to tetra-saccharide units (DP3/DP4 ratio) varies among species and therefore is characteristic of BG from a species. BG from different cereals differ in their solubility, with BG from oats being more soluble than the BG from wheat. This is thought to be related to the DP3 to DP4 ratio of the BG polymer.

[0263] In wild-type wheat grain, BG levels are greater in the whole grain than in the endosperm (Henry, 1985). BG content of wild-type whole wheat grain was about 0.6% on a weight basis, compared to about 4.2% for barley, 3.9% for oats and 2.5% for rye (Henry 1987). In wild-type wheat grain, the range was 0.4-1.4% by weight (Lazaridou *et al.*, 2007). Wheat grain BG typically has a DP3/DP4 ratio of 3-4.5 (Lazaridou *et al.*, 2007). Whilst barley BG has been associated with lowering plasma cholesterol, reducing glycemic index and reducing the risk of colon cancer, wheat BG has not been associated with these effects since wheat grain has much lower BG levels than barley. The level of BG in grain is ordinarily measured by milling the grain to wholemeal and assaying for BG by, for example, the method described in Example 1.

[0264] In wild-type wheat grain, the level of fructan is only 0.6%-2.6% by weight of the grain. As used herein, the term "fructan" means polymers of fructose which comprise fructosyl residues polymerized to a single terminal glucose unit. Fructans are synthesized from sucrose, explaining the terminal glucose. The fructose moieties are linked to each other by β-1,2 and/or β-2,6 bonds, and the glucose may be linked to the end of the chain by an α-1,2 bond as occurs in sucrose, being formed by repeated fructosyl transfer from sucrose. The enzymes involved in fructan synthesis include sucrose-sucrose fructosyl transferase (EC 2.4.1.99) which forms ketose, and either 1- or 6-fructan-fructan fructosyl transferase (EC 2.4.1.100). The degree of polymerisation (DP) varies from 3 to several hundred but is typically 3-60 and in grain of the invention mostly DP 3-10. In view of this composition, fructans are highly soluble in water and do not precipitate in 78% ethanol. The linkages between the fructosyl-residues are either exclusively of the β-1,2 type forming a linear molecule (inulin) in which the fructosyl residues are attached to the fructosyl residue of the sucrose starter, or of the β-2,6 type (levan), or both linkage types occur in branched fructans (graminans). Graminans, which comprise β-2,6-linked fructose

units with β-1,2 branch points and are therefore more complex structures, can also be present in cereals, and can be mixed with levans. The level of fructan in grain of the invention is ordinarily measured by milling the grain to wholemeal and assaying for fructan by, for example, the method described in Example 1, which is based on that of Prosky and Hoebregs (1999). The method depends on the hydrolysis of the fructans followed by determination of the released sugars.

[0265] Fructans are non-starch carbohydrates with potentially beneficial effects as a food ingredient on human health (Tungland and Meyer, 2002; Ritsema and Smeekens, 2003). The human digestive enzymes α-glucosidase, maltase, isomaltase and sucrase are not able to hydrolyse fructans because of the β-configuration of the fructan linkages. Furthermore, humans and other mammals lack, in their small intestines, the fructan exohydrolase enzymes that break down fructans and therefore dietary fructans avoid digestion in the small intestine and reach the large intestine intact. However, bacteria there are able to ferment fructans and thereby utilize them as, for example, an energy or carbon source for growth and production of short-chain fatty acids (SCFA). Dietary fructans therefore are able to stimulate the growth of beneficial bacteria such as bifidobacteria in the colon, which aids in prevention of bowel disorders such as constipation and infection by pathogenic gut bacteria. Dietary fructan also enhances nutrient absorption from diets, particularly calcium and iron, possibly via production of SCFA which in turn reduce luminal pH and modify calcium speciation and hence solubility, or exert a direct effect on the mucosal transport pathway, thereby improving the mineralization of bone and reducing the risk of iron deficiency anaemia. In addition, a high-fructan diet can improve the health of patients with diabetes and reduce the risk of colonic cancers by suppressing aberrant crypt foci which are precursors of colon cancer (Kaur and Gupta, 2002). Also, fructans have a sweet taste and are increasingly used as low-calorie sweeteners and as functional food ingredients.

[0266] Production of isolated fructan from grain of the invention is cost-effective relative to existing methods of fructan production, for example, involving the extraction of inulins from chicory. Large scale extraction of fructan can be achieved by milling the grain to wholemeal flour and then extracting the total sugars including fructans from the flour into water. This may be done at ambient temperature and the mixture then centrifuged or filtered. The supernatant is then heated to about 80°C and centrifuged to remove proteins,

then dried down. Alternatively, the extraction of flour can be done using 80% ethanol, with subsequent phase separation using water/chloroform mixtures, and the aqueous phase containing sugars and fructan dried and redissolved in water. Sucrose in the extract prepared either way may be removed enzymatically by the addition of α-glucosidase, and then hexoses (monosaccharides) removed by gel filtration to produce fructan fractions of various sizes. This would produce a fructan enriched fraction of at least 50%, preferably at least 60% or at least 70%, more preferably at least 80% fructan.

[0267] *Development of a small-scale fructan assay.* The fructan content in cereal grains and their derived food products is commonly measured using high-performance liquid chromatography (HPLC) (Huynh *et al.*, 2008) or spectrophotometry (McCleary *et al.*, 2000; McCleary *et al.*, 2013; Steegmans *et al.*, 2004). The Official AOAC Method 999.03 (AOAC, 2000b) based on spectrophotometry has been commercialized as the K-FRUCHK and K-FRUC kits by Megazyme International Limited (Bray, Ireland). These commercial kits are convenient and easy for measuring fructan levels in cereal grains (Karppinen *et al.*, 2003; Whelan *et al.*, 2011). In the K-FRUCHK assay, sucrose and low degree-of polymerisation (DP) maltosaccharides are hydrolysed to fructose and glucose. Their concentration is measured with a hexokinase/phosphor-glucose isomerise/glucose 6-phosphate dehydrogenase (HK/PGI/G6PGH) system using a spectrophotometer. After fructan hydrolysis, the total concentration of fructose and glucose is re-measured and the fructan content is then determined by the difference between the two measurements. In the K-FRUC assay, sucrose, maltose, maltodextrins and starch are hydrolysed to fructose and glucose that are further reduced by sodium borohydride to the corresponding sugar alcohols (sorbitol and mannitol). Fructose and glucose derived from fructan hydrolysis are coupled with 4-hydroxybenzoic acid hydrazide (PAHBAH) to develop colour in a boiling water bath and their absorbance is read using a spectrophotometer for calculating fructan content.

[0268] A simplified enzymatic hydrolysis followed by the HPLC analysis was recently developed for screening fructan content in a double haploid (DH) wheat population (Huynh *et al.*, 2008b). There is a need for accurate and rapid measurement of fructan content in large breeding populations with hundreds to thousands of lines. However, all the current fructan assays used in cereal grains are relatively low efficiency, allowing about 10 samples per day per worker, and require several grams of each flour sample. In order to

develop high throughput fructan assays, the inventors scaled down the K-FRUCHK and K-FRUC assays in a plate format to allow this, as described in Example 1.

[0269] Arabinoxylan is another polysaccharide found in plant cell walls, including in the cell walls in wheat grain. The levels of arabinoxylan in the grain and flour of the invention are increased relative to the corresponding wild-type grain and flour, for example by at least 1.5-fold or at least 2-fold on a weight basis. The level may be increased between 1.5-fold and 3-fold. As used herein, "arabinoxylan" (AX) refers to a linear chain backbone of β-D-xylopyranosyl residues linked through 1,4 glycosidic linkages, with α-L-arabinofuranosyl residues attached to some of the xylopyranosyl residues at O-2, O-3 and/or at both O-2,3 positions. The xylopyranosyl residues are any one of: mono-substituted at O-2 or O-3, di-substituted at O-2,3, and unsubstituted. Sidebranches may contain, in addition to arabinose residues, small amounts of xylopyranose, galactopyranose, α-D-glucuronic acid or 4-O-methyl-α-D-glucuronic residues. In cereals, the Ara/Xyl ratio may vary from 0.3 to 1.1. AX from the outer pericarp, scutellum and embryonic axis are relatively highly substituted with arabinose whereas those of the aleurone and hyaline layer are less substituted. AX may further comprise the hydrocinnamic acids, ferulic acid and p-coumaric acid, esterified to O-5 of arabinose residues linked to the O-3 of the xylose residues (Smith and Hartley, 1983). The biosynthesis of the 1,4-β-D-xylan backbone is catalyzed by 1,4-β-xylosyl transferase that uses UDP-D-xylose as substrate and transfers the xylose unit to the non-reducing end of an xylooligosaccharide chain.

[0270] The synthesis of AX in cereals involves xylotransferases that use UDP-Xyl as substrate and may involve a complex tetrasaccharide as a primer (Carpita *et al.*, 2011). Arabinoxylans are only slightly soluble in water and require alkali solvents for their efficient extraction. For example, barium hydroxide can selectively extract AX (Gruppen *et al.*, 1992). In contrast, sodium hydroxide extracts both BG and AX. The level of AX in grain is ordinarily measured by milling the grain to wholemeal and assaying for AX by, for example, the method described in Example 1.

[0271] Studies using maize, rye and wheat AX demonstrated positive effects on cecal fermentation, production of SCFA, reduction in serum cholesterol and improved absorption of calcium and magnesium (Hopkins *et al.* 2003).

[0272] As used herein, cellulose refers to a crystalline array of about 24-36 (1-4)-β-D-glucan chains that form microfibrils, found predominantly in the cell walls of plants. It is one of the most abundant polymers found in nature. The glucan chains are formed by cellulose synthases of the *CesA* gene family at the plasma membrane (Giddings *et al.*, 1980), and 24 to 36 chains are then assembled into a functional microfibril. *Arabidopsis* has 10 *CesA* genes, at least 3 of which are co-expressed during primary cell wall formation and three others during secondary cell wall formation (Carpita *et al.*, 2011), each of which add glycosyl residues to the non-reducing end of acceptor glucan chains to extend the polymers. The *CesA* genes are related to the *Csl* genes of both monocots and dicots which are involved in synthesis of other polysaccharides. For example, the CslF and CslH enzymes found only in the grasses including cereals are involved in synthesis of BG.

[0273] The food products made from the grain, food ingredients, starch granules and starch are characterized by a decreased Glycaemic Index. GI is a simple marker for the effect of carbohydrate rich foods on post-prandial glucose levels in the blood of human subjects. As used herein, "Glycaemic Index" or "GI" means a measure of the area under the curve of blood glucose concentrations after eating a portion of a test food containing 50g of carbohydrate, divided by the incremental area achieved with the same amount of carbohydrate present in an equivalent amount of glucose or white bread. GI therefore relates to the rate of digestion of foods comprising the starch and uptake of the digestion products, and is a comparison of the effect of a test food with the effect of white bread or glucose on excursions in blood glucose concentration. GI is thereby a measure of the effect of the food on post prandial serum glucose concentration and is associated with the demand for insulin for blood glucose homeostasis. One important characteristic provided by foods of the invention is a reduced GI relative to a corresponding food made with the same amount of wild-type wheat, flour, starch granules or starch as food ingredient. Furthermore, the foods of the invention may have a reduced level of final digestion and consequently be relatively low-calorie compared to a corresponding food made with the same amount of wild-type wheat as food ingredient. A low calorific product might be based on inclusion of flour produced from milled wheat grain. Such foods may have the effect of being filling, enhancing bowel health, reducing the post-prandial serum glucose and lipid concentration as well as providing for a low calorific food product.

[0274] GI of starch of the invention, or a food ingredient or food product of the invention, is readily measured using an *in vitro* assay as described in Example 9 herein. The *in vitro* assay simulates the digestion of the starch in the products as occurs upon consumption in healthy humans, and is predictive of the GI as measured in human subjects after consumption of the products.

[0275] The method of treating the subject, particularly humans, may comprise the step of administering altered wheat grain, flour, starch or a food or drink product as defined herein to the subject, in one or more doses, in an amount and for a period of time whereby the level of the one or more of bowel health or metabolic indicators improves. The indicator may change relative to consumption of a corresponding non-altered wheat starch or wheat or product thereof, within a time period of up to 24 hours, as in the case of some of the indicators such as pH, elevation of levels of SCFA, post-prandial glucose fluctuation, or it may take days such as in the case of increase in fecal bulk or improved laxation, or perhaps longer in the order of weeks or months such as in the case where the butyrate enhanced proliferation of normal colonocytes is measured. It may be desirable that administration of the altered starch or wheat or wheat product be lifelong. However, there are good prospects for compliance by the individual being treated given the relative ease with which the altered starch can be administered.

[0276] Dosages may vary depending on the condition being treated or prevented but are envisaged for humans as being at least 10g of wheat grain or starch of the invention per day, more preferably at least 15g per day, preferably at least 20 or at least 30g per day. Administration of greater than about 100 grams per day may require considerable volumes of delivery and reduce compliance. Most preferably the dosage for a human is between 10 and 100g of wheat grain of the invention, or flour, wholemeal or modified starch of the invention per day, or for adult humans between 20 and 100g per day.

[0277] The indicators of improved bowel health may comprise, but are not necessarily limited to:

i) decreased pH of the bowel contents,

ii) increased total SCFA concentration or total SCFA amount in the bowel contents,

iii) increased concentration or amount of one or more SCFAs in the bowel contents,

iv) increased fecal bulk,

v) increase in total water volume of bowel or faeces, without diarrhea,

vi) improved laxation,

vii) increase in number or activity of one or more species of probiotic bacteria,

viii) increase in fecal bile acid excretion,

ix) reduced urinary levels of putrefactive products,

x) reduced fecal levels of putrefactive products,

xi) increased proliferation of normal colonocytes,

xii) reduced inflammation in the bowel of individuals with inflamed bowel or a tendency to inflamed bowel,

xiii) reduced fecal or large bowel levels of any one of urea, creatinine and phosphate in uremic patients, and

xiv) any combination of the above.

[0278] The indicators of improved metabolic health may comprise, but are not necessarily limited to:

i) stabilisation of post-prandial glucose fluctuation,

ii) improved (lowered) glycaemic response,

iii) reduced pro-prandial plasma insulin concentration,

iv) improved blood lipid profile,

v) lowering of plasma LDL cholesterol,

vi) reduced plasma levels of one or more of urea, creatinine and phosphate in uremic patients,

vii) an improvement in a dysglucaemic response, or

viii) any combination of the above.

[0279] The pH of the bowel contents may be decreased by at least 0.1 units, preferably by at least 0.15 or 0.2 units. Each of the other indicators of bowel health or metabolic health may be improved by at least 10%, preferably at least 20%.

[0280] It will be understood that one benefit of the present invention is that it provides for products such as bread that are of particular nutritional benefit, and moreover it does so without the need to post-harvest modify the starch or other constituents of the wheat grain.

However, it may be desired to make modifications to the starch or other constituent of the grain, and the invention encompasses such a modified constituent. Methods of modification are well known and include the extraction of the starch or other constituent by conventional methods and modification of the starches to increase the resistant form. The starch may be modified by treatment with heat and/or moisture, physically (for example ball milling), enzymatically (using for example α- or β-amylase, pullalanase or the like), chemical hydrolysis (wet or dry using liquid or gaseous reagents), oxidation, cross bonding with difunctional reagents (for example sodium trimetaphosphate, phosphorus oxychloride), or carboxymethylation.

[0281] Whilst the invention may be particularly useful in the treatment or prophylaxis of humans, it is to be understood that the invention is also applicable to non-human subjects including but not limited to agricultural animals such as cows, sheep, pigs and the like, domestic animals such as dogs or cats, laboratory animals such as rabbits or rodents such as mice, rats, hamsters, or animals that might be used for sport such as horses. The method may be particularly applicable to non-ruminant mammals or animals such as mono-gastric mammals. The invention may also be applicable to other agricultural animals for example poultry including, for example, chicken, geese, ducks, turkeys, or quails, or fish.

[0282] The terms "polypeptide" and "protein" are generally used interchangeably herein. The terms "proteins" and "polypeptides" as used herein also include variants, mutants, modifications 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 60% free, more preferably at least 75% free, and more preferably at least 90% free from other components with 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 wheat cell. In an embodiment, the polypeptide has starch synthase enzyme activity, particularly SSIIa activity, and is at least 98% identical to a SSIIa polypeptide described herein.

[0283] The % identity of a polypeptide relative to a reference polypeptide can be determined by any program known in the art for aligning amino acid sequences, such as the program GAP (Needleman and Wunsch, 1970, GCG program) with a gap creation

penalty=5, and a gap extension penalty=0.3. The analysis aligns the two sequences over the full length amino acid sequence of the reference sequence. For example, if the reference sequence is the amino acid sequence set forth as SEQ ID NO:1, the alignment is along the full length of SEQ ID NO:1. A gap in an aligned sequence is regarded as a position of non-identity for each missing amino acid.

[0284] With regard to a defined polypeptide, 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 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.

[0285] Amino acid sequence deletions or insertions generally range from about 1 to 15 residues, more preferably about 1 to 10 residues and typically about 1 to 5 contiguous residues. However, they may be larger than 15 amino acids, up to the full length of the polypeptide. The polypeptide sequence may be a truncated sequence relative to the corresponding wild-type sequence or reference SEQ ID NO. For example, if the protein coding region encoding the polypeptide has a premature translation termination codon (stop codon), the resultant polypeptide will, if translated, be truncated. The extent of the truncation depends on the position of the stop codon, shortening the polypeptide by at least 5%, preferably at least 10% relative to the wild-type sequence.

[0286] The protein coding region of a gene of the invention may be disrupted by the presence of a splice-site mutation which causes mis-splicing and may result in an altered RNA transcript such that the open reading frame is disrupted. Then the amino acid sequence of the polypeptide may be identical to the wild-type up to the point of the mis-splicing or downstream of that point and then diverge from the wild-type. Such polypeptides are generally affected in their activity in the same way as a truncated

polypeptide. Examples of stop codons and splice-site mutations in an *SSIIa* gene are described in Example 11 herein.

[0287] Substitution mutants have at least one amino acid residue in the polypeptide removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis for reduced activity of the polypeptide 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 i.e. conserved amino acids. These positions are likely to be important for biological activity. These amino acids, especially those falling within a contiguous sequence of at least three other identically conserved amino acids, are preferably substituted in a relatively conservative manner in order to retain function such as SSIIa enzyme activity, or in a non-conservative manner for reduced activity. Conservative substitutions are shown in Table 3 under the heading of "exemplary substitutions". "Non-conservative amino acid substitutions" are defined herein as substitutions other than those listed in Table 3 (Exemplary conservative substitutions). Non-conservative substitutions in an SSIIa or SSIIIa polypeptide are expected to reduce the activity of the enzyme and many will correspond to an SSIIa or SSIIIa encoded by a "partial loss of function mutant *SSIIa* (or *SSIIIa*) gene".

Table 2. Amino acid sub-classification

Sub-classes	**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 3. Exemplary and Preferred Conserved Amino Acid Substitutions

Original Residue	**Exemplary conservative substitutions**	**Preferred conservative substitutions**
Ala	Val, Leu, Ile	Val
Arg	Lys, Gln, Asn	Lys
Asn	Gln, His, Lys, Arg	Gln
Asp	Glu	Glu
Cys	Ser	Ser
Gln	Asn, His, Lys,	Asn
Glu	Asp, Lys	Asp
Gly	Pro	Pro
His	Asn, Gln, Lys, Arg	Arg
Ile	Leu, Val, Met, Ala, Phe	Leu
Leu	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	Leu

[0288] In some embodiments, the present invention involves modification of gene activity, particularly of *SSIIIa* gene activity, combinations of mutant genes, 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, together with associated non-coding and regulatory regions. The term “gene” also includes mutant forms of a wild-type gene, which mutant genes may not be transcribed and/or translated, such as, for example, if a promoter region has been deleted. Associated non-coding and regulatory regions are typically located adjacent to the protein coding region on both the 5' and 3' ends for a distance of about 2 kb on either side. In this regard, the gene includes control signals such as promoters, enhancers, transcription 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 protein coding region and which are present on the mRNA are referred to as 5' non-translated sequences (5’-UTR). The sequences which are located 3' or downstream of the protein coding region and which are present on the mRNA are referred to as 3' non-translated sequences (3’-UTR). The term "gene" encompasses both cDNA and genomic forms of a gene. A “cDNA” is a DNA copy of an RNA transcript of a gene and is described herein as “corresponding to the gene”. For example, the cDNA nucleotide sequence set forth as SEQ ID NO:4 corresponds to the *SSIIa-A* gene whose wild-type sequence is set forth as SEQ ID NO:7. The term "gene" includes synthetic or fusion molecules encoding the proteins of the invention described herein. Genes are ordinarily present in the wheat genome as double-stranded DNA. A chimeric gene may be introduced into an appropriate vector for extrachromosomal maintenance in a cell or for integration into the host genome. As used herein, genes or genotypes are referred to in italicised form (e.g. *SSIIa* or *SSIIIa*) while proteins, enzymes or phenotypes are referred to in non-italicised form (SSIIa or SSIIIa).

[0289] As used herein, the term “genotype” refers to the genetic makeup of a wheat cell, tissue, plant, plant part or plant product. The genetic makeup will be identical to that of the wheat plant from which the product was obtained. As used herein, the term “phenotype” refers to an observable characteristic, or set of multiple characteristics, of the cell, tissue, plant, plant part or plant product which result from the interaction between the plant’s genotype and the environment under which the plant was grown.

[0290] A genomic form or clone of a gene containing the coding 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 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 an encoded polypeptide.

[0291] The present invention 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 and includes for example cDNA, mRNA, tRNA, siRNA, shRNA, hpRNA, and single or double-stranded DNA. It may be DNA or RNA of cellular, genomic or synthetic origin. Preferably the polynucleotide is solely DNA or solely RNA as occurs in a cell. 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 includes 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 in an RNA molecule. "Complementary" means two polynucleotides are capable of basepairing along part of their lengths, or along the full length of one or both (fully complementary).

[0292] 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 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 to be transcribed in the cell.

[0293] The present invention refers to use of oligonucleotides which may be used as "probes" or "primers". As used herein, "oligonucleotides" are polynucleotides up to 50 nucleotides in length, preferably 15-50 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, typically comprised of 10-30 or 15-25 nucleotides which are identical to, or complementary to, part of an *SSIIa* or *SSIIIa* gene or cDNA corresponding to an *SSIIa* or *SSIIIa* gene. 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. Polynucleotides used as a probe are typically conjugated with a detectable label such as a radioisotope, an enzyme, biotin, a fluorescent molecule or a chemiluminescent molecule. Oligonucleotides and probes of the invention are useful in methods of detecting an allele of a *SSIIa*, *SSIIIa* or other gene associated with a trait of interest, for example modified starch. Such methods employ nucleic acid hybridization and in many instances include oligonucleotide primer extension by a suitable polymerase, for example as used in PCR for detection or identification of wild-type or mutant alleles. Preferred oligonucleotides and probes hybridise to a *SSIIa* or *SSIIIa* gene sequence from wheat or other cereals, including any of the sequences disclosed herein, for example SEQ ID NOs: 15 to 49. Preferred oligonucleotide pairs are those that span one or more introns, or a part of an intron and therefore may be used to amplify an intron sequence in a PCR reaction. Numerous examples are provided in the Examples herein.

[0294] The terms "polynucleotide variant" and "variant" and the like refer to polynucleotides displaying substantial sequence identity with a reference polynucleotide

sequence and which are able to function in an analogous manner to, or with the same activity as, the reference sequence. These terms also encompass polynucleotides that are distinguished from a reference polynucleotide by the addition, deletion or substitution of at least one nucleotide, or that have, when compared to naturally occurring molecules, one or more mutations. 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 hybridising agents. The terms "polynucleotide variant" and "variant" also include naturally occurring allelic variants. 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). Preferably, a polynucleotide variant of the invention which encodes a polypeptide with enzyme activity is at least 90% in length relative to the wild-type, up to the full length of the gene.

[0295] A variant of an oligonucleotide of the invention includes molecules of varying sizes which are capable of hybridising, for example, to the wheat genome at a position 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 hybridise to the target region. Furthermore, a few nucleotides may be substituted without influencing the ability of the oligonucleotide to hybridise to the target region. In addition, variants may readily be designed which hybridise close (for example, but not limited to, within 50 nucleotides) to the region of the plant genome where the specific oligonucleotides defined herein hybridise.

[0296] By "corresponds to" or "corresponding to" in the context of polynucleotides or polypeptides is meant a polynucleotide (a) having a nucleotide sequence that is substantially identical or complementary to at least a portion of, preferably all of (fully complementary), a reference polynucleotide sequence or (b) encoding an amino acid sequence identical to an amino acid sequence in a polypeptide. This phrase also includes

within its scope a polypeptide having an amino acid sequence that is substantially identical to a sequence of amino acids in a reference polypeptide. Terms used to describe sequence relationships between two or more polynucleotides or polypeptides include "reference sequence", "sequence identity", "percentage of sequence identity", "substantial identity" and "identical", and are defined with respect to a defined minimum number of nucleotides or amino acid residues or preferably 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 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.

[0297] The % identity of a polynucleotide to a reference polynucleotide can be determined by any program known in the art for this purpose, such as for example, GAP (Needleman and Wunsch, 1970, GCG program) with a gap creation penalty=5, and a gap extension penalty=0.3. Reference also may be made to the BLAST family of programs as for example disclosed by Altschul *et al.,* 1997. A detailed discussion of sequence analysis can be found in Unit 19.3 of Ausubel *et al.*, 1994-1998, Chapter 15. Unless stated otherwise, the alignment is carried out along the full length of the reference sequence.

[0298] Nucleotide or amino acid sequences are indicated as "essentially similar" when such sequences have a sequence identity of at least 98%, more particularly at least about 98.5%, quite particularly about 99%, especially about 99.5%, more especially about 99.8%, and includes when the sequences are identical. It is clear that when RNA sequences are described as essentially similar 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.

[0299] With regard to the defined polynucleotides, it will be appreciated that higher % identity figures 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 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.

[0300] In some embodiments, 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. The higher the stringency, the higher will be the degree of complementarity between a target nucleotide sequence and the labelled polynucleotide sequence. "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 Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6, herein incorporated by reference. Specific hybridization conditions referred to herein are as follows: 1) low stringency hybridization conditions in 6 X sodium chloride/sodium citrate (SSC) at about 45°C, followed by two washes in 0.2 X SSC, 0.1% SDS at 50-55°C; 2) medium stringency hybridization conditions 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 in 6 X SSC at about 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 0.5 M sodium phosphate, 7% SDS at 65°C, followed by one or more washes at 0.2 X SSC, 1% SDS at 65°C.

[0301] As used herein, a "chimeric gene" or "genetic construct" refers to any gene that is not a native gene in its native location i.e. it has been artificially manipulated, including a chimeric gene or genetic construct which is integrated into the wheat genome. Typically a

chimeric gene or genetic construct comprises regulatory and transcribed or protein coding sequences that are not found together in nature. Accordingly, a chimeric gene or genetic construct 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 produced in an unmodified plant at the same developmental stage as the plant under investigation, preferably a wheat plant, such as starch or an *SSIIa* or *SSIIIa* gene or SSIIIa polypeptide. An "endogenous gene" refers to a native gene in its natural location in the genome of an organism, preferably an *SSIIa* or *SSIIIa* gene in a wheat plant. 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, preferably the wheat genome, but which does not naturally occur in the cell. These include modified forms of gene sequences found in that cell so long as the introduced gene contains some modification, e.g. an introduced mutation or the presence of a selectable marker gene, relative to the naturally-occurring gene. Foreign or exogenous genes may be genes found in nature that are inserted into a non-native organism, native genes introduced into a new location within the native host, or chimeric genes or genetic constructs. The term "genetically modified" includes introducing genes into cells, mutating genes in cells and artificially altering or modulating the regulation of a gene in a cell by modifying the genome, or organisms to which these acts have been done or their progeny or parts such as grain.

[0302] 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.

[0303] 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 regulates the expression of the genetic sequence. This may be a naturally occurring cis-acting sequence in its native context, for example regulating a wheat *SSIIa* or *SSIIIa* gene, or a sequence in a genetic construct which when positioned appropriately relative to an expressible genetic sequence, regulates its expression. Such a cis-regulatory region may be capable of activating, silencing, enhancing, repressing or otherwise altering the level of expression and/or cell-type-specificity and/or developmental specificity of a gene sequence at the transcriptional or post-transcriptional level. For example, the presence of an intron in the 5'-leader (UTR) of genes has been shown to enhance expression of genes in monocotyledonous plants such as wheat (Tanaka *et al.*, 1990). Another type of cis-acting sequence is a matrix attachment region (MAR) which may influence gene expression by anchoring active chromatin domains to the nuclear matrix.

[0304] By "vector" is meant a nucleic acid molecule, preferably a DNA molecule derived from a plasmid or plant virus, into which a nucleic acid sequence may be inserted. The vector may also include a selection marker such as an antibiotic resistance gene that can be used for selection of suitable bacterial or plant transformants, or sequences that enhance transformation of prokaryotic or eukaryotic (especially wheat) cells such as T-DNA or P-DNA sequences. Examples of such resistance genes and sequences are well known to those of skill in the art.

[0305] 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, and are well known in the art. 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) or based on a growth advantage in the presence of a metabolizable substrate. Exemplary selectable marker genes for selection of plant transformants include, but are not limited to, a *hyg* gene which confers hygromycin B resistance; a neomycin phosphotransferase (*npt*) gene conferring resistance to kanamycin and the like as, for example, described by Potrykus *et al.*, 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 WO87/05327, an acetyl transferase 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.,* 1988, a *bar* gene conferring resistance against bialaphos as, for example, described in WO91/02071; or a nitrilase gene such as *bxn* from *Klebsiella ozaenae* which confers resistance to bromoxynil (Stalker *et al.,* 1988). 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.*, 1985), which may be employed in calcium-sensitive bioluminescence detection; a green fluorescent protein gene (GFP, Niedz *et al.,* 1995) or one of its variants; a luciferase (*luc*) gene (Ow *et al.,* 1986), which allows for bioluminescence detection, and others known in the art.

[0306] In some embodiments, the level of an enzyme activity is modulated by decreasing the level of expression of genes encoding the enzyme in the wheat plant, or increasing the level of expression of a nucleotide sequence that codes for the enzyme in a wheat plant. Increasing expression can be achieved at the level of transcription by using promoters of different strengths or inducible promoters, which are capable of controlling the level of transcript expressed from the coding sequence. Heterologous sequences may be introduced which encode transcription factors that modulate or enhance expression of genes whose products down-regulate starch synthesis such as SSIIa. The level of expression of the gene may be modulated by altering the copy number per cell of a construct comprising the coding sequence and a transcriptional control element that is operably connected thereto and that is functional in the cell. Alternatively, 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 is one which results in a substantial increase in amylose content in the wheat plant. This may be detected simply by testing the transformants.

[0307] Reducing gene expression may also be achieved through introduction and transcription of a "gene-silencing chimeric gene" introduced into the wheat plant. The gene-silencing chimeric gene is preferably introduced stably into the wheat genome, preferably the wheat nuclear genome, so that it is stably inherited in progeny. As used herein "gene-silencing effect" refers to the reduction of expression of a target nucleic acid in a wheat cell, preferably an endosperm cell during seed development while the plant is growing, which can be achieved by introduction of a silencing RNA. In a preferred embodiment, a gene-silencing chimeric gene is introduced which encodes an RNA molecule which reduces expression of one or more endogenous genes, for example genes other than the *SSIIa* or *SSIIIa* genes, or preferably the three endogenous *SSIIa* or *SSIIIa* genes. Such reduction may be the result of reduction of transcription, including by methylation of promoter regions via chromatin remodelling, or post-transcriptional modification of the RNA molecules, including via RNA degradation, or both. Gene-silencing should not necessarily be interpreted as an abolishing of the expression of the target nucleic acid or gene. It is sufficient that the level expression of the target nucleic acid in the presence of the silencing RNA is lower that in the absence thereof. The level of expression of the targeted gene may be reduced by at least about 40% or at least about 45% or at least about 50% or at least about 55% or at least about 60% or at least about 65% or at least about 70% or at least about 75% or at least about 80% or at least about 85% or at least about 90% or at least about 95% or effectively abolished to an undetectable level.

[0308] Antisense techniques may be used to reduce gene expression in wheat cells. 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, preferably an *SSIIa* or *SSIIIa* gene. 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, 1999).

[0309] As used herein, "artificially introduced dsRNA molecule" refers to the introduction of double-stranded RNA (dsRNA) molecule, which preferably is synthesised in the wheat cell by transcription from a chimeric gene encoding such dsRNA molecule. RNA interference (RNAi) is particularly useful for specifically reducing the expression of

a gene or inhibiting the production of a particular protein, also in wheat (see, for example, Regina *et al.*, 2006). 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, and its complement, thereby forming a dsRNA. Conveniently, the dsRNA can be produced from a single promoter in the 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 a related (to a *SSIIa* or *SSIIIa* gene) or unrelated sequence 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; Smith *et al.,* 2000; WO 99/32619; WO 99/53050; WO 99/49029; and WO 01/34815.

[0310] The DNA encoding the dsRNA typically comprises both sense and antisense sequences arranged as an inverted repeat. In a preferred embodiment, the sense and antisense sequences are separated by a spacer region which may (or may not) comprise 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). 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 larger than about 200 bp, for example between 200 and 1000 bp). A hpRNA can also be rather small with the double-stranded portion ranging in size from about 30 to about 42 bp, but not much longer than 94 bp (see WO04/073390). The presence of the double stranded RNA region is thought to trigger a response from an endogenous plant system that destroys both the double stranded RNA and also the homologous RNA transcript from the target plant gene(s), efficiently reducing or eliminating the activity of the target gene.

[0311] The length of the sense and antisense sequences that hybridise should each be at least 19 or at least 21 contiguous nucleotides, preferably at least 30 or 50 nucleotides, and more preferably at least 100, 200, 500 or 1000 nucleotides. The full-length sequence corresponding to the entire gene transcript may be used. 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 for the overall sequence identity. The RNA molecule may of course comprise unrelated sequences which may function to stabilize the molecule. The promoter used to express the dsRNA-forming construct may be any type of promoter that is expressed in the cells which express the target gene, preferably a promoter which is preferentially expressed in the endosperm of the developing wheat grain relative to non-grain tissues of the wheat plant. When the target gene is *SSIIa*, *SSIIIa* or other gene expressed selectively in the endosperm, an endosperm specific promoter is preferred which is not expressed in leaf or stem tissues, so as to not affect expression of the target gene(s) in other tissues.

[0312] As used herein, "silencing RNAs" are RNA molecules that have 21 to 24 contiguous nucleotides that are complementary to a region of the mRNA transcribed from the target gene, preferably *SSIIa* or *SSIIIa*. The sequence of the 21 to 24 nucleotides is preferably fully complementary to a sequence of 21 to 24 contiguous nucleotides of the mRNA i.e. identical to the complement of the 21 to 24 nucleotides of the region of the mRNA. However, miRNA sequences which have up to five mismatches in region of the mRNA may also be used (Palatnik *et al.,* 2003), and basepairing may involve one or two G-U basepairs. When not all of the 21 to 24 nucleotides of the silencing RNA are able to basepair with the mRNA, it is preferred that there are only one or two mismatches between the 21 to 24 nucleotides of the silencing RNA and the region of the mRNA. With respect to the miRNAs, it is preferred that any mismatches, up to the maximum of five, are found towards the 3' end of the miRNA. In a preferred embodiment, there are not more than one or two mismatches between the sequences of the silencing RNA and its target mRNA.

[0313] Silencing RNAs derive from longer RNA molecules that are encoded by the chimeric DNAs of the invention. The longer RNA molecules, also referred to herein as "precursor RNAs", are the initial products produced by transcription from the chimeric DNAs in the wheat cells and have partially double-stranded character formed by intra-molecular basepairing between complementary regions. The precursor RNAs are processed by a specialized class of RNAses, commonly called "Dicer(s)", into the silencing RNAs, typically of 21 to 24 nucleotides long. Silencing RNAs as used herein include short interfering RNAs (siRNAs) and microRNAs (miRNAs), which differ in their biosynthesis. SiRNAs derive from fully or partially double-stranded RNAs having at least 21 contiguous basepairs, including possible G-U basepairs, without mismatches or non-basepaired

nucleotides bulging out from the double-stranded region. These double-stranded RNAs are formed from either a single, self-complementary transcript which forms by folding back on itself and forming a stem-loop structure, referred to herein as a "hairpin RNA", or from two separate RNAs which are at least partly complementary and that hybridize to form a double-stranded RNA region. MiRNAs are produced by processing of longer, single-stranded transcripts that include complementary regions that are not fully complementary and so form an imperfectly basepaired structure, so having mismatched or non-basepaired nucleotides within the partly double-stranded structure. The basepaired structure may also include G-U basepairs. Processing of the precursor RNAs to form miRNAs leads to the preferential accumulation of one or more distinct, small RNAs each having a specific sequence, the miRNA(s). They are derived from one strand of the precursor RNA, typically the "antisense" strand of the precursor RNA, whereas processing of the long complementary precursor RNA to form siRNAs produces a population of siRNAs which are not uniform in sequence but correspond to many portions and from both strands of the precursor.

[0314] MiRNA precursor RNAs of the invention, also termed herein as "artificial miRNA precursors", are typically derived from naturally occurring miRNA precursors by altering the nucleotide sequence of the miRNA portion of the naturally-occurring precursor so that it is complementary, preferably fully complementary, to the 21 to 24 nucleotide region of the target mRNA, and altering the nucleotide sequence of the complementary region of the miRNA precursor that basepairs to the miRNA sequence to maintain basepairing. The remainder of the miRNA precursor RNA may be unaltered and so have the same sequence as the naturally occurring miRNA precursor, or it may also be altered in sequence by nucleotide substitutions, nucleotide insertions, or preferably nucleotide deletions, or any combination thereof. The remainder of the miRNA precursor RNA is thought to be involved in recognition of the structure by the Dicer enzyme called Dicer-like 1 (DCL1), and therefore it is preferred that few if any changes are made to the remainder of the structure. For example, basepaired nucleotides may be substituted for other basepaired nucleotides without major change to the overall structure. The naturally occurring miRNA precursor from which the artificial miRNA precursor of the invention is derived may be from wheat, another plant such as another cereal plant, or from non-plant sources. Examples of such precursor RNAs are the rice mi395 precursor, the *Arabidopsis*

mi159b precursor, or the mi172 precursor. The use of artificial miRNAs have been demonstrated in plants, for example Alvarez *et al.,* 2006; Parizotto *et al.,* 2004; Schwab *et al.,* 2006.

[0315] Another molecular biological approach that may be used to down-regulate endogenous 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 co-suppression approaches.

[0316] 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.

[0317] A number of techniques are available for the introduction of nucleic acid molecules into a wheat cell, well known to workers in the art. The term "transformation" as used herein means alteration of the genotype of a cell, for example a bacterium or a plant, particularly a wheat plant, by the introduction of a foreign or exogenous nucleic acid. By "transformant" is meant an organism so altered. Introduction of DNA into a wheat plant by crossing parental plants or by mutagenesis *per se* is not included in transformation. The nucleic acid molecule may be replicated as an extrachromosomal element or is preferably stably integrated into the genome of the plant. 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. In an

embodiment, a transgene is integrated in the wheat nuclear genome which in hexaploid wheat includes the A, B and D subgenomes, herein referred to as the A, B and D "genomes".

[0318] The most commonly used methods to produce fertile, transgenic wheat plants comprise two steps: the delivery of DNA into regenerable wheat cells and plant regeneration through *in vitro* tissue culture. Two methods are commonly used to deliver the DNA: T-DNA transfer using *Agrobacterium tumefaciens* or related bacteria and direct introduction of DNA via particle bombardment, although other methods have been used to integrate DNA sequences into wheat or other cereals. 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. Such techniques for wheat are well known in the art.

[0319] Transformed wheat plants can be produced by introducing a nucleic acid construct according to the invention into a recipient cell and growing a new plant that comprises and expresses a polynucleotide according to the invention. The process of growing a new plant from a transformed cell which is in cell culture is referred to herein as "regeneration". Regenerable wheat cells include cells of mature embryos, meristematic tissue such as the mesophyll cells of the leaf base, or preferably from the scutella of immature embryos, obtained 12-20 days post-anthesis, or callus derived from any of these. The most commonly used route to recover regenerated wheat plants is somatic embryogenesis using media such as MS-agar supplemented with an auxin such as 2,4-D and a low level of cytokinin, see Sparks and Jones , 2004).

[0320] *Agrobacterium*-mediated transformation of wheat may be performed by the methods of Cheng *et al.,* 1997; Weir *et al.,* 2001; Kanna and Daggard, 2003 or Wu *et al.,* 2003. Any *Agrobacterium* strain with sufficient virulence may be used, preferably strains having additional virulence gene functions such as LBA4404, AGL0 or AGL1 (Lazo *et al.,* 1991) or versions of C58. Bacteria related to *Agrobacterium* may also be used. The DNA that is transferred (T-DNA) from the *Agrobacterium* to the recipient wheat cells is comprised in a genetic construct (chimeric plasmid) that contains one or two border regions of a T-DNA region of a wild-type Ti plasmid flanking the nucleic acid to be transferred. The genetic construct may contain two or more T-DNAs, for example where

one T-DNA contains the gene of interest and a second T-DNA contains a selectable marker gene, providing for independent insertion of the two T-DNAs and possible segregation of the selectable marker gene away from the transgene of interest. The T-DNA vector is preferably a "super-binary" plasmid as known in the art.

[0321] Any wheat type that is regenerable may be used; varieties Bob White, Fielder, Veery-5, Cadenza and Florida have been reported with success. Transformation events in one of these more readily regenerable varieties may be transferred to any other wheat cultivars including elite varieties by standard backcrossing. Other methods involving the use of *Agrobacterium* include: co-cultivation of *Agrobacterium* with cultured isolated protoplasts; transformation of seeds, apices or meristems with *Agrobacterium*, or inoculation *in planta* such as the floral-dip method for *Arabidopsis* as described by Bechtold *et al.*, 1993.

[0322] Another method commonly used for introducing the nucleic acid construct into a plant cell is high velocity biolistic 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.,* 1987.

[0323] Preferred selectable marker genes for use in the transformation of wheat include the *Streptomyces hygroscopicus bar* gene or *pat* gene in conjunction with selection using the herbicide glufosinate ammonium, the *hpt* gene in conjunction with the antibiotic hygromycin, or the *nptII* gene with kanamycin or G418. Alternatively, positively selectable markers such as the *manA* gene encoding phosphomannose isomerase (PMI) with the sugar mannose-6-phosphate as sole C source may be used.

[0324] The present invention is further described by the following non-limiting Examples.

EXAMPLE 1: Materials and methods

[0325] *Plant materials.* Three wheat cultivars which each comprised single null mutations in an *SSIIa* gene on the A, B or D genomes were kindly provided by Dr M Yamamori, National Institute of Agrobiological Resources, Tsukuba, Japan. They were Chousen 57 (C57) comprising a null mutation in *SSIIa-A* and therefore lacking SSIIa-A

polypeptide (SGP-A1), Kanto 79 (K79) comprising a null mutation in *SSIIa-B* and therefore lacking SSIIa-B polypeptide (SGP-B1), and Turkey 116 (T116) comprising a null mutation in *SSIIa-D* and therefore lacking the SGP-D1 polypeptide (Yamamori *et al.*, 2000).

[0326] Chinese Spring (CS) nullisomic/tetrasomic lines for homologous group seven chromosomes, designated N7AT7D, N7BT7D and N7DT7B (Sears and Miller, 1985) were kindly supplied by Dr E. Lagudah (CSIRO Agriculture, Canberra, Australia).

[0327] Wheat plants including C57, K79, T116, three Australian wheat cultivars Sunco, EGA Hume and Westonia were grown at the CSIRO Agriculture, Canberra, in a glasshouse with natural light and at temperatures of 18°C (night) and 24°C (day). Mature grain of each line was harvested and air-dried to a moisture content of approximately 9%. Unless otherwise specified, 5g of dried grain per line was milled using a Udy Cyclone mill (Fort Collins, CO, USA) with 0.5 mm mesh screen to generate wholemeal flour or using a Brabender Quadrumat Junior mill (Brabender® GmbH & Co. KG, Duisburg, Germany) to obtain white flour.

[0328] For analysis of proteins and starch in developing grain and mature grain as described in Example 12`, mutant and wild-type wheat plants (*ssIIa* and *SSIIa*, respectively) were obtained from a double haploid population reported by Konik-Rose *et al.* (2007). Twenty to forty developing endosperms were collected at 15 days post anthesis (DPA) in tubes on dry ice and stored at -80^{o}C for the analyses of RNA, soluble proteins and starch granule bound proteins as described in Example 12. For analysis of proteins and starch properties in grain, grain was harvested at maturity from glasshouse or field-grown plants.

[0329] *DNA analysis of wheat plants.* For detecting the presence or absence of mutant *ssIIa* or wild-type *SSIIa* alleles by PCR on genomic DNA samples, young leaves were harvested from plants and genomic DNA extracted using a Fast DNA Kit (BIO101 system, Q-BIO gene). For marker-assisted breeding, primer pairs JKSS2AP1F (5'-TGCGTTTACCCCACAGAGCA CA-3' (SEQ ID NO:15) located between nucleotides 91 and 113 of nucleotide sequence Accession No. AB201445) and JKSS2AP2R (5'-TGCCAAAGGTCCGGAATCATGG-3' (SEQ ID NO:16) located between nucleotides

1225 and 1246 of AB201445) were used for the A genome *SSIIa* gene (Figure 2); primers JKSS2BP7F (5'- GCGGACCAGGTTGTCGTC- 3' (SEQ ID NO:17) located between nucleotides 5978 and 5995 of nucleotide sequence Accession No. AB201446) and JLTSS2BPR1 (5' CTGGCTCACGATCCAGGGCATC-3' (SEQ ID NO:18) located between nucleotides 6313 and 6335 of AB201446) for the B genome *SSIIa* gene (Figure 3); and primers JTSS2D3F (5'-GTACCAAGGTATGGGGACTATGAA-3' (SEQ ID NO:19) located between nucleotides 2369 and 2392 of nucleotide sequence Accession No. AB201447) and JTSS2D4R (5'-GTTGGAGAGATACCTCAACAGC-3' (SEQ ID NO:20) locating between nucleotides 2774 and 2796 of AB201447) were used for the D genome *SSIIa* gene of wheat (Figure 4).

[0330] The PCR reactions contained 50 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.125 mM of each dNTP, 10 pmol of primers, 0.5 M glycine betaine, 1 µl of dimethylsulphoxide (DMSO), and 1.5–3.5 U of Hot-star Taq polymerase (QIAGEN) in reaction volumes of 20 µl. The amplification reactions were conducted using a HYBAID PCR Express (Integrated Sciences) with one cycle of 95°C for 5 min, 35 cycles of melting at 94°C for 45 s, annealing temperature of 52°C (A genome) or 60°C (for B and D genome) for 30 s, and extension at 72°C for 2 min 30 s, then 1 cycle of 72°C for 10 min followed by cooling to 25°C. The resultant PCR fragments were separated on 1% or 2% agarose gels and visualized (UVitec) after ethidium staining. Other, standard amplifications used 59°C for the annealing temperature but otherwise used the same PCR conditions, unless stated otherwise.

[0331] Southern blot hybridization analysis was performed on DNA from a larger scale (9 ml) extraction from lyophilized ground tissue (Stacey and Isaac, 1994). DNA samples were adjusted to 0.2 mg/ml and digested with restriction enzymes such as *Bam*HI and *Eco*RI. Restriction enzyme digestion, gel electrophoresis and vacuum blotting are carried out as described by Stacey and Isaac, (1994). ^{32}P-labeled probes were produced from cDNA and used in hybridisation to Southern blots. Hybridising sequences were detected by autoradiography according to the method of Jolly *et al.* (1996).

[0332] *RNA extraction and quantitative real-time PCR (qRT-PCR).* Total RNA from endosperms at 15 DPA was extracted using a NucleoSpin® RNA Plant Kit (Macherey-Nagel) and quantified using Nanodrop 1000 (Thermo Scientific). Amounts of 0.5 µg of

RNA templates were used for cDNA synthesis in 50 µl reactions at 50°C using SuperScript III reverse transcriptase (Invitrogen). The cDNA template (100 ng) was used in a 10 µl qRT-PCR reaction with the annealing temperature at 58°C using RT-PCR primers (Table 4). As a quantitation control, a pair of primers was used which amplified a region located in an exon at the 3' end of a tubulin gene. The amplification reactions were conducted in a Rotor-Gene 6000 (Corbett) using a Rotor-Gene™ SYBR® Green PCR Kit (QIAGEN). Comparative quantitation was analysed using amplification of the tubulin gene fragment as a reference amplification in the Real Time Rotary Analyzer Software (Corbett). For each sample, triplicates of qRT-PCR reactions were performed.

Table 4. QRT-PCR primers used for RNA expression determination

Cereal	Name	Oligonucleotide Sequence	SEQ ID NO	Reference
Barley	ZLBSSI-1RTR3	AAGGTCTCCACCGTGTCTCGAAG	21	This study
	ZLBSSI-1RTF3	GACGTACAGTTTGTCATGCTTGG	22	This study
	ZLBSSIIaF	CTGCTGGACAGGATATGGAAGT	23	This study
	ZLBSSIIaR	GTATCACCATAACGGAGCGACT	24	This study
	ARHv2aF1	CAATCACTGATGGTGTAACCAAA	25	Regina *et al.*,
	ARHv2aR3	CCTTCATGTTGGTCAATAGCAGC	26	Regina *et al.*,
	ARHv2bF1	CAGAATGGACAAAGAATCATCC	27	Regina *et al.*,
	ARHv2bR1	GAAAATACATCCATGCCTCCATC	28	Regina *et al.*,
Wheat	SSIFw	AGGGTACAGGGTGGGCGTTCT	29	Sestili *et al.*, 2010
	SSIR	GTAGGGTTGGTCCACGAAGG	30	Sestili *et al.*, 2010
	SSIIFw	TTCGACCCCTTCAACCACTC	31	Sestili *et al.*, 2010
	SSIIR	ACGTCCTCGTAGAGCTTGGC	32	Sestili *et al.*, 2010
	SBEIIaFw	TGACGAATCTTGGAAAATGG	33	Sestili *et al.*, 2010
	SBEIIaR	GGCGGCATTTATCATAACTATTG	34	Sestili *et al.*, 2010
	SBEIIbFw	GTAGATGCGGTCGTTTACTTGA	35	Sestili *et al.*, 2010
	SBEIIbR	CCAGCCACCTTCTGTTTGTT	36	Sestili *et al.*, 2010
Rice	JLRSSI-RTF	GGGCCTTCATGGATCAACC	37	Ohdan *et al.*, 2005
	JLRSSI-RTR	CCGCTTCAAGCATCCTCATC	38	Ohdan *et al.*, 2005
	JLRSSIIa-RTF	CTGGACAGGATCTGGAAGTGAA	39	This study
	JLRSSIIa-RTR	GGAACCTCAACAGCAGCCTTAC	40	This study
	JLRSBEIIa-RTF	GCCAATGCCAGGAAGATGA	41	Ohdan *et al.*, 2005
	JLRSBEIIa-RTR	GCGCAACATAGGATGGGTTT	42	Ohdan *et al.*, 2005
	sbe2b-f	TACGAATTCTCCAGCGGAATGAG	43	Nishi *et al.*, 2001
	sbe2b-r	TACGGTACCCAAGATGTACAGA	44	Nishi *et al.*, 2001
	α-tublin-f	GGAAATACATGGCTTGCTGCTT	45	Toyota *et al.*,
	α-tublin-r	TCTCTTCGTCTTGATGGTTGCA	46	Toyota *et al.*,
Common	JLRHvTaTub-RTF	CAGGCTTGTATCCCAGGTCA	47	This study
	JLRHvTaTub-RTR	GCGTAGGAGGAAAGCATGAA	48	This study

[0333] *Isolation of soluble and starch granule bound proteins from developing endosperm.* Developing endosperms from developing seeds harvested at 15 DPA were homogenised and suspended in pre-chilled soluble protein extraction buffer at 1.5 µl/mg (0.25 M K_2HPO_4, pH 7.5, 0.05 M EDTA, 20% glycerol, Sigma protease inhibitor cocktail and 0.5 M DTT). The homogenate was centrifuged at 16,000 g for 15 min at 4°C. The supernatant containing soluble proteins was used for the estimation of protein concentration using Coomassie Plus Protein Assay Reagent (Bio-Rad). If desired, samples were stored at -20°C prior to analysis. The pellets kept from the soluble protein preparation were directly treated with proteinase K after washing with water, and then starch was

purified as follows. The starch granule bound proteins were prepared from purified starch according to Rahman *et al.*, (1995) with minor alterations. Starch granules were boiled for 5-10 min in protein denaturing extraction buffer (50 mM Tris buffer, pH 6.8, 10 % glycerol, 5 % SDS, 5 % β-mercaptoethanol, and bromophenol blue) at a ratio of 15 µl/mg starch. After centrifugation at 13,000 g for 20 min, the supernatant was used for SDS-PAGE analysis.

[0334] *Isolation of starch and extraction of starch granule bound proteins from mature grains* Whole grains (100-150 mg) from each plant were ground in a ball bearing machine at a speed of 30 rpm for 30 s using a WIG-L-BUG Mixer MSD (USA). Wholemeal products were first treated with 12.5 mM NaOH, filtered through 0.5 mm nylon sieves, washed with water three times, and then incubated with 0.5 mg proteinase K in 1 ml of 50 mM phosphate buffer at 37°C for 2 hrs. The starch pellets obtained by centrifugation at 5,000 g were suspended and washed with water for 3 times following with centrifugations after each wash. After washing with acetone, the residual starches were air-dried at 37°C overnight. Preparation of starch granule bound proteins from mature grain starches were the same as that described earlier for developing endosperm starches.

[0335] *SDS-PAGE and gel staining.* For the quantitation of protein contents in starch, an equal amount of starches (4 mg) were used for the extraction of starch granule bound proteins. The same volume of supernatant containing total proteins was loaded for each sample into NuPAGE Novex 4-12% Bis-Tris Gels (Life technologies). This allowed for the detection of variation in protein binding patterns from the same amount of starches. Samples containing 20 µg total proteins were used for soluble proteins. The SDS-PAGE gels were run and detected as previously described (Butardo *et al.* 2012).

[0336] *Immunoblotting.* Anti-serum against GBSSI, SSI, SSIIa, SBEIIa and SBEIIb from previous studies are listed and enumerated in Table 5, including their antigen sources and specificities. Western blotting and detection was carried out as previously-described (Butardo *et al.* 2012) using the same protein standards as above.

Table 5. Polyclonal antibodies used to detect cereal starch synthase proteins

Antibody	Dilution	Antigen source	Specificity	Reference
Anti-GBSSI	1:3000	wheat	barley, wheat, rice	Li *et al.*, 1999
Anti-SSI	1:4000	wheat	barley, wheat, rice	Rahman *et al.*, 1995
Anti-SSIIa	1:500	rice	barley, wheat, rice	Kosar-Hashemi *et al.*, 2007
Anti-SBEI	1:2000	wheat	barley, wheat, rice	Butardo *et al.*, 2012
Anti-SBEIIa	1:2000	wheat, rice	barley, wheat, rice	Regina *et al.*, 2005
Anti-SBEIIb	1:3000	wheat, rice	barley, wheat, rice	Regina *et al.*, 2005

[0337] *Quantitation of protein bands on SDS-PAGE gels and immunoblots.* To quantitate and compare the abundance of proteins between different genotypes, two protein bands (80 kDa and 60 kDa) in 5 µL MagicMark™ XP protein ladders (Invitrogen) were used as references. After visualising the protein bands, SDS-PAGE gels and immunoblots were scanned (Epson Perfection 2450 PHOTO; Epson America Inc., CA, USA) to image files for band intensity analysis using the Quantity One software package following the prescribed methods (Bio-Rad). Band 80 kDa was used for the quantitation of SBEIIa, SBEIIb and SSIIa, band 60 kDa for SSI and GBSSI.

[0338] *Mass spectrometry.* In-gel proteolytic digestion can be done on selected protein bands from Coomassie Blue stained SDS-PAGE gels. Ion trap tandem MS can be conducted as described by Butardo *et al.* (2012). Proteins can be identified by the correlation of uninterrupted MS to entries in SwissProt/TREMBL, through ProteinLynx Global Server (Version 1, Micromass) (Colgrave *et al.* 2013).

[0339] *Grain weight.* Grain was harvested from plants at maturity, which was taken as when the plants were completely yellowed. The heads were harvested and stored at 37°C for at least two weeks to ensure complete drying, then stored at room temperature if further storage was required, and then threshed to provide mature grain. It was this grain or wholemeal obtained from it that was analysed for parameters described herein that depend on the grain weight, as well as the grain weight itself. Grain weight for each wheat line was determined from the total weight of 100 grains. The moisture content of grain was measured with a MPA FT-NIR spectrometer (BRUKER); this was typically about 9% on a

weight basis for mature grain. Parameters that depend on the grain weight such as starch content, BG content, fructan content etc as described herein were calculated on a dry weight basis, assuming a 9% moisture content (w/w) if not measured by NIR.

[0340] *Wheat grain lipid analysis.* Total lipids from samples of wholemeal of about 300 mg were extracted with a mixture of chloroform/ methanol/ 0.1 M KCl (at a ratio of 2:1:1, v/v/v). Fatty acid methyl esters (FAME) were prepared by incubating lipid samples in 1 N Methanolic-HCl (Supelco, Bellefonte, PA) at 80°C for 2 h. TAG and polar membrane lipid pools were fractionated from total lipids by thin layer chromatography (TLC) (Silica gel 60, Merck, Germany) using a solvent mixture of hexane: diethylether: acetic acid (70:30:1, v/v/v) and individual membrane lipid classes were separated by TLC using a solvent mixture of chloroform/methanol/acetic acid/ water (90/15/10/3, v/v/v/v). Authentic lipid standards were loaded and were run in separate lanes on the same plates for identification of lipid classes. Silica bands, containing individual class of lipid were used to prepare FAME as mentioned above and were analyzed by gas chromatography GC-FID 7890A (Agilent Technologies, Palo Alto, CA, USA) that was fitted with a 30 m BPX70 column (SGE, Austin, TX, USA) for quantifying individual fatty acids on the basis of peak area of the known amount of heptadecanoin that was added as an internal standard.

[0341] *Starch extraction.* Unless stated otherwise, starch was extracted from grain samples by first grinding grain (10 g) to wholemeal using a Cyclone mill machine (Cyclote 1093, Tecator, Sweden). Starch was isolated from the wholemeal by a protease extraction method (Morrison *et al.*, 1984), and washed with water using 10 ml of water per gram of wholemeal, at room temperature, with removal of the tailings. The starch was then freeze-dried and weighed for analysis. Starch is also isolated on a small scale from developing wheat grain using the method of Regina *et al.*, (2006).

[0342] *Starch content.* The total starch content of grain was assayed by AACC method 76.13, using the total starch analysis kit (K-TSTA) supplied by Megazyme (Bray, Co Wicklow, Republic of Ireland) and calculated on a weight basis as a percentage of the mature, unmilled grain weight. Subtraction of the starch weight from the total grain weight to give a total non-starch content of the grain determined whether the reduction in total weight was due to a reduction in starch content.

[0343] *Amylose content.* Unless otherwise stated, the amylose content of starch samples was determined in triplicate by the iodometric (iodine binding) method of Morrison and Laignelet (1983) with slight modifications as follows. Approximately 2 mg of starch was weighed accurately (accurate to 0.1 mg) into a 2 ml screw-capped tube fitted with a rubber washer in the lid. To remove lipid, 1 ml of 85% (v/v) methanol was mixed with the starch and the tube heated in a 65°C water bath for 1 hour with occasional vortexing. After centrifugation at 13,000g for 5 min, the supernatant was carefully removed and the extraction step repeated. The starch was then dried at 65°C for 1 hour and dissolved in urea-dimethyl sulphoxide solution (UDMSO; 9 volumes of dimethyl sulphoxide to 1 volume of 6 M urea), using 1 ml of UDMSO *per* 2 mg of starch. The mixture was immediately vortexed vigorously and incubated in a 95°C water bath for 1 hour with intermittent vortexing for complete dissolution of the starch. An aliquot of the starch-UDMSO solution (50 µl) was treated with 20 µl of I_2-KI reagent that contained 2 mg iodine and 20 mg potassium iodide *per* ml of water. The mixture was made up to 1 ml with water. The absorbance of the mixture at 620 nm was measured by transferring 200 µl to microplate and reading the absorbance using an Emax Precision Microplate Reader (Molecular Devices, USA). Standard samples containing from 0 to 100% amylose and 100% to 0% amylopectin were made from potato amylose (Sigma catalogue No. A-0512) and potato amylopectin (Sigma, catalogue No. A-8515) and treated as for the test samples. The amylose content (percentage amylose) was determined from the absorbance values using a regression equation derived from the absorbances for the standard samples.

[0344] The amylose contents of starch samples were also determined, when stated, by debranching starch samples and then measured using size-exclusion chromatography (SEC) as described previously (Butardo *et al.* 2012; Castro *et al.* 2005). By this method, the short chains resulting from amylopectin debranching were separated from the longer amylose chains and the relative amounts determined. Pullulan standards (Shodex P-82) calibrated with the Mark–Houwink–Sakaruda equation were used for the estimation of the molecular weight from the elution time. Samples were prepared and analysed in triplicate.

[0345] Analysis of the amylose/amylopectin ratio of non-debranched starches may also be carried out according to Case *et al.,* (1998) or by an HPLC method using 90% DMSO for separating debranched starches as described by Batey and Curtin, (1996).

[0346] *Resistant Starch (RS) content.* The RS content of grain was determined in triplicate using the RS analysis kit (K-RSTARCH) supplied by Megazyme (Bray, Co Wicklow, Republic of Ireland) and calculated on a weight basis as a percentage of the starch. Instead of 100 mg sample proposed in the RS analysis kit, a reduced amount of wholemeal (40 mg) was used for each assay in this work in a 15 ml conical bottom capped tubes (Cat No: 188271, Greiner bio-one) using *pro rata* amounts of solutions and buffers from the kit. Standard samples containing from 0 to 20 mg/ml glucose were made from glucose (K-RSTARCH kit) and treated as for the test samples. The RS content on a weight basis and non-resistant starch content for the test samples were determined from the absorbance values using a regression equation derived from the absorbance for the standard samples. The RS content was then calculated as the weight of RS as a percentage of the weight of the total starch content.

[0347] The level of RS in food samples such as bread may also be measured *in vitro* as described in WO2012/058730. That method describes the sample preparation and *in vitro* digestion of starch in foods, as normally eaten. The method has two sections: firstly, starch in the food is hydrolysed under simulated physiological conditions; secondly, by-products are removed through washing and the RS determined after homogenization and drying of the sample. Starch quantitated at the end of the digestion treatment represented the RS content of the food.

[0348] *β-Glucan (BG).* BG levels were determined in triplicate using the kit (K-BGLU) supplied by Megazyme (Bray, Co, Wicklow, Republic of Ireland).

[0349] *Fructan content.* Fructan extraction and assay were performed in 2 ml tubes or 96 well plates (2 ml well) using a modified Megazyme fructan kit (K-FRUC) assay procedure as follows. Wheat wholemeal (40 mg) was mixed with 1 ml water (80°C) and incubated with shaking (1200 rpm) at 80°C for 30 min. After cooling to room temperature, the tubes were centrifuged for 5 min, and 20 µl of supernatant containing fructans and other sugars removed for fructan assay. Sucrose, maltose, maltodextrins and starch in the supernatant were hydrolysed to glucose and fructose by adding 20 µl of Enzyme Solution containing sucrase, amylase and maltase from the K-FRUC kit, and incubation of the mixtures at 40°C with shaking (1000 rpm) for 30 min. Glucose and fructose in the samples were then reduced by adding 20 µl of 10 mg/ml alkaline borohydride solution and incubation at 40°C

with shaking (1000 rpm) for 30 min. Fructans in this solution were hydrolysed with fructanases (40°C for 30 min with shaking at 1000 rpm) to glucose and fructose. p-hydroxybenzoic acid hydrazide (PAHBAH) was added to develop the colour complex at 98°C for 6 min. After cooling the samples, the colour complex was measured at 410 nm using a spectrophotometer and the absorbance value was converted to fructan content using a standard curve which contained from 0 to 0.27 mg/ml fructose (Megazyme, K-FRUC kit) and treated as for the test samples after hydrolysis with fructanase. The fructan content (percentage fructan) for the test samples was determined from the absorbance values using a regression equation derived from the absorbances for the standard samples.

[0350] *Scaled down fructan assay in plate format.* For a scaled down fructan assay, all of the amounts for the enzyme solutions, buffers and reagents in the Megazyme kits were scaled down 10-fold and the reactions were conducted in a 96-well plate. Wholemeal samples of 20 mg for samples with high fructan content, or 40 mg samples with lower fructan levels of about 0.5-2%, were used. Pre-wetting flours with ethanol was not necessary for fructan extraction - the wholemeal was well-dispersed in hot water by vortexing before extracting fructans. For the fructan extraction, 20 min extraction time was sufficient. The hydrolysis reactions were performed in 1.1 ml 96-well plates sealed with caps at 40°C for 30 min with shaking at 1,000 rpm using a BioShake iQ and a 96-well adaptor (Q Instruments, Jena, Germany). In the modified K-FRUC assay, the plates after fructan hydrolysis and addition of p-hydroxybenzoic acid hydrazide (PAHBAH) were sealed with caps and tightly clamped into a custom made plate holder for colour development at 100°C for 6 min using a water bath (WiseBath, Thermoline Scientific, Wetherill Park, NSW, Australia). Hydrolysed samples (250 µl) were transferred into a 96-well flat bottom microtitre plate (UV-Star® Microplate or PS-Microplate, Greiner Bio-One, Germany) for reading absorbance at 340 nm (for K-FRUCHK) or 410 nm (for K-FRUC) using a Multiskan Spectrum plate reader (Thermo Scientific, Finland).

[0351] *Total arabinoxylan (AX).* AX was measured using 20 mg samples of wholemeal in 2 ml screw capped tubes. Each sample was mixed with 1 ml of 0.5 M sulphuric acid, vortexed, and the mixtures incubated at 99°C with shaking (1000 rpm) for 30 min. The tubes were then cooled in ice water for 5 min. The tubes were centrifuged at 10,000g for 5 min and supernatant (800 µl) from each tube was transferred to a 96-well plate. If desired,

these plates were stored at -20°C before further treatment. For dilution, 100 µl aliquots of supernatant were transferred to another 96-well plate (Greiner bio-one masterblock) and 900 µl Milli Q water was added to each well. The diluted supernatant (100 µl) was transferred to an assay plate (BioRad Titre tube Microtubes Racked, catalog #223-9390). For making xylose standards, 100 µl of standard solutions were made having concentrations at 30, 50, 75, 100, 150 and 200 µg/ml using a 2 mg/ml stock solution (Sigma, catalog No. X-3877). 0.5 ml of freshly made phloroglucinol reagent (PGR, see below) was added to each well. The plates were sealed with MicroCap strips (National Scientific, TN3346-08C), clamped, and incubated at 100°C for 25 min in a fume hood. The samples were then mixed well by inverting the plates. After that, 200 µl samples were transferred to a UV star plate in a fume hood. The absorbance of each sample was measured at 510 and 552 nm using a plate spectrophotometer eg Thermo multiscan.

[0352] The phloroglucinol reagent (PGR) was prepared freshly for each assay in a fumehood. For this, two solutions were made separately and then mixed. Solution 1 was made by dissolving 0.6 g Phloroglucinol (Sigma, catalog No. 7933) in 2.4 ml absolute ethanol for a few minutes in a 50 ml tube. Solution 2 comprised 55 ml glacial acetic acid with 1.1 ml concentrated hydrochloric acid (HCl) added slowly into the acetic acid. Solution 1 was transferred into a 250 ml bottle. The 50 ml tube containing residues of Solution 1 was then rinsed with Solution 2 to quantitatively transfer all of the Solution 1, and then all of Solution 2 mixed with the Solution 1 in the bottle. Finally, 0.6 ml glucose solution (70 mg/ml in Milli Q water) was added into the mixture of Solutions 1 and 2.

[0353] *Cellulose content.* Cellulose assay was performed in 2 ml tubes or 96 well plates (2 ml well) using 50 mg samples of wholemeal. Lignin, hemicellulose and solubilised starch in the wholemeal were removed by adding 600 µl acetic nitric reagent (10:1 (v/v) mixture of 80% acetic acid: 70% nitric acid) and incubation of the mixtures at 99°C with shaking (1000 rpm) for 1 hr. After cooling, the samples were centrifuged at maximum speed for 5 min and supernatants were discarded. Each pellet was washed with 1 ml water, centrifuged at maximum speed for 5 min and each supernatant discarded. For solubilising crystalline cellulose in each pellet, 1 ml of 72% H_2SO_4 was added to each sample tube. Samples were diluted based on the estimated cellulose content and cellulose standards. For developing colour, 100 µl of anthrone reagent (0.2% anthrone in 72% H_2SO_4) was added to

each sample tube, and the mixtures incubated at 98°C for 10 min. The absorbance of the treated samples was read at 620 nm using a spectrophotometer and the cellulose content was calculated by reference to a standard curve which used 0 to 0.75 mg/ml cellulose (Sigma: catalog No. G-6413).

[0354] *Chain length distribution analysis.* Determination of the chain length distribution of amylopectin was conducted by fluorescence-activated capillary electrophoresis (FACE) after debranching of the starch samples using a capillary electrophoresis unit according to Morell *et al.,* (1998). Samples were prepared as previously described (O'Shea and Morell, 1996).

[0355] *Starch gelatinisation.* The gelatinisation temperature profiles of starch samples were measured in 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), for example using conditions as reported by Batey *et al.,* (1997). The parameters measured included peak viscosity (the maximum hot paste viscosity), holding strength, final viscosity and pasting temperature. 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 and following collection of the gelatinized material.

[0356] *Starch granule morphology.* Starch granule morphology was examined by microscopy. Purified starch granule suspensions in water were examined under both normal and polarized light using a Leica-DMR compound microscope to determine the starch granule morphology. Scanning electron microscopy was carried out using a Joel JSM 35C instrument. Purified starches were sputter-coated with gold and scanned at 15kV at room temperature.

[0357] *Analysis of protein expression in endosperm.* Specific expression of SBEI, SBEIIa and SBEIIb proteins in endosperm, in particular the level of expression or accumulation of these proteins, was analysed by Western blot procedures. Endosperm was dissected away from all maternal tissues and samples of approximately 0.2 mg were homogenized in 600 µl of 50 mM potassium phosphate buffer (42 mM K_2HPO_4 and 8 mM

KH_2PO_4), pH 7.5, containing 5 mM EDTA, 20% glycerol, 5 mM DTT and 1 mM Pefabloc. The ground samples were centrifuged for 10 min at 13,000g and the supernatant aliquoted and frozen at –80°C until use. For total protein estimation, a BSA standard curve was set up using 0, 20, 40, 60, 80 and 100 µl aliquots of 0.25 mg/ml BSA standard. The samples (3 µl) were made up to 100 µl with distilled water and 1 ml of Coomassie Plus Protein reagent was added to each. The absorbance was read after 5 min at 595 nm, using the zero BSA sample from the standard curve as the blank, and the protein levels in the samples determined. Samples containing 20 µg total protein from each endosperm were run on an 8% non-denaturing polyacrylamide gel containing 0.34 M Tris-HCl (pH 8.8), acrylamide (8.0%), ammonium persulphate (0.06%) and TEMED (0.1%). Following electrophoresis, the proteins were transferred to a nitrocellulose membrane according to Morell *et al.,* 1997 and immuno-reacted with SBEIIa, SBEIIb or SBEI specific antibodies (Table 5). Antiserum against wheat SBEIIa protein (anti-wBEIIa) was generated using a synthetic peptide having the amino acid sequence of the N-terminal sequence of mature wheat SBEIIa, AASPGKVLVPDGESDDL (SEQ ID NO: 49) (Rahman *et al.,* 2001). Antiserum against wheat SBEIIb (anti-wBEIIb) was generated in an analogous manner using the N-terminal synthetic peptide, AGGPSGEVMI (SEQ ID NO: 50) (Regina *et al.,* (2005). This peptide was thought to represent the N-terminal sequence of the mature SBEIIb peptide and furthermore was identical to the N-terminus of the barley SBEIIb protein (Sun *et al.,* 1998). A polyclonal antibody against wheat SBEI was synthesised in an analogous manner using the N-terminal synthetic peptide VSAPRDYTMATAEDGV (SEQ ID NO:51) (Morell *et al.*, 1997). Such antisera were obtained from rabbits immunised with the synthetic peptides according to standard methods.

[0358] *Statistical analyses.* Statistical analysis of the amylose data was carried out using the 16th edition of Genstat for Windows (VSN International Ltd, Herts, UK). Other data were subjected to statistical analyses (*t* test and one-way ANOVA, with Tukey post-test) using GraphPad Prism Version 5.01. Error bars represent Standard error of mean (SEM). The statistical significance was defined at $P<0.05$ and $P<0.01$.

EXAMPLE 2. Identification of cDNA sequences and genomic DNA sequences for wheat *SSIIb* and *SSIIc* genes and the encoded polypeptides and comparison to *SSIIa*

[0359] To identify genes encoding starch synthase II isoenzymes (SSIIb and SSIIc) in wheat corresponding to SSIIb and SSIIc in rice (Ohdan *et al.*, 2005) and compare them to SSIIa, the NCBI database was searched using the cDNA sequences from rice, namely Accession No. AF419099 for SSIIa, AF395537 for SSIIb and AF383878 for SSIIc. The wheat homologs were identified as follows. For the wheat *SSIIa* genes, three homoeologous cDNA sequences corresponding to the *SSIIa* genes in wheat were identified. These were Accession No: AF155217 (SEQ ID NO:4) for *SSIIa-A* on the A genome, AJ269504 (SEQ ID NO:5) for *SSIIa-B* on the B genome and AJ269502 (SEQ ID NO:6) for *SSIIa-D* on the D genome. The genomic DNA nucleotide sequences corresponding to these three homoeologous cDNA sequences were identified: Accession No. AB201445 for the *SSIIa-A* gene (SEQ ID NO:7), AB201446 for the *SSIIa-B* gene (SEQ ID NO:8) and AB201447 for the *SSIIa-D* gene (SEQ ID NO:9). Searching the IWGSC database, the locations of the three corresponding homoeologous genes were identified on chromosome 7AS (Traes_7AS_53CAFB43A, 7A:52346437 - 52346905 bp, reverse strand), 7BS (IWGSC: Chromosome 7BS, Traes_7BS_7BEAF5EC0, 7B: 31821573 - 31821749 bp forward strand) and 7DS (IWGSC: Chromosome 7DS, Traes_7DS_E6C8AF743, IWGSC_CSS_7DS_scaff_3877787: 1 to 396 bp, 5137 to 5419 bp forward strand), respectively. The amino acid sequences were, respectively: Accession No: AAD53263 for SSIIa-A, CAB96627 for SSIIa-B and CAB86618 for SSIIa-D polypeptides.

[0360] When the SSIIa amino acid and corresponding nucleotide sequences were compared pairwise by BLAST over the full length sequences, the homoeologous sequences were 95-96% identical for each comparison. They were therefore readily distinguished from each other.

[0361] Two cDNA sequences were identified in the NCBI database encoding wheat *SSIIb* genes, which were Accession Nos. AK332724 from the A genome and EU333947 from the D genome. No corresponding genomic DNA sequences were identified in the NCBI database, however, the genomic DNA sequences were found in the IWGSC database. Three homoeologous genomic DNA sequences encoding SSIIb were identified,

namely the *SSIIb-A* gene on chromosome 6AL (homology with IWGSC: Chromosome 6AL, Traes_6AL_AE01DC0EA, 6A: 187503905 bp to 187505233 bp, forward strand through BLAST search at EnsemblPlants website) (cDNA sequence SEQ ID NO:12), the *SSIIb-B* gene on chromosome 6BL (Chromosome 6BL, gene: Traes_6BL_61D83E262, 6B:162116364 - 162116691 bp, reverse strand through BLAST search at EnsemblPlants website) (cDNA sequence SEQ ID NO:13) and the *SSIIb-D* gene on chromosome 6DL (homology with IWGSC: Chromosome 6DL, gene: Traes_6DL_19F1042C7, 6D: 147050072 - 147051031 bp, reverse strand through BLAST search at EnsemblPlants website) (cDNA sequence SEQ ID NO:14). One amino acid sequence (Accession No: ABY56824, SEQ ID NO:11) was identified in the NCBI database which was 100% identical with the amino acid sequences deduced from EU333947; it was therefore the SSIIb-D polypeptide sequence. A full length amino acid sequence (SEQ ID NO:10) was deduced from the nucleotide sequence of AK332724 for the SSIIb-A polypeptide which had 90% homology with ABY56824 from the D genome SSIIb. An amino acid sequence for the SSIIb-B polypeptide was deduced from the DNA fragment from IWGSC (Traes_6BL_61D83E262, 6B:162116364 - 162116691 bp, reverse strand). The full length SSIIb-A and SSIIb-D amino acid sequences were approximately 90% identical. When compared pairwise, the three corresponding cDNA nucleotide sequences were 91-95% identical. When compared to the SSIIa amino acid or nucleotide sequences, the SSIIb sequences were 71-79% identical to the corresponding SSIIa paralog. Any SSII sequences could therefore be readily identified as SSIIa or SSIIb from either the amino acid or nucleotide sequences.

[0362] For the wheat *SSIIc* genes, one cDNA sequence (Accession No: EU307274) was identified in the NCBI database which corresponded to a gene located on wheat chromosome 1DL (homology with IWGSC: Chromosome 1DL, gene: Traes_1DL_F667ED844, IWGSC_CSS_1DL_scaff_2205619:1950-3041 bp, forward strand through BLAST search at EnsemblPlants website), this therefore corresponded to *SSIIc-D*. The cDNA sequence for another *SSIIc* gene was identifed through searching the IWGSC database, that gene was located on chromosome 1AL (IWGSC: Chromosome 1AL, gene: Traes_1AL_729BF3204, 1A: 68687585 – 68688377, forward strand through BLAST search at EnsemblPlants website). The cDNA sequence had 98% identity with the sequence from nucleotides 1679 to 2469 of Accession number: EU307274. A sequence

from chromosome 1BL was also identified, which was a partial length cDNA sequence for *SSIIc-B* (IWGSC: Chromosome 1BL, gene: Traes_1BL_447468BDE, 1B: 31475067-314776087 bp, forward strand through BLAST search at EnsemblPlants website). One amino acid sequence (Accession No: ABY639) was identified in the NCBI database, which had a 100% identity with the amino acid sequence deduced from the cDNA sequence of Accession No. EU307274. Two partial length amino acid sequences were also deduced from the nucleotide sequences of the genomic DNA fragments for *SSIIc* from the A and B genomes. When compared pairwise, the SSIIc sequences were close to 98% identical to each other. They were quite divergent to the SSIIa sequences.

[0363] It was concluded that the *SSIIa* genes and SSIIa polypeptide sequences could readily be distinguished from the corresponding SSIIb and SSIIc sequences.

EXAMPLE 3. Genome specific DNA markers for marker-assisted breeding

[0364] In order to generate triple-null *ssIIa* mutant plants which were isogenic with wild-type plants in several different genetic backgrounds, plants of the three wheat lines C57 (null for *SSIIa-A*), K79 (null for *SSIIa-B*) and T116 (null for *SSIIa-D*) (Yamamori *et al.*, 2000) were used in a series of crosses, backcrosses and inter-crosses. To detect and track the mutations, each of which are recessive, in the breeding program with molecular markers, genome-specific DNA markers based on the *SSIIa* gene sequences were designed and used. The specific mutations in C57, K79 and T116 SSIIa genes on the A, B and D genomes, respectively, were reported by Shimbata *et al.*, (2005). Each of the mutations was a deletion or an insertion of DNA within the respective *SSIIa* genes (Figures 2 to 4) and therefore these mutations were ideal for designing molecular markers. A DNA marker was designed for each gene by locating a forward oligonucleotide primer upstream of each of the mutation sites and a reverse primer after each of the mutation sites, sequences are described in Example 1 “DNA analysis of wheat plants”.

[0365] These primers were used to amplify DNA fragments specific for each genome from the wild-type and the *ssIIa* null mutant plants. For the A genome *SSIIa* gene, a 1072 bp fragment was amplified from wild-type and a 778 bp fragment from the *ssIIa*-A null mutant gene. For the B genome *SSIIa* gene, a 374 bp fragment was amplified from wild-type and a 522 bp fragment from the *ssIIa*-B null mutant gene. For the D genome *SSIIa*

gene, a 427 bp fragment was amplified from wild-type and a 364 bp fragment from the *ssIIa*-D null mutant gene. These genome-specific fragments were readily distinguished by their size by gel electrophoresis and therefore could be used as co-dominant DNA markers to detect the mutant and wild-type alleles. Other specific primer pairs based on the *SSIIa* gene sequence could easily be designed to provide alternative molecular markers.

EXAMPLE 4. Generation of triple-null *ssIIa* mutants in different genetic backgrounds

[0366] In order to generate triple-null *ssIIa* mutant plants which were isogenic in several different genetic backgrounds, plants of the three wheat lines C57 (null for *SSIIa-A*), K79 (null for *SSIIa-B*) and T116 (null for *SSIIa-D*) (Yamamori *et al.*, 2000) were used in a series of crosses, backcrosses, intercrosses and progeny selections, shown schematically in Figures 5-7. The DNA markers described in Example 1 were used for screening the progeny plants in each generation, allowing for distinguishing the *ssIIa* null mutant alleles and the corresponding wild-type alleles in wheat varieties Sunco, EGA Hume or Westonia used as the recurrent parents. Plants of C57, K79 and T116 were first crossed with plants of wheat cultivar Sunco, using the Sunco plants as the female plants, to produce C57-Sunco F1, K79-Sunco F1 and T116-Sunco F1. Double null *ssIIa* mutants for C57-K79-Sunco F1 and K79-T116-Sunco F1 were then produced by crossing the single null mutants in the Sunco background followed by selfing of the progeny to produce F2 plants from the crosses. The DNA markers were used to select progeny which were heterozygous for two null *ssIIa* mutations. These mutants were then used in three successive back-crosses to Sunco as the recurrent parent to produce BC3 heterozygotes having two null mutations. The BC3 plants were then crossed and the progeny selfed, with selection of the triple-null *ssIIa* mutants (C57-K79-T116-Sunco BC3 F2) in the Sunco genetic background (Figure 5).

[0367] *Producing BC3F8 seeds for ssIIa null mutants and wild-type wheat lines in cv. EGA Hume, Sunco and Westonia backgrounds.* To produce plants and grain in two different genetic backgrounds other than Sunco, double mutants in Sunco (C57-K79-Sunco F1 or F2, K79-T116-Sunco F1 or F2) were used as pollen donors in crosses to plants of the cultivars EGA Hume and Westonia (Figures 6 and 7), with use of the DNA markers in each generation to detect and select the mutant alleles in the progeny. Selected double mutant *ssIIa* progeny were used in three successive back-crosses to EGA Hume or

Westonia as recurrent parent, resulting in BC3 plants. Double mutants were crossed and selfed, producing 466 F2 progeny, from which a total of 21 triple-null *ssIIa* plants (designated as "abd" genotype) were selected (Figures 6 and 7). The 466 progeny included wild-type, single null *ssIIa* and double null *ssIIa* genotypes as well as all combinations of heterozygotes for the three *SSIIa* genes.

[0368] Following the production and selection of the 21 triple-null mutants covering the three genetic backgrounds, three generations of single seed descent (SSD) were performed to generate increased homozygosity in each of the three genetic backgrounds, providing the BC3F3, BC3F4 and BC3F5 generations of grain. The BC3F5 grain were further bulked up in three growing generations to produce 10 to 20 g of grain of the BC3F8 generation of each of the lines.

[0369] Triple wild-type *SSIIa* segregants (ABD genotype) were also generated from the crosses and selected as control lines. In each generation, the DNA markers for all three genomes were used for selecting *ssIIa* mutants or wild-type *SSIIa* alleles for each genome for each generation. Eventually, 4, 6 and 11 triple-null mutant *ssIIa* lines of the BC3F8 generation were generated for the EGA Hume, Sunco and Westonia genetic backgrounds, respectively, and 5 wild-type *SSIIa* BC3F8 lines were generated for each of the EGA Hume, Sunco and Westonia genetic backgrounds. These were grown at the same time under the same growing conditions, grain harvested at plant maturity, and the grain dried to about 9% moisture content (on a weight basis). These grain lots were analysed for various parameters including seed weight, starch content, amylose content, total dietary fibre, lipid content etc. as described below.

EXAMPLE 5. Analysis of grain and starch parameters

[0370] *Grain weight.* The average grain weight (mg per grain) of the triple-null *ssIIa* mutants and the wild-type grain in three genetic backgrounds was calculated by measuring the weight of 100 grains from each line. Average per grain weight ranged from 25 mg to 36 mg for *ssIIa* null mutants and 29 mg to 48 mg for the wild-type lines (Table 6, Figure 8). The plants were grown under far from ideal growing conditions, which explained the low weights even for the wild-type controls. The mean data are shown in Figure 9. Compared with the wild-type lines, the triple-null mutant *ssIIa* grain had lower grain

weight, the differences being significant ($P<0.05$) for each genetic background including Sunco (Table 7, Figure 9). The mutants in Sunco, EGA Hume, and Westonia had 25%, 15% and 30% lower grain weight, respectively. This was not surprising, given that *SSIIa* encodes a starch synthase which is involved in starch production and it was known that mutants in *SSIIa* produced less starch (Yamamoto *et al.*, 2000; Konik-Rose *et al.*, 2007).

[0371] There were no statistically significant difference between the grain weights of the Sunco and Westonia mutants. The EGA Hume mutant grain had significantly heavier grain weight than the triple-null *ssIIa* mutants in the other two genetic backgrounds.

[0372] *Lipid content.* The total fatty acid content (lipid content) was measured as described in Example 1. The data are shown in Table 5 and Figure 8 for individual lines. It was noted that there were significant increases in the TFA content in the triple-null *ssIIa* lines compared to the wild-type. In particular grain from three Sunco mutant lines (JTSBC3F7_190, JTSBC3F7_287 and JTSBC3F7_294) had substantially increased lipid content as a percentage of grain weight.

[0373] When the lipid content was calculated on a per grain basis, it was observed that the mutant lines exhibited an increased level of lipid in mg per grain (Figures 15 and 16).

[0374] *Amylose content.* Amylose content as a proportion of the starch in the triple-null *ssIIa* mutants and wild-type grain in the three genetic backgrounds was measured by the iodine binding assay as described in Example 1. The data are provided in Table 6 and Figure 10 and the means shown in Table 7 and Figure 11 (Sunco). The amylose content for the triple-null mutants ranged from 36.6% to 64% and for the wild-type grain from 22.6% to 31.0%. Compared with the wild-type grain, the null *ssIIa* mutants had greater amylose content as a proportion of total starch and the differences were statistically significant. The Sunco, EGA Hume and Westonia mutant grain had 187%, 135%, and 165% of the level of amylose compared to the corresponding wild-type, respectively. Comparing the triple-null mutant grain in the three genetic backgrounds, the Sunco grain contained significantly higher proportions of amylose than the other two null mutant grain samples. There were no statistically significant differences between the EGA Hume and Westonia mutants. Likewise, there were no statistically significant differences between the three wild-type grain samples. Grain from three of the 6 Sunco triple-null mutant lines

contained 61.6%, 64% and 55.1% amylose as a proportion of the starch in the grain. These values were much higher than seen previously for *ssIIa* mutant grain in hexaploid wheat (Yamamoto *et al.*, 2000; Konik-Rose *et al.*, 2007) and were therefore unexpected and surprising to the inventors.

[0375] When the amylose content was calculated on a per grain basis (mg amylose per grain), it was observed that the mutant lines did not generally exhibited an increased level of amylose in mg per grain (Figures 17 and 18) but rather exhibited a decreased level of amylopectin and therefore decrease total starch content. The inventors concluded that this was due to the mutations in the three *SSIIa* genes and therefore the loss of SSIIa enzyme activity during development of the endosperm as the plants were growing.

[0376] *Starch content.* The starch content in the grain for three triple-null *ssIIa* mutants and three wild-type lines was measured as described in Example 1. The data are presented in Table 6 and Figure 10 and the means are shown in Table 7 (Sunco) and Figure 11. The starch content ranging from 30.4% to 70.0% for the triple-null *ssIIa* mutants and from 58.1% to 74.3% for the wild-type grain. Compared with the starch content of the wild-type lines, the EGA Hume, Sunco and Westonia mutant grain had on average 15%, 28% and 18% less starch, respectively, than their corresponding wild-type lines. These differences were statistically significant ($p<0.05$). The Sunco mutant grain contained significantly less starch than the *ssIIa* mutant grain in the other two genetic backgrounds. The starch content of the EGA Hume mutant grain was not significantly different to the Westonia grain. Grain from three Sunco mutant lines (JTSBC3F7_190, JTSBC3F7_287 and JTSBC3F7_294) had low starch content at 42.5%, 34.8% and 30.4%, respectively. These same lines also had the highest amylose content as a proportion of their starch, indicating that amylopectin synthesis was most reduced in these lines. When calculated on a mg starch per grain basis, the reduced starch content in the *ssIIa* mutant grain was evident (Figures 17 and 18), again caused by the loss of SSIIa activity.

[0377] *β-glucan content.* The β-glucan (BG) content of the triple-null *ssIIa* mutant grain and the wild-type grain was measured as described in Example 1. The data are shown in Table 6 and Figure 12 as a percentage weight/weight of the whole grain, and the means shown in Table 7 (Sunco) and Figure 13. The BG content ranged from 1.3% to 3.3% for the triple-null *ssIIa* mutants and from 0.3% to 0.8% for wild-type grain. Compared with

the wild-type grain, the mutant EGA Hume, Sunco and Westonia had 144%, 245% and 177% more BG, respectively. This increase of about 1.5 to 2.5-fold was surprising to the inventors as there was no previous report of such a feature in hexaploid wheat. The Sunco mutant grain had significantly more BG than the EGA Hume and Westonia mutant grain ($P<0.05$). Grain of three Sunco mutant lines (JTSBC3F7_190, JTSBC3F7_287 and JTSBC3F7_294) which had the highest levels of amylose also contained the highest BG content, of 2.5%, 3.3% and 3.2%, respectively. This demonstrated the correlation between increased amylose content and increased BG content in the *ssIIa* mutants, each on a weight basis.

[0378] When calculated on a per grain basis, it was observed that the mutant grain had significantly increased levels of BG (Figures 19 and 20). The inventors considered that this was due to a diversion of carbon, coming into the grain as di-saccharides or mono-saccharides, from amylopectin into BG relative to the wild-type.

[0379] *Fructan content.* The fructan content of the triple-null *ssIIa* mutant grain and the wild-type grain was measured as described in Example 1. The data are shown in Table 6 and Figure 14 as a percentage weight/weight of the whole grain, and the means shown in Table 7 for Sunco. The fructan content ranged from 3.1% to 10.8% for the triple-null *ssIIa* mutants and from 0.7% to 1.5% for the wild-type grain. Compared with the wild-type grain, the EGA Hume, Sunco and Westonia mutant grain had 242%, 521% and 376% more fructan, respectively. The Sunco mutant grain had significantly more fructan than the EGA Hume and Westonia mutant grain ($P<0.05$). The three high amylose Sunco mutant lines (JTSBC3F7_190, JTSBC3F7_287 and JTSBC3F7_294) also contained the highest fructan content of 7.7%, 10.8% and 10.5%, respectively. Such levels of fructan on a weight basis had never previously been reported in hexaploid wheat grain. This demonstrated the correlation between not only increased amylose and increased BG but also the increased fructan in the *ssIIa* mutants.

[0380] When calculated on a per grain basis, it was observed that the mutant grain had significantly increased levels of fructan (Figures 21 and 22). The inventors considered that this was due to a diversion of carbon, coming into the grain as di-saccharides or mono-saccharides, from amylopectin into fructan relative to the wild-type during development of the endosperm.

[0381] *Arabinoxylan content.* The arabinoxylan (AX) content of the triple-null *ssIIa* mutant grain and the wild-type grain was measured as described in Example 1. The data are shown in Table 6 and Figure 14 as a percentage weight/weight of the whole grain, and the means shown in Table 7 for Sunco. The AX content ranged from 6.7% to 8.8% for the triple-null *ssIIa* mutants and from 4.3% to 5.7% for wild-type grain. Compared with the wild-type grain, the EGA Hume, Sunco and Westonia mutant grain had 35%, 65% and 43% more AX, respectively. The Sunco mutant grain had significantly more AX than the EGA Hume and Westonia mutant grain ($P<0.05$). The three high amylose Sunco mutant lines (JTSBC3F7_190, JTSBC3F7_287 and JTSBC3F7_294) contained the highest AX content of 8.7%, 8.5% and 8.4%, respectively. This demonstrated the correlation between the four parameters, namely increased amylose, increased BG, increased fructan and increased AX in the *ssIIa* mutants. Arabinoxylan contents on a per grain basis were also significantly increased (Figures 21 and 22).

[0382] *Cellulose content.* The cellulose content of the triple-null *ssIIa* mutant grain and the wild-type grain was measured as described in Example 1. The data are shown in Table 6 and Figure 14 as a percentage weight/weight of the whole grain, and the means shown in Table 7 for Sunco. The cellulose content ranged from 2.6% to 4.6% for the triple-null *ssIIa* mutants and from 2.0% to 3.4% for the wild-type grain. Compared with the wild-type lines, the mutant EGA Hume, Sunco and Westonia had 19%, 43% and 29% more cellulose, respectively. There were no significant differences in cellulose content between the three null mutants. The three high amylose Sunco lines (JTSBC3F7_190, JTSBC3F7_287 and JTSBC3F7_294) also contained high cellulose content of 4.3%, 3.9% and 4.6%, respectively. Cellulose contents were not significantly increased on a per grain basis (Figures 21 and 22).

[0383] *Total fibre content.* The total fibre content for the triple-null *ssIIa* mutant grain and the wild-type grain was calculated as the sum of the β-glucan (BG), fructan, arabinoxylan (AX) and cellulose contents (each as a percentage of grain weight). The data are provided in Table 6 and Figure 12 as a percentage weight/weight of the whole grain and the means are provided in Table 7 and Figure 13. Total fibre content in the grain ranged from 15.9% to 27.5% for *ssIIa* null mutants and from 8.5% to 10.4% for wild-type grain. Compared with the wild-type grain, the mutants in EGA Hume, Sunco and Westonia

had 68%, 125% and 88% greater total fibre content, respectively. The Sunco mutant grain had significantly more total fibre than the EGA Hume and Westonia mutant grain ($P<0.05$). There were no significant differences in the total fibre content between the EGA Hume and Westonia mutant grains. The three high amylose Sunco mutant lines (JTSBC3F7_190, JTSBC3F7_287 and JTSBC3F7_294) contained the highest total fibre content at 23.2%, 26.5% and 27.5%, respectively. Total fibre content was also significantly increased on a per grain basis (Figures 19 and 20).

[0384] *Resistant starch (RS) content.* The RS content as a percentage of the starch in the triple-null *ssIIa* mutants and wild-type grain in the Sunco genetic background was measured using a commercial resistant starch analysis kit as described in Example 1. The data are provided in Table 8 and means shown in Figure 23. The RS content for the triple-null mutants ranged from 1.0% to 3.8% and for the wild-type grain from 0.4% to 0.8%. Compared with the wild-type grain, the *ssIIa* mutants had about 5-fold greater RS content and the difference was statistically significant. Grain from three of the 6 triple-null *ssIIa* mutant lines in the Sunco genetic background contained 3.8%, 2.8% and 3.1% RS as a percentage of the starch in the grain (Figure 23, upper panel). The levels of RS calculated as mg per grain were also substantially increased (Figure 23, lower panel).

[0385] *Discussion.* A number of starch properties have previously been reported to be modified in triple-null *ssIIa* hexaploid wheat including starch granule morphology, amylose content, amylopectin chain length distribution, crystallinity and starch gelatinization temperature, RVA and swelling power (Yamamori *et al.* 2000; Yamamori *et al.* 2006; Konik-Rose *et al.* 2007). The present study found that the genetic background of *ssIIa* null mutations had an effect on grain composition parameters including, to the surprise of the inventors, the amylose content in the starch which was increased above 45% in some lines. Three back-crossed populations were generated in different genetic backgrounds, using commercially grown wheat cultivars, and genotyped using DNA markers for the *ssIIa* null mutations. Four to eleven lines of the triple-null *ssIIa* mutants in each genetic background and five wild-type lines from each of the three breeding populations were selected and analysed.

[0386] Through the analysis of the seed weight and grain composition in each genetic background, three high amylose, high fibre, high AX and BG, and high fructan lines were

identified. These three lines were from crosses with the same genetic background, namely Sunco. Wheat lines containing about 60% amylose, more than 23% total fibre and more than 7% fructan were identified and selected. Such levels have never before been reported for hexaploid wheat and were entirely unexpected to the inventors. The increases were also observed on a per grain basis. These high amylose *ssIIa* null mutants also contained increased BG, AX and cellulose, demonstrating the correlation of these parameters. However, these three mutant lines also showed reduced starch content and seed weight, due to substantially reduced amylopectin synthesis.

Table 6. Grain parameters for *ssIIa* triple mutants (abd) and SSIIa wild-type (ABD) wheat in three genetic backgrounds

Wheat line ID	Genotype	Genetic background	grain weight (mg)	Amylose %	Total Starch %	Total fibre %	Beta Glucan %	Fructan %	AX %	Cellulose %	Lipid content %
SS533	abd	EGA Hume	35.76	38.4	67.98	16.67	1.54	3.87	7.66	3.60	3.31
SS299	abd	EGA Hume	36.04	36.6	63.03	17.01	1.29	4.55	7.48	3.68	3.42
SS412	abd	EGA Hume	33.23	39.6	62.57	16.87	1.45	4.88	7.52	3.02	3.74
SS393	abd	EGA Hume	33.00	43.0	64.47	18.14	1.78	5.82	7.73	2.81	3.76
SS344	abd	Sunco	30.25	37.0	63.75	16.96	1.54	3.36	8.81	3.25	3.27
SS497	abd	Sunco	32.00	45.4	65.16	18.26	1.96	3.75	9.48	3.08	3.66
SS435	abd	Sunco	28.83	45.0	56.79	19.62	1.72	4.79	9.69	3.42	3.84
SS274	abd	Sunco	24.95	64.0	46.65	25.14	2.42	8.45	9.57	4.70	5.13
SS110	abd	Sunco	27.81	55.1	33.37	28.94	3.09	11.56	9.24	5.04	5.74
SS047	abd	Sunco	27.16	61.6	38.22	28.74	3.20	11.86	9.38	4.30	5.72
SS104	abd	Westonia	32.90	37.7	76.88	17.62	1.53	4.05	7.78	4.26	3.53
SS224	abd	Westonia	28.94	39.4	70.36	16.97	1.40	4.42	7.31	3.85	3.80
SS403	abd	Westonia	28.84	49.9	62.34	17.96	1.87	4.73	8.11	3.25	3.99
SS446	abd	Westonia	30.45	49.1	59.25	18.56	1.97	4.87	8.38	3.34	4.16
SS066	abd	Westonia	28.35	46.4	56.33	19.91	1.87	5.00	8.62	4.43	4.30
SS324	abd	Westonia	30.82	50.5	56.38	19.21	1.97	5.56	8.10	3.58	4.17
SS135	abd	Westonia	31.76	43.68	58.92	18.74	1.80	5.84	7.36	3.75	4.15
SS131	abd	Westonia	29.78	43.66	54.10	20.53	1.73	6.10	8.32	4.38	4.57

Wheat line ID	Genotype	Genetic background	grain weight (mg)	Amylose %	Total Starch %	Total fibre %	Beta Glucan %	Fructan %	AX %	Cellulose %	Lipid content %
SS306	abd	Westonia	32.54	42.35	56.12	19.97	1.81	6.25	7.69	4.22	3.96
SS627	abd	Westonia	33.94	38.77	57.25	19.81	1.83	7.10	7.32	3.56	4.36
SS028	abd	Westonia	34.78	43.81	55.22	21.28	1.67	7.91	7.51	4.19	4.61
SS199	ABD	EGA Hume	39.68	30.1	69.90	9.95	0.61	1.13	4.70	3.51	2.30
SS581	ABD	EGA Hume	43.59	29.9	70.10	10.68	0.72	1.23	6.02	2.70	2.13
SS386	ABD	EGA Hume	39.40	27.0	73.00	10.18	0.59	1.47	5.51	2.62	2.34
SS366	ABD	EGA Hume	43.81	31.9	68.10	10.14	0.55	1.52	5.73	2.35	2.09
SS427	ABD	EGA Hume	37.72	27.61	72.39	11.13	0.63	1.63	6.25	2.63	2.26
SS077	ABD	Sunco	43.63	31.0	69.00	10.77	0.57	0.85	5.95	3.41	2.10
SS421	ABD	Sunco	41.35	29.8	70.20	9.59	0.66	1.07	5.41	2.46	2.23
SS532	ABD	Sunco	39.98	27.1	72.90	9.97	0.81	1.18	5.29	2.69	2.26
SS293	ABD	Sunco	28.93	26.2	73.80	10.09	0.52	1.32	5.68	2.57	2.27
SS048	ABD	Sunco	36.03	23.44	76.56	11.12	0.81	1.46	6.10	2.75	2.21
SS073	ABD	Westonia	40.88	30.5	69.50	10.22	0.68	0.78	5.46	3.30	2.34
SS422	ABD	Westonia	41.29	30.5	69.50	10.79	0.73	1.07	5.57	3.42	2.30
SS628	ABD	Westonia	47.72	22.59	77.41	11.32	0.70	1.21	5.71	3.69	2.32
SS584	ABD	Westonia	46.16	25.66	74.34	9.30	0.53	1.31	5.27	2.19	2.41
SS415	ABD	Westonia	44.06	24.81	75.19	10.01	0.55	1.53	5.37	2.56	2.49

Table 7. Mean and standard deviations (std) of grain parameters for three *ssIIa* triple mutants (abd) compared to wild-type (ABD) wheat in Sunco genetic backgrounds

		Seed weight (mg)	**Amylose (iodine) %**	**Amylo-pectin %**	**Total Starch %**	**Total fibre %**	**Beta Glucan %**	**Fructan %**	**Arabino-xylan %**	**Cellulose %**	**total lipid %**
Sunco-abd	mean	26.64	60.23	39.77	39.41	27.60	2.90	10.62	9.40	4.68	5.53
Sunco-WT	mean	37.98	27.51	72.49	70.80	10.31	0.67	1.17	5.68	2.78	2.22
Sunco-abd	std	1.50	4.60	4.60	6.72	2.14	0.42	1.89	0.17	0.37	0.35
Sunco-WT	std	5.77	2.99	2.99	4.51	0.62	0.14	0.24	0.34	0.37	0.07

Table 8. Resistant starch content of starch in the *ssIIa* mutant grain in the Sunco background

wheat ID	**Line name**	**RS (%)**	**std**	**RS (%)**	**std**	per seed weight (mg)	**RS per seed (mg)**		**mean**	**std**
SS344	JTSBC3F7_255	1.04	0.13	2.56	0.94	30.25	0.31		0.71	0.23
SS497	JTSBC3F7_109	2.45	0.05			32.00	0.78			
SS435	JTSBC3F7_260	2.1	0.15			28.83	0.61			
SS274	JTSBC3F7_190	3.79	0.22			24.95	0.95			
SS110	JTSBC3F7_294	2.81	0.22			27.81	0.78			
SS047	JTSBC3F7_287	3.14	0.49			27.16	0.85			
SS077wt	JTSBC3F7_300	0.41	0.18	0.55	0.16	43.63	0.18		0.20	0.04
SS421wt	JTSBC3F7_008	0.64	0.11			41.35	0.26			
SS532wt	JTSBC3F7_182	0.38	0.11			39.98	0.15			
SS293wt	JTSBC3F7_021	0.75	0.63			28.93	0.22			
SS048wt	JTSBC3F7_005	0.59	**0.5**			36.03	0.21			
l.s.d.				0.97					0.23	

*Per seed weight was the average seed weight of 100 seeds

EXAMPLE 6. Combination of *SSIIa* mutations and other starch synthesis gene mutations or inhibitory constructs.

[0387] Wheat plants which were triple-null mutant for the three *SSIIa* genes as described in Example 1 were crossed with plants comprising a hairpin RNA construct designated hp-SBEIIa and having reduced starch branching enzyme II (SBEII) activity in the endosperm (Regina *et al.*, 2006). The hp-SBEIIa parental plants showed a high amylose phenotype in grain starch (Regina *et al.*, 2006). F1 plants were obtained and selfed to produce F2 progeny grain. Screening of 288 F2 grains was carried out by PCR using three different primer pairs described by Konik-Rose *et al.* (2007), each primer pair being specific for a *ssIIa* mutant gene from a particular genome. This resulted in the identification of one homozygous, triple-null *ssIIa* grain, designated YDH7, which lacked the wild-type alleles for each of the three *SSIIa* genes. Further testing of YDH7 DNA confirmed the presence of the hp-SBEIIa genetic construct in this grain in addition to the three mutated *ssIIa* genes. The grain was germinated to produce progeny plants and grain, designated herein as the YDH7 line.

[0388] In a similar fashion, plants comprising triple-null mutations in *SSIIa* are crossed with plants of a *sbeI* triple-null wheat line described in Regina *et al.*, 2004. DNA extracted from F2 half seeds are screened for the null mutations in each of the *SBEI* genes using a PCR based cleaved amplified products (CAPs) marker designed from the intron 2 region of the wheat *SBEI* gene. The marker generates a 460 bp DNA fragment from the *SBEI-D* gene from the D genome, a 390 bp DNA fragment from the *SBEI-B* gene from the B genome and a 200 bp DNA fragment from the *SBEI-A* gene from the A genome. Apart from these *SBEI* genome-specific fragments, this CAPS marker also produces a 250 bp DNA fragment that is non-specific to the *SBEI* genes and is useful as an internal control in the PCR reactions. Grains are identified which lack the *SBEI*-specific DNA fragments amplified from the wild-type genes, indicating that they are triple-null mutants for *sbeI*. These triple-null grains are then tested by PCR for the presence of the *ssIIa* null mutations. These grains are sown to produce plants and grain which contain the combination of *ssIIa* and *sbeI* mutations, homozygous for each of the mutant alleles.

EXAMPLE 7: Analysis of starch granules and starch properties

[0389] Starch granules in wholemeal produced from triple mutant *ssIIa* grain were examined. The properties of the starch extracted from the wholemeal were also analysed, as follows. For these analyses, five *ssIIa* mutant wheat lines, designated A24, B22, B29, B63 and E24, were selected from the double haploid population of Konik-Rose *et al.* (2007) and analysed for starch and granule properties. Grain samples (20g) from each line and from a corresponding wild-type line were milled to wholemeal in a Quadramat Jnr. Mill (Brabender, Cyrulla's Instruments, Sydney, Australia) after conditioning the grains to a moisture content of 14%. Starch was isolated was isolated from the wholemeal samples by a protease extraction method (Morrison *et al.*, 1984) followed by water washing and removal of the tailings.

[0390] *Changes in starch granule morphology and birefringence.* Starch granule morphology and their birefringence under polarised light were examined for granules in the wholemeal samples. Scanning electron microscopy was used to identify gross changes in starch granule size and structure. Compared to the wild-type granules, starch granules from endosperms lacking SSIIa displayed significant morphological alterations. They were highly irregular in shape and many of the A granules (>10 μm diameter) appeared to be sickle shaped. In contrast, both A and B (< 10 μm diameter) starch granules in the wild-type wholemeal were smooth surfaced and spherical or ellipsoid in shape.

[0391] When observed microscopically under polarised light, wild-type starch granules typically showed a strong birefringence pattern. However, the incidence of birefringence was greatly reduced for granules from the triple-null *ssIIa* grain. Less than 10% of the starch granules from mutant *ssIIa* grains were birefringent when visualized under polarized light. In contrast, about 94% of the wild-type starch granules exhibited full birefringence. Loss of birefringence therefore correlated with the lack of SSIIa and increased amylose content.

[0392] *Chain length distribution of starch by FACE.* Chain length distribution of isoamylase de-branched starch was determined by fluorophore assisted carbohydrate electrophoresis (FACE) as described in Example 1. This technique provided a high resolution analysis of the distribution of chain lengths in the range from DP 1 to 50 and the relative

frequency of chains of different lengths in the modified starch compared to wild-type starch. From the molar difference plot (Figure 24) in which the normalized chain length distribution of the wild-type starch was subtracted from the normalized distribution of the triple mutant *ssIIa* starch, it was observed that there was a marked increase in the proportion of chain lengths of DP 7-10 and a substantial decrease in the chain lengths of DP 11-24 in starch from the triple-null *ssIIa* grain. There was also a slight increase in chain lengths of DP 26-36.

[0393] *Molecular weight of amylopectin and amylose.* The molecular weight distribution of starch from the mutant grain was determined by size exclusion chromatography (SEC) after isoamylase debranching. The isoamylase debranching treatment cleaves each of the α-1,6-linkages in amylopectin to release the separate chains but leaves the amylose mostly untouched, therefore allowing separation of the amylose (eluting first) and the glucan chains from the amylopectin on the basis of size. Figure 25 shows the resultant SEC traces for the debranched starches according to their degree of polymerisation. The relative amount of amylose (first peak) in the starch of the triple-null *ssIIa* mutant grain was substantially increased relative to the wild-type starch, and the amount of amylopectin (peak III) was much reduced compared to wild-type. The average molecular weight of the amylose in the starch of the triple-null *ssIIa* mutant grain appeared to be reduced compared to that of amylose in the wild-type starch, with amylose peak positions at 274 kDa versus 330 kDa for the wild-type (Figure 25).

[0394] *Starch Swelling Power (SSP).* Starch swelling power of gelatinized starch was determined following the small scale test of Konik-Rose *et al.*, (2001) which measured the uptake of water during gelatinization of starch. The estimated value of SSP was significantly lower for starch from the triple-null *ssIIa* grain with a figure of 6.85 compared to starch from the wild-type at 11.79.

[0395] *Starch Pasting properties.* Starch paste viscosity parameters were determined using a Rapid Visco Analyzer (RVA) essentially as described in Regina *et al.*, (2004). The temperature profile for the RVA comprised the following stages: hold at 60°C for 2 min, heat to 95°C over 6 min, hold at 95°C for 4 min, cool to 50°C over 4 min, and hold at 50°C for 4 min. The results showed that the peak and final viscosities were significantly lower in starch

from the triple-null *ssIIa* grain (105.5 and 208.6, respectively) compared to the wild-type wheat starch (232.3 and 350.6, respectively).

[0396] *Starch gelatinisation properties.* Gelatinisation properties of the starch were studied using differential scanning calorimetry (DSC) as described in Regina *et al.*, (2004). DSC was carried out on a Perkin Elmer Pyris 1 differential scanning calorimeter. Starch and water were premixed at a ratio of 1:2 and approximately 50 mg weighed into a DSC pan which was sealed and left to equilibrate overnight. A heating rate of 10°C per minute was used to heat the test and reference samples from 30 to 130°C. Data was analysed using the software available with the instrument. The results clearly showed a reduced end gelatinisation temperature (61.5°C) for starch from the triple-null *ssIIa* grain compared to the control (67.1°C). The peak gelatinisation temperature was also lower in the triple-null *ssIIa* starch (55.4°C) compared to the control starch (61.3°C). Therefore, starch from the triple-null *ssIIa* grain has significantly lower gelatinisation temperature and reduced enthalpy of gelatinisation.

[0397] *Grain compositional changes.* Proximate composition of triple-null *ssIIa* wheat grain was analysed to determine the changes induced in grain components by loss of SSIIa activity. The sucrose level in the *ssIIa* wholemeal flour was increased relative to that from a wild-type wheat, NB1. The overall sugar content was also higher in the *ssIIa* mutant starch compared to wild-type starches. Wholemeal from the *ssIIa* grain and a second grain comprising an hp-SBEIIa genetic construct (Regina *et al.*, 2006) had an increased level of protein of >19% in the grain while wild-type wholemeal had a level within the range of 11-13%. The total dietary fibre (TDF) was higher in the *ssIIa* starch with a level of 19.2% compared to the wild-type value of 11.0%. The level of other grain components such as neutral non-starch polysaccharides (NNSP), total anti-oxidants and total lipids was comparatively higher in the *ssIIa* flour compared to that of the wild-type wheat. For NNSP, the highest value of 13.8% was recorded for the *ssIIa* mutant compared to 8.3% in the wild-type NB1. Total starch content was about 53% in the *ssIIa* mutant grain compared to >60% in NB1 and YDH7.

EXAMPLE 8: Production of breads and other food products

[0398] Food products such as bread and breakfast cereals are effective ways of delivering the modified wheat starch into the diet. To show that the high amylose wheat could readily be incorporated into breads and breakfast cereals and to examine the factors that allowed retention of the food quality, samples of flour were produced, analysed and used in baking or extrusion experiments. Small-scale bread bakes were carried out using YDH7 flour with three levels of addition, 30%, 60% and 100% in comparison with the bakes from flours from grain comprising the hp-SBEIIa genetic construct and the wild-type NB1. Increasing the addition level of either YDH7 or hp-SBEIIa flour resulted in significant increases in RS and reductions in GI.

[0399] An extruded breakfast cereal was prepared from wholemeal from the *ssIIa* mutant wheat and compared to a corresponding breakfast cereal from wild-type wheat. The breakfast cereal contained an increased level of RS (4.3%) when using the triple-null *ssIIa* wheat compared to the wild-type wheat (1.3%). Increasing the melt temperature from 110°C to 140°C in the extruded process slightly reduced the RS content when using the triple-null *ssIIa* wheat. The results also showed that the wholemeal breakfast cereals from the triple-null *ssIIa* wheat had a lower HI (hydrolysis index for estimating GI value) value of 72 compared to that from the wild-type wheat at 82.

[0400] The following methods are employed for the production of bread from *ssIIa* wheat at a larger scale. Quantities of wheat grain are conditioned to 16.5% moisture content overnight before milling and sieving to achieve a final average particle size of about 150 μm. The protein and moisture content of the milled samples are determined by infrared reflectance (NIR) according to AACC Method 39-11 (1999), or by the Dumas method using an air-oven according to AACC Method 44- 15 A ($AACC_5$ 1999).

[0401] *Micro Z-arm Mixing.* Optimum water absorption values of wheat flours are determined with a Z-arm Mixer, for example using 4g of test flour per mix (Gras *et al.*, (2001); Bekes *et al.*, (2002) with constant angular velocity with shaft speeds for the fast and slow blades of 96 and 64 rpm, respectively. Mixing is carried out for about 20 minutes. Before adding water to the flour, the baseline is automatically recorded for 30 sec by mixing

only the solid components. The water addition is carried out in one step using an automatic water pump. The following parameters are determined from the individual mixing experiments by taking the averages: WA%- Water Absorption is determined at 500 Brabender Unit (BU) dough consistency; Dough Development Time (DDT): time to peak resistance (sec).

[0402] *Mixograms.* To determine optimal dough mixing parameters with the modified wheat flour, samples with variable water absorption corresponding to water absorption determined by the Z-arm mixer, are mixed in a Mixograph keeping the total dough mass constant. For each of the flour samples, the following parameters are recorded: MT - mixing time (sec); PR - Mixograph peak resistance (Arbitrary Units, AU); BWPR - band width at peak resistance (Arbitrary Units, AU); RBD - resistance breakdown (%); BWBD - bandwidth breakdown (%); TMBW - time to maximum bandwidth (s); and MBW - maximum bandwidth (Arbitrary Units, A.U.). Dough extensibility parameters are measured as follows: doughs are mixed to peak dough development in a Mixograph. Extension tests at 1 cm/sec are carried out on a TA.XT2i texture analyser with a modified geometry Kieffer dough and gluten extensibility rig (Mann *et al.*, 2003). Dough samples for extension testing (~1.0 g / test) are moulded with a Kieffer moulder and rested at 30°C and 90% RH for 45 min. before extension testing. The R_Max and Ext_Rmax are determined from the data with the help of Exceed Expert software (Smewing, TX2 texture analyzer handbook, SMS Ltd: Surrey, UK, 1995).

[0403] An illustrative recipe based on 14 g of flour as 100% is as follows: flour 100%, salt 2%, dry yeast 1.5%, vegetable oil 2%, and improver (ascorbic acid 100 ppm, fungal amylose 15 ppm, xylanase 40 ppm, soy flour 0.3%, obtained from Goodman Fielder Pty Ltd, Australia) 1.5%. The water addition level is based on the Z-arm water absorption values that are adjusted for the full formula. Flour (14 g) and the other ingredients are mixed to peak dough development time in a Mixograph. The moulding and panning is carried out with two proofing steps at 40C at 85% RH. Baking is carried out in a Rotel oven for 15 min at 190°C. Loaf volume (determined by the canola seed displacement method) and weight measurements are taken after cooling the loaves on a rack for 2 hours. Net water loss is measured by weighing the loaves over time.

[0404] The flour or wholemeal may be blended with flour or wholemeal from non-modified wheats or other cereals such as barley to provide desired dough and bread-making or nutritional qualities. For example, flour from cvs Chara or Glenlea has a high dough strength while that from cv Janz has a medium dough strength. In particular, the levels of high and low molecular weight glutenin subunits in the flour is positively correlated with dough strength, and further influenced by the nature of the alleles present.

[0405] Flour from line YDH7 was used at 100%, 60% and 30% addition levels according to the methods described above for dough preparation and bread making. That is, either 100% of the flour came from the YDH7 or control flour, or 60% or 30% by weight of YDH7 flour was blended with the Baking Control (B. extra) flour. Percentages were of total flour in the bread formulation. The unmodified wheat flour was from wild-type cv. NB1. The grain samples were milled in a Brabender Quadramat Junior mill. All flour blends had their water absorption determined on a Z-arm mixer and optimal mixing times determined on Mixograph as described above. These conditions were used in preparing the test bake loaves.

[0406] *Mixing Properties.* Apart from the control sample using entirely wild-type flour (Baking Control, NB1), all other flour samples gave elevated water absorption values. Increased incorporation levels of flour from the YDH7 line also led to a decrease in the optimal Mixograph mixing times. In keeping with the water absorption data, the breads including flour from the YDH7 line all showed a reduction in specific loaf volume (loaf volume/loaf weight), correlating with increasing levels of addition of the YDH7 flour.

[0407] These studies showed that breads with commercial potential, including acceptable crumb structure, texture and appearance, could be obtained using the modified *ssIIa* wheat flour blended with control flour samples. Furthermore, high amylose *ssIIa* wheats may be used in combination with preferred genetic background characteristics (e.g. preferred high and low molecular weight glutenins), or modifications in the food processing such as, for example, the addition of improvers such as gluten, ascorbate or emulsifiers, or the use of differing bread-making styles (e.g. sponge and dough bread-making, sour dough, mixed grain, or wholemeal) to provide a range of products with particular utility and nutritional efficacy for improved bowel and metabolic health.

[0408] *Other food products:* Yellow alkaline noodles (YAN) (100% flour, 32% water, 1% Na_2CO_3) are prepared in a Hobart mixer using the standard BRI Research Noodle Manufacturing Method (AFL 029). Noodle sheet is formed in the stainless steel rollers of an Otake noodle machine. After resting (30 min) the noodle sheet is reduced and cut into strands. The dimensions of the noodles are 1.5 x 1.5 mm.

[0409] Instant noodles (100% flour, 32% water, 1% NaCl and 0.2% Na_2CO_3) are prepared in a Hobart mixer using the standard BRI Research Noodle Manufacturing method (AFL 028). Noodle sheet is formed in the stainless steel rollers of an Otake noodle machine. After resting (5 min) the noodle sheet is reduced and cut into strands. The dimensions of the noodles are 1.0 x 1.5 x 25mm. The noodle strands are steamed for 3.5 min and then fried in oil at 150°C for 45 sec.

[0410] Sponge and Dough (S&D) bread. The BRI Research sponge and dough baking involves a two-step process. In the first step, the sponge is made by mixing part of the total flour with water, yeast and yeast food. The sponge is allowed to ferment for 4 h. In the second step, the sponge is incorporated with the rest of the flour, water and other ingredients to make dough. The sponge stage of the process is made with 200 g of flour and is given 4 h fermentation. The dough is prepared by mixing the remaining 100 g of flour and other ingredients with the fermented sponge.

[0411] Pasta- Spaghetti. The method used for pasta production is as described in Sissons *et al.,* (2007). Flours from *ssIIa* modified wheat and wild-type wheat are mixed with Manildra semolina at various percentages (test sample: 0, 20, 40, 60, 80, 100%) to obtain flour mixes for small scale pasta preparation. The samples are corrected to 30% moisture. The desired amount of water is added to the samples and mixed briefly before being transferred into a 50 g farinograph bowl for a further 2 min mix. The resulting dough, which resembles coffee-bean-size crumbs, is transferred into a stainless steel chamber and rested under a pressure of 7000 kPa for 9 min at 50°C. The pasta is then extruded at a constant rate and cut into lengths of approximately 48 cm. The pasta is dried using a temperature and humidity cabinet. The drying cycle uses a holding temperature of 25°C followed by an increase to 65°C for 45 min then a period of about 13 h at 50°C followed by cooling. Humidity is controlled during the cycle. Dried pasta is cut into 7 cm strands for subsequent tests.

EXAMPLE 9: *In vitro* measurements of Glycaemic Index (GI) of food samples

[0412] The Glycemic Index (GI) of food samples was measured *in vitro* as follows. The *in vitro* method simulates what happens to food samples when consumed by human subjects and is predictive of *in vivo* GI measurement. Food samples were homogenised with a domestic food processor. An amount of sample representing approximately 50 mg of carbohydrate was weighed into a 120 ml plastic sample container and 100 µl of carbonate buffer added without α-amylase. Approximately 15-20 seconds after the addition of carbonate buffer, 5 ml of Pepsin solution (65 mg of pepsin (Sigma) dissolved in 65 ml of HCl 0.02 M, pH 2.0, made up on the day of use) was added, and the mixture incubated at 37°C for 30 minutes in a reciprocating water bath at 70 rpm. Following incubation, the sample was neutralised with 5 ml of NaOH (0.02 M) and 25 ml of acetate buffer 0.2 M, pH 6 added. 5 ml of enzyme mixture containing 2 mg/mL of pancreatin (α-amylase, Sigma) and 28U/mL of amyloglucosidase from *Aspergillus niger* (AMG, Sigma) dissolved in Na acetate buffer (sodium acetate buffer, 0.2 M, pH 6.0, containing 0.20 M calcium chloride and 0.49 mM magnesium chloride) was then added, and the mixture incubated for 2-5 minutes. 1 ml of solution was transferred from each flask into a 1.5 ml tube and centrifuged at 3000 rpm for 10 minutes. The supernatant was transferred to a new tube and stored in a freezer. The remainder of each sample was covered with aluminium foil and the containers incubated at 37°C for 5 hours in a water bath. A further 1 ml of solution was then collected from each flask, centrifuged and the supernatant transferred as before. Supernatants were stored in a freezer until the absorbances could be read.

[0413] All supernatants were thawed and centrifuged at 3000 rpm for 10 min. Samples were diluted as necessary (1 in 10 dilution usually sufficient), 10 µl of supernatant transferred from each sample to 96-well microtitre plates in duplicate or triplicate. A standard curve for each microtitre plate was prepared using glucose (0 mg, 0.0625 mg, 0.125 mg, 0.25 mg, 0.5 mg and 1.0 mg). 20 µl of Glucose Trinder reagent (Microgenetics Diagnostics Pty Ltd, Lidcombe, NSW) was added to each well and the plates incubated at room temperature for approximately 20 minutes. The absorbance of each sample was measured at 505 nm using a plate reader and the amount of glucose calculated with reference to the standard curve.

[0414] Bread loaves baked using flour from the YDH7 wheat line were tested for GI using the method described above along with bread made from the non-transformed wild-type wheat, as well as blends of the two flours using incorporation levels of 60% and 30% YDH7 flour, the remainder 40% or 70% flour being from the wild-type grain. Increased incorporation of YDH7 flour resulted in significant reductions in GI as measured by the *in vitro* test.

EXAMPLE 10: Processing of high amylose wheat and resultant RS levels

[0415] A small scale study was conducted to determine the resistant starch (RS) content in processed grain from the YDH7 wheat which had been rolled or flaked. The technique involved conditioning the grains to a moisture level of 25% for one hour, followed by steaming the grains. Following steaming, the grains were flaked using a small-scale roller. The flakes were then roasted in an oven at 120°C for 35 min. Two roller widths and three steaming timings were used on approximately 200 g of samples from high amylose wheat having reduced SSIIa and wild-type, control wheat (cv. Hartog). The roller widths tested were 0.05 mm and 0.15 mm. The steaming timings tested were 60', 45' and 35'.

[0416] This study showed a clear and substantial increase in the amount of RS in processed high amylose wheat compared to the control. There also appeared to be some effect of the processing conditions on the RS level. For example with the high amylose grain, increased steaming times led to a slight reduction in the level of RS, most likely due to increased starch gelatinization during steaming. The wider roller gap generated a higher RS level except at the longest steaming time. This could have been due to increased shear damage of the starch granules when the grains were rolled at narrower gaps, reducing RS levels slightly. Narrower roller gaps also led to higher RS levels in the Hartog control, albeit at much lower overall RS levels. In contrast to the high amylose results, increased steaming times led to higher RS levels, possibly due to increased starch gelatinization at longer steaming times contributing to more starch retrogradation during subsequent processing and cooling.

EXAMPLE 11: Generation and identification of additional *ssIIa* mutations

[0417] *Mutagenesis of wheat by heavy ion bombardment.* Mutagenesis by radiation such as by heavy ion bombardment (HIB) readily produces deletion mutations at practical

frequencies in the wheat genome. A mutagenised wheat population was generated in the wheat variety Chara, a commonly used commercial variety, by HIB of wheat seeds. Two sources of heavy ions were used, namely carbon and neon, for mutagenesis which was conducted at Riken Nishina Centre, Wako, Saitama, Japan. Mutagenised seeds were sown in the greenhouse to obtain the M1 plants. These were then selfed to produce the M2 generation. DNA samples were isolated from each of approximately 15,000 M2 plants, each from a different M1 plant.

[0418] Each DNA is individually screened for mutations in each of the *SSIIa* genes by PCR using genome specific oligonucleotide primers which produce amplified fragments for each of the *SSIIa* genes on the A, B and D genomes. Such diagnostic PCR primers can readily be designed by those skilled in the art by comparing the three genomic nucleotide sequences (SEQ ID NOs:7, 8 and 9) and choosing oligonucleotide sequences which anneal specifically to one genomic sequence but not the other two, or where the resultant amplified fragments are cleaved differentially by restriction enzyme digestion to produce different sized fragments for the three genomic *SSIIa* genes. Each of the PCR reactions on wild-type (non-mutagenised) DNA samples thereby yield 3 distinct amplification products which corresponded to the amplified regions of *SSIIa* genes on the A, B and D genomes, whereas the absence of one of the fragments in the PCRs from mutagenised M2 DNA samples indicates the absence of the corresponding region in one of the genomes, i.e. the presence of a mutant allele in which at least part of the gene was deleted. Such mutant alleles would certainly be null alleles. When screened in such a manner using *SBEIIa*- and *SBEIIb*-gene specific primers, the 15,000 M2 plants identified a total of 34 mutants which were deletion mutants for the *SBEIIa* and/or *SBEIIb* genes (WO2012/058730), indicating that the frequency of deletion mutations for a gene of interest in this mutagenized M2 populations was about 1 per 1000 lines. Screening of the M2 lines therefore identifies about 15 mutants each having a deletion mutation of, or in, an *SSIIa* gene. Since the *SSIIa* genes on the A, B and D genomes are distinguished by the diagnostic PCR reactions, the mutant alleles are assigned to one of the genomes according to which amplification product was absent. About 5 mutants are identified for each of the *SSIIa-A*, *SSIIa-B* and *SSIIa-D* genes in this manner from the population of 15,000 M2 plants.

[0419] The extent of the chromosome deletion in each of the mutants is determined by microsatellite mapping. Microsatellite markers previously mapped to the short arm of chromosomes 7A, 7B and 7D, the chromosomal locations of the *SSIIa* genes, are tested on these mutants to determine the presence or absence of each marker in each mutant. Mutant plants in which either all or most of the specific chromosome microsatellite markers were retained, based on the production of the appropriate amplification product in the reactions, are inferred to be relatively small deletion mutants. Such mutants were preferred, considering that it was less likely that other, important genes were affected by the mutations.

[0420] *Crossing of mutants.* Mutant plants that were homozygous for smaller deletions of or in an *SSIIa* gene as judged by the microsatellite marker analysis are selected for crossing to generate progeny plants and grain which have mutant *ssIIa* alleles on multiple genomes. F1 progeny plants from the crosses are selfed, and F2 seed obtained and analysed for their *SSIIa* genotype. Such mutants can also be crossed with mutants comprising point mutations in *SSIIa* genes to produce triple gene mutants having combinations of deletions and point *ssIIa* mutations.

[0421] *Point mutations.* Point mutations including single nucleotide polymorphisms (SNP) can be identified in publically available libraries of mutagenized wheat plants. Such libraries include, for example, one available from John Innes Centre, UK. A wheat *SSIIa* nucleotide sequence (Genbank Accession No: AB201445) was used to interrogate the John Innes Centre wheat database using BLAST software and lines comprising SNPs in each of the three *SSIIa* genes were identified. The SNPs were categorized into three classes. The first group comprised mutants which had a mutated *SSIIa* gene comprising a new stop codon in the protein coding region of the gene. These mutations were predicted to cause premature termination of translation of the SSIIa protein encoded by that gene. Premature termination mutations, also known as nonsense mutations or "stop codon-gained mutations", are almost always null mutations provided the mutation is not close to the 3' end of the protein coding region, although even those may be null mutations. The second group of mutants comprised lines which had a nucleotide polymorphism in a splice site of an *SSIIa* gene, in either a splice donor site or a splice acceptor site. Such mutations were expected to cause mis-splicing of the RNA transcript from the *SSIIa* gene and severely affect the mRNA; splice-site mutations are most often null mutations. The third group consisted of mutants which comprised a point

mutation in one of the *SSIIa* genes which resulted in an amino acid substitution in the encoded SSIIa polypeptide; these are termed "missense mutations". The impact of each missense mutation on the structure of the encoded protein is predicted using Blosum 62 and Pam 250 matrices.

[0422] The *SSIIa* gene mutants which were identified in the first and second groups are listed in Table 9. For the *SSIIa-A* gene, 5 nonsense mutations, 2 splice-site mutations and 49 missense mutations were identified from the database. Two of the nonsense mutations, identified in different pools, were identical. For the *SSIIa-B* gene, 2 nonsense mutations, 7 splice site variants and 22 missense mutations, were identified. For the *SSIIa-D* gene, 2 splice mutations and 49 missense mutations were identified. Several mutants had more than one polymorphism in an *SSIIa* gene, including some which had a polymorphism in an intron as well as a polymorphism in a protein coding region.

[0423] *Identification of mutations by TILLING.* A population of mutated plant lines was developed after sodium azide mutagenesis of seeds of the wheat cultivar Sunstate, using the method described in WO2014/028980. Five thousand M1 plants were grown to maturity, allowing them to self-fertilise. M2 seeds were harvested separately from each plant, thereby producing the mutagenized population of 5000 lines. DNA was extracted from a leaf section (~2 cm) from plants from each of the lines. Wheat leaf DNAs were quantified using a plate reader (FLUOstar Omega, BMG LABTECH) and normalized to 10 ng/µl using a robot machine (Corbett Life Science). For TILLING, DNA samples from sets of 8 plants were pooled together in each 96 plate well. PCR reactions were conducted to amplify segments of the *SSIIa* genes, for example with one cycle of 95°C for 5 min, then 35 cycles of melting at 94°C for 45 s, annealing temperature of 60 to 62°C for 30 s, and extension at 72°C for 2 min 30 s, then 1 cycle of 72°C for 10 min, followed by cooling to 25°C. The resultant PCR fragments (5 µl) were separated on 1 or 2% agarose gels and visualized (UVitec) after ethidium staining for examining the quality of PCR amplification.

[0424] Heteroduplexing of PCR fragments to the wild-type RNA transcript is carried out using a PCR machine with 1 cycle of 99°C for 5 min, 70 cycles of annealing starting at 70°C and reducing at a rate of 0.6°C per cycle, for 20 s, followed by cooling to 25°C. The heteroduplexes are digested with the *Cel*I enzyme, using 5 µl of heteroduplexed PCR product

with 5 µl of 2x buffer mixture and 0.5 µl of *Cel*I enzyme. The buffer mixture is composed of 20 mM HEPES, pH 7.5, 20 mM $MgSO_4$, 20 mM KCl, 0.004% Triton X-100 and 0.4 µg/ml BSA. The assembled reactions are mixed and incubated at 45°C for 15-30 min. The digested DNA samples are then loaded onto a DNA fragment analyser (Advanced Analytical Technologies) for separating DNA fragments according to the Mutation Discovery kit (DNF-910-K1000T). Alternatively, sets of 10 amplification products are pooled after normalization of the PCR products, and sequenced with one DNA pool per flow cell. The sequence data is analysed to select lines having *ssIIa* gene polymorphisms. SNP assays are designed for each polymorphism based on kaspar technology, and genotyping is performed on the M1 lines in each pool that is positive for a particular polymorphism. Thereby, the individual mutant line containing each mutant gene is identified and the mutant *SSIIa* sequences confirmed.

[0425] Plants containing a point mutation in a single SSIIa gene are crossed with plants containing mutations in the other two *SSIIa* genes to generate triple-null *ssIIa* mutants.

Table 9. *ssIIa* mutants identified in the JIC mutagenized library

Line	Chromo-some	Mutation type	Mutation Position	Chromosome position	WT base	Mutant base	cDNA position	Amino acid change	Codon change
ssIIa-A mutants									
Kronos2261	7AS	Stop codon gained	322	52346758	C	T	1013	W269*	tgG/tgA
Cadenza0110	7AS	Stop codon gained	114	52346550	G	A	1221	Q339*	Cag/Tag
Cadenza0403	7AS	Stop codon gained	114	52346550	G	A	1221	Q339*	Cag/Tag
Cadenza1738	7AS	Stop codon gained	3460	52349896	C	T	563	W119*	tgG/tgA
Cadenza1097	7AS	Stop codon gained	323	52346759	C	T	1012	W269*	tGg/tAg
Cadenza1237	7AS	Splice acceptor site variant	5153	52351589	C	T	N/A	N/A	N/A
Cadenza0413	7AS	Splice region variant & intron variant	5081	52351517	G	A	N/A	N/A	N/A
ssIIa-B mutants									
Cadenza1731	7BS	Stop codon gained	1811	31821582	G	A	342	W82*	tGg/tAg
Kronos3900	7BS	Stop codon gained	1812	31821583	G	A	343	W82*	tgG/tgA
Kronos2209	7BS	Splice acceptor variant	1172	31820943	G	A	N/A	N/A	N/A
Cadenza1031	7BS	Splice region variant & intron variant	40	31819811	G	A	N/A	N/A	N/A
Cadenza1788	7BS	Splice region variant & intron variant	41	31819812	G	A	N/A	N/A	N/A
Kronos2375	7BS	Splice region variant, intron variant	1001	31820772	C	T	N/A	N/A	N/A
Kronos2187	7BS	Splice region variant, intron variant	1088	31820859	G	A	N/A	N/A	N/A
Kronos3229	7BS	Splice region variant, intron variant	1167	31820938	C	T	N/A	N/A	N/A
Cadenza1176	7BS	Splice region variant & intron variant	1378	31821149	C	T	N/A	N/A	N/A
ssIIa-D mutants									
Cadenza0627	7DS	Splice region variant, intron variant	555				N/A	N/A	N/A
Cadenza1762	7DS	Splice region variant, intron variant	1612				N/A	N/A	N/A

EXAMPLE 12: Analysis of proteins in *ssIIa* mutant grain

[0426] *Expression of starch biosynthetic genes in ssIIa mutants.* Five *ssIIa* mutant wheat lines, designated A24, B22, B29, B63 and E24, were selected from the double haploid population of Konik-Rose *et al.* (2007) and the content of several starch biosynthetic mRNAs and proteins analysed as follows. The level of mRNA expression in developing grains at 15 DPA of the *ssIIa* mutant wheat plants was compared to the wild-type by quantitative reverse transcription PCR (qRT-PCR) as described in Example 1. Quantitation of RNA in each sample was done by reference to a constitutively-expressed tubulin gene. The qRT-PCR data revealed that the level of *ssIIa* mRNA in the homozygous triple mutant *ssIIa* endosperm was significantly lower than that in the corresponding wild-type grains ($p<0.01$), being only about 8% of the level relative to the corresponding wild-type wheat endosperm. In contrast, the levels of *SSI, SBEIIa* and *SBEIIb* transcripts in the mutant grain was about the same as in the corresponding wild-type developing grain. This indicated a specific effect on *ssIIa* mRNA in the mutant endosperm.

[0427] *Analyses of the abundance of granule bound proteins in the starch of mature grain.* To compare protein band intensity on a protein gel and the amount of protein sample loaded on the gel, one to four mg of starch as starch granules from mature *ssIIa* mutant and *SSIIa* wild-type grains were used for the extraction of starch granule bound proteins. The proteins were separated on a protein gel as described in Example 1. Four proteins with molecular weight of at least 60 kDa were observed to be bound to the starch granules in the wild-type mature grain. In the *ssIIa* mutant grain, a single protein, identified as GBSSI, was observed at approximately 60 kDa. When the protein bands were quantitated, there was a positive correlation between the protein band intensity and the amount of starch used for protein extraction for each of SSIIa, SBEII and SSI from the wild-type grain and for GBSSI in the mutant grain. Due to the low levels of SSIIa, SBEII and SSI proteins inside the starch granules, an amount of four mg starch was selected for extracting starch granule bound proteins for the further analysis described as follows.

[0428] Four starch granule bound proteins having a molecular weight of about 60 kDa or greater were observed when the protein extracts of wild-type wheat grain were subjected to

gel electrophoresis and stained with Sypro. They were identified as SSIIa (~88 kDa), SBEIIa and SBEIIb (each ~83 kDa), SSI (~75 kDa) and GBSSI (~60 kDa) polypeptides by immunonblot analyses. SBEIIa and SBEIIb migrated at almost the same position on the gels, but the abundance of SBEIIb inside the wild-type starch granules was much greater than SBEIIa as judged by the Sypro staining.

[0429] When the proteins from the *ssIIa* mutant wheat starch granules were analysed by gel electrophoresis, SSIIa and SBEIIa proteins were not detected. SSI and SBEIIb proteins were not detected by Sypro staining but could be detected by immunoblot analyses although their abundance was so low that accurate quantitation could not be made (Figure 26).

[0430] *Analysis of the protein abundance of starch biosynthetic enzymes in the soluble stroma and inside the starch granules of developing endosperm.* To determine whether the low concentration of SSI, SBEIIa and SBEIIb inside the starch granules in the mature, mutant grain was due to a lower rate of synthesis during grain development, at the time when SSIIa protein was absent in the mutant grain, the SSI, SBEIIa and SBEIIb proteins in the soluble stroma fraction and inside the starch granules of the developing endosperms were quantitated at 15 DPA. Quantitation was done by immunodetection. When the soluble proteins of the developing endosperms were analysed, significantly increased amounts of both SBEIIb and SSI were present in the *ssIIa* mutant grain compared to the *SSIIa* wild-type developing grain at the same stage (15 DPA). In contrast, the level of SBEIIa was similar in the *ssIIa* mutant and *SSIIa* wild-type grain. SBEIIa was more abundant in the soluble fraction of the amyloplasts than SBEIIb. It was concluded that the reduced levels of SSI, SBEIIa and SBEIIb proteins inside the starch granules was not due to reduced expression levels but instead reflected a reduced binding in the starch granules, indicating a localisation away from the starch granules and into the soluble fraction in the stroma.

[0431] When the starch granule bound proteins in the developing grain were analysed, no SSIIa protein was detected in the mutant wheat *ssIIa* grain by immunoblot analyses. The level of SBEIIb was decreased to about 25% compared to the wild-type. The level of SSI inside the starch granules of the mutant *ssIIa* was also approximately 25% of the wild-type

level. Similar patterns of reduction in the amount of starch granule bound proteins were observed in the mature endosperms. However, on a per starch basis, the concentration of starch biosynthetic enzymes in starch granule bound proteins of developing endosperms was higher than that in mature grains as a result of the dilution effect of higher starch levels in mature grain endosperm.

EXAMPLE 13. Sequence analysis of genes encoding SSIIIa and SSIIIb in hexaploid wheat and the enzymes for the A, B and D genomes

[0432] To identify gene and polypeptide sequences for SSIIIa and SSIIIb in hexaploid wheat, selected nucleotide sequences for wheat cDNAs encoding SSIIIa (Genbank No: AF258608) and SSIIIb (Genbank No: EU333946) were used to search the wheat genomic EnsemblPlant database using BLASTn. Between 15 and 20 cDNA sequences were identified and obtained for each of the three *SSIIIa* genes and the three *SSIIIb* genes on the A, B and D genomes. These genomic DNA sequences were assembled with sequence analysis software, CodonCode Aligner, to obtain assembled genomic sequence contigs for the six genes. Two to four contigs were obtained for each of the six genes. Those contigs were then arranged in order by sequence comparisons using the cDNA sequences AF258608 and EU333946. The cDNA sequences were deduced from the genomic DNA sequences by comparing with the AF258608 and EU333946 cDNA sequences. The open reading frames were analysed using the pWRAW32 software to obtain the predicted amino acid sequences. Genomic DNA sequences, cDNA sequences and polypeptide sequences were aligned using the Bioedit Sequence Alignment Editor. The SSIIIa-D gene and SSIIIb-A gene sequences were partial length sequences, encoding only part of the protein coding region. Therefore the full length *Aegilops tauschii* SSIIIa cDNA (Accession No. AF258609; Li *et al* 2000) and polypeptide sequences were used as representative of the SSIIIa-D genome sequence, since *A. tauschii* is a presumed progenitor of the D genome in hexaploid wheat.

[0433] Each of the *SSIIIa* and *SSIIIb* genes have 16 exons and 15 introns. The exon sequences were deduced by comparing the genomic and cDNA sequences.

[0434] The nucleotide sequences of the SSIIIa cDNAs were compared to determine the extent of sequence identity between *SSIIIa* genes and between *SSIIIa* and *SSIIIb* genes. The results (Table 10) showed 96-97% sequence identity among three homoeologous *SSIIIa* genes, but 80% identity or less between *SSIIIa* gene cDNAs and *SSIIIb* gene cDNAs. The SSIIIa and SSIIIb genes could therefore be readily distinguished.

[0435] The amino acid sequences of the SSIIIa polypeptides were compared to determine the extent of sequence identity between SSIIIa polypeptides and between SSIIIa and SSIIIb polypeptides. The results comparing the amino acid sequences of the polypeptides showed that the three SSIIIa polypeptides were 94-97% identical when compared pairwise, and less than 80% identical to SSIIIb polypeptides.

Table 10. Nucleotide and amino acid sequence identity between SSIIIa and SSIIIb cDNAs and polypeptides

	SSIIIa-A	**SSIIIa-B**	**SSIIIa-D**	**SSIIIb-A**	**SSIIIb-B**	**SSIIIb-D**
Identity between nucleotide sequences (%)						
SSIIIa-A		97	97		79	78
SSIIIa-B			96		78	
SSIIIa-D					80	
SSIIIb-A						
SSIIIb-B				98		
SSIIIb-D				98	98	
Identity between amino acid sequences						
SSIIIa-A		94.2	96.9	68	58	61
SSIIIa-B			95.5		59	
SSIIIa-D					59	
SSIIIb-A						
SSIIIb-B				88.3		
SSIIIb-D				92.6	95.7	

EXAMPLE 14: Screening for *ssIIIa* gene mutations by using TILLING technology

Mutagenesis of wheat seeds

[0436] Mature seed of the Australia wheat line WAWHT2750 was treated with the chemical mutagen ethyl methanesulfonate (EMS) to produce a M2 mutant population as follows. Dry seeds were divided into treatment groups of ~300 grains wrapped in cheesecloth and soaked in an EMS solution (0.7% (w/v) with gentle agitation overnight (~18 h). Treated seeds were washed extensively under running tap water for at least 2 h and then sown in pots at the rate of about 20 kernels per pot in a greenhouse maintained at 16–18°C. After seedling development, pots were then transferred outdoors for 2 weeks before the M1 seedlings were transplanted to the field. M2 seeds were harvested as five separate spikes per M1 plant, with each spike being threshed and bagged individually.

DNA extraction from plant leaves

[0437] One M2 seed from each spike was sown in a 100-cell (10 x 10) seedling tray. Leaves of ~20-days-old seedlings were collected in a 96-well format and dried in a closed box with silica gel. DNA extraction was performed according to the manufacturer's instructions on an ABI6100 PrepStation (Applied Biosystems, Melbourne, VIC, Australia), with the modifications of adding a centrifugation step after the cell lysis and adding RNase A to the elution buffer. All buffers were purchased from Applied Biosystems, Australia. Briefly, dried leaves were crushed into a powder using two ball bearings per sample on a Mix-Miller (MM300; Retsch, Germany). Extraction buffer (600 µl) was added to each sample and incubated at room temperature for 10 min before centrifuging at 5000 rpm for 10 min. The supernatant (~550 µl) was loaded onto the DNA binding plate, and DNA was bound to the membrane by vacuum suction and washed in 600 µl washing buffer (three times), and eluted first in 100 µl of elution buffer 1 and then in 100 µl of elution buffer 2 (added RNase A to 50 µg ml−1). The DNA concentrations were normalized to 5 to 15 ng/µl after gel electrophoresis with a known mass standard. DNA samples from 3 or 4 individual spikes were pooled for initial screening.

Screening for mutations of wheat SSIIIa genes

[0438] To provide for screening of *SSIIIa* mutations by TILLING, which is an efficient way to detect single nucleotide changes after mutagenesis, primers were designed for specifically amplifying DNA fragments from the 3' part of exon 3 and part of intron 3 for the *SSIIIa* genes of each of the A, B and D genomes (Figure 27). That part of exon 3 was selected for screening because the nucleotide sequences of the A, B and D genomes in that region contained numerous polymorphisms that allowed the nucleotide sequences to be readily assigned to the A, B or D genome *SSIIIa* genes, and because that region encoded more tryptophan and glutamine residues than other parts of the genes. Trp and Gln codons can both form a stop codon by a single nucleotide substitution. The primers designed and used for amplifying the A genome fragment were wSSIIIa-ABF1 (5' CCAGAGGTAGTGAAAGCTGATTCAG 3', SEQ ID NO:70, located from nucleotide 4586 of wheat SSIIIa genomic DNA, Genbank Accession No. AF258609) and wSSIIIa-AR1 (5' GAATACAAATGATCTTTAAGCGTCCCT 3', SEQ ID NO:71, located at

nucleotide 5474 of Accession No. AF258609). For the B genome, the primers were ZLwSSIIIa-ABF1 and wSSIIIa-BR1 (5' CATGCCATATTTGTAGGAGCACTATGCC 3', SEQ ID NO:72, located at nucleotide 5370 of Accession No. AF258609). For the D genome, the primers were wSSIIIa-DF1 (5' GTGGATCCGCAAGCACTAAAGG 3', SEQ ID NO:73 located at nucleotide 4505 of Accession No. AF258609) and wSSIIIa-DR1 (5' GAATACAAATGATCTTTAAGCGTCTG 3', SEQ ID NO:74, located at nucleotide 5475 of Accession No. AF258609) (Figure 2).

[0439] Amplification was performed in 10 µl volumes each containing 2 µl of pooled DNA, 1 µl 10x Pfu buffer (Stratagene, La Jolla, CA), 0.2 mM dNTPs, 0.4 µM primers and 0.25 U PfuUltraII Fusion HS DNA Polymerase (Stratagene). Polymerase chain reaction was conducted using a thermal cycler (MasterCycler 5333; Eppendorf, North Ryde, NSW, Australia) using the cycling conditions as follows: 95°C for 2 min, followed by 6 cycles of touchdown at 94°C for 30 s, an annealing step starting at 72°C for 30 s and decreasing 1°C per cycle, and 72°C for 1 min, then 35 cycles of PCR (94°C for 20 s, 60 or 65oC for 20 s, and 72°C for 15 s), and finally extension at 72°C for 1 min. The annealing temperature used was 65oC for the A and B genome amplifications and 60oC for the D genome. After PCR amplification, the DNA products were denatured and annealed in the thermal cycler using the conditions: 95°C for 8 min, 85°C for 1 min, followed by 99 cycles of 84°C for 30 s, decreasing 0.5°C per cycle. The annealed DNA samples were then digested with Cel I enzyme prepared according to Till *et al.* (2004a) except that in the last step, the dialyzed extract was mixed with glycerol to give an enzyme solution in 50% (v/v) glycerol. Each digestion reaction was performed at 45°C for 30 min in a 20 µL reaction volume containing 8 µl of PCR product, 2 µl of Cel I enzyme, and 10 µL of 2x buffer [20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, pH 7.5, 20 mM MgSO4, 20 mM KCl, 0.004% (v/v) TritonX-100 (SIGMA, Castle Hill, NSW, Australia), and 0.4 µg/ml bovine serum albumin]. The digested samples were then electrophoresed in a thin 2% agarose gel and stained with ethidium bromide. Images were analyzed visually for the presence of cleavage products using Adobe Photoshop software (Adobe Systems Inc., San Jose, CA).

[0440] When pooled samples were identified as showing Cel I cleavage bands, individual DNA samples from the pool were tested together with a wild-type DNA sample. The PCR products of all identified, putative mutants were sequenced to confirm the presence of a

mutation and to determine the position and nature of each mutation. The mutations were confirmed by sequencing of genomic DNA from individual plants, as follows.

Identifying mutations of ssIIIa genes by sequencing

[0441] For genomic DNA sequencing of mutants showing Cel I cleavage bands, genomic DNA from individual wheat plants was amplified by PCR using the 3 pairs of primers, namely wSSIIIa-ABF1/wSSIIIa-AR1, wSSIIIa-ABF1/wSSIIIa-AB1 and wSSIIIa-DF1/wSSIIIa-DR1. Each PCR reaction of 20 µl used 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 Advantage 2 Taq polymerase mix (Clontech). 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, 60°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 products were treated with Exonuclease I-Shrimp alkaline phosphatase to remove primers and dNTPs as described (Affymetrix). DNA sequencing was performed using an automated ABI system with dye terminators according to standard procedures. The fragments of the three *SSIIIa* genes were distinguished because of polymorphisms in the amplified region, see Figure 27.

[0442] DNA sequences were analysed using the CodonCode suite of programs to detect single-nucleotide polymorphisms (SNPs) in genomic DNAs of an *SSIIIa* gene in the A, B or D genomes.

[0443] Numerous plants having SNPs in an *SSIIIa* gene were identified in the mutagenized wheat population of approximately 2000 M2 wheat lines. SNPs were identified for each of the *SSIIIa* genes on the A, B and D genomes, including some having introduced premature stop codons (translation termination) in the protein coding regions which were presumed to be null mutations. Plants with the following exemplary null mutations were selected and used in plant crosses to combine the mutations: a mutation in the A genome that provided a stop codon at amino acid position 865 (designated ssIIIa-A-Q865stop), a mutation in the A genome that provided a stop codon at amino acid position 931 (designated ssIIIa-A-W931stop), a mutation in the B genome that provided a stop

codon at amino acid position 758 (designated ssIIIa-B-W758stop) and a mutation in the D genome that provided a stop codon at position 923 (designated ssIIIa-D-W923stop).

EXAMPLE 15. Producing triple null *ssIIIa* mutants by crossing single null mutants

[0444] For producing triple null mutants for the *SSIIIa* gene, a plant having a B genome null mutation (wSSIIIa-B-W758stop) was first crossed with a plant having a D genome null mutation (wSSIIIa-D-W923stop) to produce F1 seeds which were heterozygous for both of the null mutations. F1 plants obtained from these seeds were then crossed with plants having one or other of the two selected A-genome *ssIIIa* null mutations, containing either wSSIIIa-A-Q865stop or wSSIIIa-A-W931stop.

[0445] To detect F2 and subsequent generations of progeny plants containing all three mutations, PCR fragments across the mutation sites were amplified from DNA obtained from the progeny plants using the same three sets of primers as identified above. To do this, 2 cm portions of young leaves were collected into 96-well plates and freeze-dried. Genomic DNA was extracted from each leaf sample using a rapid DNA extraction method, as follows. Dry leaf sections were ground with a Mixer Mill MM400 (Retsch). Leaf powder was mixed with 375 µl of extraction buffer (0.1 M Tris-HCl, pH8.0; 0.05 M EDTA, pH 8.0; 1.25% SDS). Samples were then incubated in an oven at 65°C for at least one hour, and then cooled down to room temperature. Samples were gently mixed with 187 µl of 6M ammonium acetate and cooled at -20°C for 30 min. After centrifugation for 30 min at 3000 g, 340 µl supernatant from each sample was transferred to a new plate containing 220 µl isopropanol per well. DNA was precipitated for 5 minutes at room temperature and collected by centrifugation for 30 min at 3000 g. After discarding the supernatant, the pellets were air dried at room temperature for 1 min and each washed with 180 µl of 70% (v/v) ethanol. DNA samples were centrifuged for 30 minutes at 3000 g. The genomic DNA in each pellet was then dissolved in 225 µl MilliQ water overnight at 4°C. After centrifuging for 20 minutes at 3000 g, DNA samples (150 µl) were transferred to a new microtiter plate and stored at -20°C for further analysis.

[0446] For genotyping for the presence or absence of the *ssIIIa* mutations designated SSIIIa-A-Q865stop and SSIIIa-A-W931stop, primers wSSIIIa-ABF1 and wSSIIIa-AR1

were used for PCR amplification, and primers wSSIIIa-AF3 and wSSIIIa-AF2 were used for sequencing, respectively. For SSIIIa-B-W758stop, primers wSSIIIa-ABF1 and wSSIIIa-BR1 were used for the PCR amplification and primer wSSIIIa-BR2 was used for the sequencing. For SSIIIa-D-W923stop, primers wSSIIIa-DF1 and wSSIIIa-DR1 were used for the PCR amplification and primer wSSIII-DR1 was used for the sequencing. PCR amplifications were carried out as above using a HYBAID PCR Express (Integrated Sciences) except using GoTaq Hot Start Polymerase (Promega) instead of Advantage 2 Taq polymerase. To confirm that these primer pairs produced genome-specific amplification products, DNA fragments were amplified for each of the A, B and D genomes from three nullisomic-tetrasomic lines of chromosome 1 of cultivar Chinese Spring (Figure 28). Using primers wSSIIIa-ABF1 and wSSIIIa-AR1 for the A genome, wSSIIIa-ABF1 and wSSIIIa-BR1 for the B genome, and wSSIIIa-DF1 and wSSIIIa-DR1 for the D genome, the sizes of the PCR fragments were 916 bp, 808 bp and 997 bp for the A, B and D genome SSIIIa fragments, respectively.

[0447] Plants having these single null mutations were selected for the crossing program to generate triple-null *ssIIIa* and triple double null (*ssIIa-ssIIIa*, Example 15) progeny seeds and plants. Two null mutants in the A genome (wSSIIIa-A-Q865stop and wSSIIIa-A-W931stop), one null mutant in the B genome (wSSIIIa-B-W758stop) and one null mutant in the D genome (wSSIIIa-D-W923stop) were selected. wSSIIIa-A-Q865stop was caused by a C to T mutation whereas the other three were G to A mutations, relative to the sense strand. All four mutations were confirmed by DNA sequencing in progeny plants and in the crossing populations.

[0448] Two triple null *ssIIIa* mutant lines were generated through combining the single null mutations, differing in the selected A genome mutation. Five triple null homozygous plants and five near isogenic wild-type homozygous plants were selected from F4 or F5 populations for each of the crossing populations, which were named as Q865 ssIIIa triple null, Q865 SSIIIa wild-type, W931 ssIIIa triple null and W931 SSIIIa wild-type, and lines established from these plants. The near isogenic wild-type plants were selected from the same crossing populations as the triple nulls in order to minimize any effect from the genetic background. Seed weight and selected grain compositions were then analysed using F5 (for Q865 ssIIIa triple null, Q865 SSIIIa wild-type) or F6 (W931 ssIIIa triple null

and W931 SSIIIa wild-type) seeds, as described below. By this means, multiple lines which were homozygous, triple null ssIIIa mutant were obtained, as well as corresponding triple *SSIIIa* wild-type plants. One of the triple null *ssIIIa* mutant lines was designated 2_27_8, comprising the ssIIIa-A-W931stop mutation as well as the ssIIIa-B-W758stop and ssIIIa-D-W923stop mutations, and was used in some subsequent experiments.

EXAMPLE 16. Production of triple null mutants for both *ssIIa* and *ssIIIa* genes

[0449] Plants of a *ssIIa* triple null mutant wheat line designated SS028 (Examples 4, 5), which was homozygous for three *ssIIa* null mutations and having a genetic background of the cultivar Westonia, and the wheat *ssIIIa* null mutant designated 2-27-8 were crossed to produce F1 seeds. F1 plants were produced from the seed and self-fertilised to generate F2 seeds. Subsequent progeny generations to the F5 generation were produced, with screening to identify plants which were homozygous for all three of the *ssIIa* mutations and all three of the *ssIIIa* mutations ("double-triple nulls"). The primers used in the screening to detect mutations of *ssIIIa* genes from the three genomes were the same as described above. For marker-assisted breeding of the *ssIIa* gene mutations, primer pairs JKSS2AP1F (5'-TGCGTTTACCCCACAGAGCA CA-3' (SEQ ID NO:15) located between nucleotides 91 and 113 of nucleotide sequence Accession No. AB201445) and JKSS2AP2R (5'-TGCCAAAGGTCCGGAATCATGG-3' (SEQ ID NO:16) located between nucleotides 1225 and 1246 of AB201445) were used for the A genome *SSIIa* gene, primers JKSS2BP7F (5'-GCGGACCAGGTTGTCGTC-3' (SEQ ID NO:17) located between nucleotides 5978 and 5995 of nucleotide sequence Accession No. AB201446) and JLTSS2BPR1 (5' CTGGCTCACGATCCAGGGCATC-3' (SEQ ID NO:18) located between nucleotides 6313 and 6335 of AB201446) for the B genome *SSIIa* gene, and primers JTSS2D3F (5'-GTACCAAGGTATGGGGACTATGAA-3' (SEQ ID NO:19) located between nucleotides 2369 and 2392 of nucleotide sequence Accession No. AB201447) and JTSS2D4R (5'-GTTGGAGAGATACCTCAACAGC-3' (SEQ ID NO:20) locating between nucleotides 2774 and 2796 of AB201447) were used for the D genome *SSIIa* gene of wheat. These primers were used to amplify DNA fragments specific for each genome from the wild-type and the ssIIa null mutant plants. For the A genome *SSIIa* gene, a 1072 bp fragment was amplified from wild-type and a 778 bp fragment from the ssIIa-A null mutant gene. For the B genome *SSIIa* gene, a 374 bp fragment was amplified from

wild-type and a 522 bp fragment from the ssIIa-B null mutant gene. For the D genome *SSIIa* gene, a 427 bp fragment was amplified from wild-type and a 364 bp fragment from the ssIIa-D null mutant gene. These genome-specific fragments were readily distinguished by their size by gel electrophoresis and therefore could be used as co-dominant DNA markers to detect the mutant and wild-type alleles. Other specific primer pairs based on the *SSIIa* gene sequence could easily be designed to provide alternative molecular markers.

[0450] Near isogenic, fully wild-type *SSIIa /SSIIIa* lines ("double wild-type") were also identified from the same breeding populations and were used as controls in comparing grain characteristics. The F6 seeds from 11 lines of *ssIIa-ssIIIa* double triple null mutants and 5 lines of *SSIIa-SSIIIa* double wild-type were used for the analyses of their grain weight and grain composition.

EXAMPLE 17. Analyses of triple null *ssIIIa* and triple-double null *ssIIa-ssIIIa* grain.

Total starch content, amylose content and resistant starch content

[0451] Wheat grains were first ground to wholemeal using a Cyclone mill machine (Cyclote 1093, Tecator, Sweden). Total starch content was assayed using AOAC method 996.11 (K-TSTA 07/11, Megazyme) using 20 mg of wholemeal for each of three replicate samples (Konik-Rose *et al.*, 2007). Starch was isolated by a protease extraction method (Morrison *et al.*, 1984) followed by water washing and removal of the tailings. Starch was then freeze-dried. 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) and calculated on a weight basis as a percentage of the total starch content. Resistant starch (RS) content was analysed using AOAC method 2002.02 (K-RSTAR 08/11, Megazyme) using 20 mg of wheat wholemeal for each of three replicate samples, and calculated as a percentage of the total starch content.

ß-glucan content, fructan content and total dietary fibre content

[0452] ß-Glucan was measured using the AOAC method 995.16 (K-BGLU 07/11, Megazyme) using 20 mg of wholemeal for each of three replicate samples. Fructan was measured using the AOAC method 999.03 (K-FRUC 03/14, Megazyme) using 40 mg of

wholemeal for each of three replicate samples. Total dietary fibre (TDF) was measured using the AOAC method 985.29 (K-TDFR 06/14, Megazyme) using 100 mg of wholemeal for each of three replicate samples. Each were calculated on a weight basis as a percentage of the grain weight. Total fibre content was calculated as the sum of the ß-glucan, arabinoxylan, frucatn and cellulose contents.

Grain weight

[0453] Grain weights were determined as the average grain weight of at least 20 grains for each measurement, with three measurements for each line. If enough grain was available, the average grain weight of 100 grains was determined.

[0454] Statistical analyses were performed using Genstat version 9. Analysis of variance was performed for grain weight, starch content, amylose content, ß-glucan content, RS content, fructan content, total dietary fibre (TDF) content and total fibre content (TDF + fructan) to obtain the least significant difference (LSD, $P < 0.05$), looking at variation between the genotypes.

Seed weight of wheat ssIIIa triple null mutants

[0455] Seed weights were measured for 5 lines of each of the four groups of F5 seeds for Q865stop ssIIIa triple null and Q865 SSIIIa wild-type lines, and F6 seeds for W931 ssIIIa triple null and W931 SSIIIa wild-type lines. There was no significant difference in average seed weight of Q865stop ssIIIa triple null grain and SSIIIa wild-type grain or between W931stop ssIIIa triple null and W931 SSIIIa wildtype (Table 11). Average seed weights were between 34 mg to 38 mg for all four groups.

Starch content, amylose content and RS content of wheat ssIIIa triple null mutants

[0456] Grain from both of the ssIIIa triple null mutant lines contained significantly less starch, and more amylose and RS, than their respective wild-types (Table 11). The *ssIIIa* mutant grain contained approximately 10% less starch (total starch content) and 15% less amylopectin than wild-type grain (Table 11). However, grain of both *ssIIIa* mutant lines contained more amylose and more RS than wild-type grain (Table 11), exhibiting 30% more amylose than wild-type grain. The Q865stop triple null ssIIIa grain and the

W931stop triple null grain contained approximately 2.7 and 3.6 times greater RS content than the wild-type grain, respectively.

ß-glucan content, fructan content, TDF content and total fibre content of wheat ssIIIa triple null mutants

[0457] Grain from both of the *ssIIIa* triple null mutant lines contained significantly more ß-glucan on a weight basis than their respective wild-types. The *ssIIIa* triple null mutant grain contained about 1.5 to 1.6-fold more ß-glucan (% weight) comparing to the corresponding wild-type grain (Table 11). The average fructan content in the ssIIIa-Q865 triple null grain was increased significantly on a weight basis relative to the corresponding wild-type grain, but not in the ssIIIa-W931 triple null mutant grain (Table 11). The ssIIIa-Q865 triple null mutant grain contained 1.4-fold more fructan (% weight) comparing to the wild-type grain, while the ssIIIa-W931 triple null mutant grain contained approximately 1.2-fold more fructan comparing to the wild-type grain (Table 11) although the latter was not statistically significant. The average TDF in the ssIIIa-W931 triple null grain was increased relative to the wild-type, but not in the ssIIIa-Q865 grain. The ssIIIa-W931 triple null mutant grain contained approximately 10% more TDF than the wild-type grain, while there was no significant difference between the SSIIIa-Q865 triple null grain and the wild-type for TDF content. The total fibre content in both lines of *ssIIIa* null mutant grain was significantly increased. The ssIIIa-Q865 triple null and ssIIIa-W931 triple null mutant grain contained 5% or 10% more total fibre than the wild-type grain, respectively.

[0458] It was concluded that the triple null *ssIIIa* mutant grain was significantly affected in multiple grain parameters, in selected lines, including increases selected from ß-glucan, fructan, TDF and total fibre contents, without any significant reduction in grain weight.

Seed weight of ssIIa-ssIIIa double triple null mutants

[0459] There was a reduction in average seed weight of the *ssIIa-ssIIIa* double triple null mutant grain compared to the corresponding wildtype lines (Table 12), but the difference was not statistically significant in this experiment. However, the average seed weight of the ssIIa-ssIIIa double triple null mutant grain was significantly greater than that of the ssIIa triple null mutant grain, one of the parental lines (Table 12). The increased average

seed weight relative to the *ssIIa* triple null grain was unexpected since both SSIIa and SSIIIa are starch synthases, and removal of two starch synthases was expected to reduce starch content more than removal of SSIIa alone, thereby reducing grain weight further. Surprisingly however, the addition of the *ssIIIa* mutations appeared to partially alleviate the reduced grain weight caused by the *ssIIa* mutations and loss of SSIIa activity.

Starch content, amylose content and RS content of ssIIa-ssIIIa double triple null mutant wheat grain

[0460] The *ssIIa-ssIIIa* double triple null mutant grain contained significantly less total starch, including less amylopectin, and more amylose and RS on a weight basis than the corresponding wild-type grain (Table 12). The grain of the *ssIIa-ssIIIa* double triple null mutants contained approximately 20% less starch and 20% less amylopectin than the wild-type grain (Table 12). Despite the reduction in starch content, the *ssIIa-ssIIIa* double triple null mutant grain contained more amylose and more RS than the wild-type grain (Table 12). Indeed, the *ssIIa-ssIIIa* double triple null mutant grain contained 50% and 23% more amylose relative to the wild-type grain or the *ssIIIa* triple null mutant grain, respectively. Most significantly and importantly, the *ssIIa-ssIIIa* double triple null mutant grain contained approximately 8 and 2.4-fold more RS than the wild-type grain and *ssIIIa* triple null mutant grain, respectively. However the amylose content was approximately 89% of that of the *ssIIa* triple null mutant, one of the parental lines (Table 12). Among grain obtained from 11 *ssIIa-ssIIIa* double triple null mutant lines, three had similar levels of amylose content relative to the *ssIIa* triple null mutant grain.

ß-glucan, fructan, TDF and total fibre contents of ssIIa-ssIIIa double triple null grain

[0461] The ß-glucan, fructan, TDF and total fibre contents of the double triple null mutant grain and control grain were measured using the methods as described above. The *ssIIa-ssIIIa* double triple null triple mutant grain contained significantly more ß-glucan, fructan, TDF and total fibre than that of the corresponding wild-type grain (Table 12). The *ssIIa-ssIIIa* double triple null triple mutant grain contained 2.9-fold more ß-glucan realtive to the wild-type grain (Table 12), which content was 1.38- and 1.27-fold more than the *ssIIIa* (SSIIa wild-type) and *ssIIa* (SSIIIa wild-type) triple null mutant grains, respectively.

The *ssIIa-ssIIIa* double triple null triple mutant grain contained approximately 4-fold more fructan comparing to the wild-type grain, which content was 4.78-fold and 1.67-fold more than the *ssIIIa* and *ssIIa* triple null mutant grain, respectively (Table 12). The *ssIIa-ssIIIa* double triple null mutant grain contained approximately 1.4-fold more TDF relative to the wild-type grain, which content was 1.28-fold more than the *ssIIIa* triple null mutant grain (Table 12). The *ssIIa-ssIIIa* double triple null mutant grain contained 1.6-fold more total fibre relative to the wild-type grain, which content was 1.47- and 1.3-fold more than the ssIIIa and ssIIa triple null mutant grains, respectively (Table 12). Each of these components was present at significantly greater levels in the double triple null mutant grain compared to the triple null ssIIa grain and the triple null ssIIIa grain, to a surprising extent. It was concluded that the combination of the *ssIIa* and *ssIIIa* mutations in the homozygous state enhanced each of these fibre components in hexaploid wheat grain.

Contents of components in ssIIIa triple null mutant grain on a per caryopsis basis

[0462] To further investigate these grain components, the levels in the triple mutant grain and the double triple mutant grain were determined on a per caryopsis basis. The *ssIIIa* triple null mutant contained less starch, and more amylose, RS, ß-glucan and fructan on a per caryopsis basis comparing to the corresponding wild-type grain although the extent of the increases in amylose and fructan contents were less in the ssIIIa-w931 triple null line (Table 13). The ssIIIa-Q865 null mutant grain had a starch content of 92% relative to the wild-type, a similar amount of amylopectin, and 1.21-, 2.49-, 1.49- and 1.44-fold more amylose, RS, ß-glucan and fructan content on a per caryopsis comparing to the wild-type grain, respectively (Table 13). The ssIIIa-W931 null mutant grain had a starch content of 80% and an amylopectin content of 89% relative to the wild-type grain, and 1.04-, 2.86-, 1.43- and 1.05-fold more amylose, RS, ß-glucan and fructan content on a per caryopsis comparing to the wild-type grain (Table 13). The TDF and total fibre contents were not significantly different on a per caryopsis basis between the *ssIIa-ssIIIa* triple null mutant grain and the wild-type grain, even though the contents on a weight basis were increased.

Contents of components in ssIIa-ssIIIa double triple null mutant grain on a per caryopsis basis

[0463] When determined on a per caryopsis basis, the *ssIIa-ssIIIa* double triple null mutant grain contained less starch (73% relative to wild-type) and amylopectin (88%), and more amylose (1.11 fold increase), RS (5.76 fold increase), ß-glucan (2.69 fold increase), fructan (3.67 fold increase), TDF (1.32 fold increase) and total fibre (1.49 fold increase) relative to the wild-type grain (Table 13). In comparison to the *ssIIIa* triple null mutant grain, the *ssIIa-ssIIIa* double triple null mutant grain contained less starch (81%) and amylopectin (87%), similar content in amylose (99%), and more RS (1.97 fold increase) and ß-glucan (1.30 fold), fructan (4.46 fold), TDF (1.19 fold) and total fibre (1.37 fold) on a per caryopsis basis (Table 13). When compared to the *ssIIa* triple null mutant grain, the *ssIIa-ssIIIa* double triple null mutant grains contained a similar content of RS (99.5%), and more starch (1.12 fold increased), amylose (1.11 fold), amylopectin (1.12 fold), ß-glucan (1.34 fold), fructan (1.76 fold), and total fibre (1.41 fold) on a per caryopsis base (Table 13). Therefore, the combination of the *ssIIa* and *ssIIIa* null mutant alleles increased the amounts of these components per grain, which was most surprising considering that both genes encode starch synthases. The lack of two starch synthases would have been expected to reduce these components in the grain, particularly the starch content and the amylose content, relative to a lack of only one of the enzymes.

Table 11. Characteristics of *ssIIIa* triple null grain relative to SSIIIa wild-type grain

Genotype	No of lines	Seed weight (mg)	Starch content (%)	Amylose content (%)	Amylopectin content (%)	RS content (%)	β-glucan content (%)	Fructan content (%)	TDF content (%)	Total fibre content (%)
ssIIIa-Q865 triple null	5	34.2±4.2[a]	60.3±2.0[b]	43.6±1.6[a]	56.4±1.6[a]	1.0±0.3[a]	1.8±0.1[a]	2.1±0.4[a]	17.4±0.4[a]	19.5±0.6[a]
SSIIIa-Q865 wild-type	5	34.1±6.3[a]	65.5±2.8[a]	33.0±0.6[b]	67.0±0.6[b]	0.4±0.1[b]	1.2±0.1[b]	1.5±0.1[b]	16.9±0.7[a]	18.4±0.7[b]
L.s.d.		4.6	2.5	1.2	1.2	0.3	0.1	0.3	0.9	1.0
% of wild-type		100.4	92.0	132.1	84.2	269.8	148.9	143.6	102.6	105.9
ssIIIa-W931 triple null	5	33.9±4.7[a]	56.2±2.1[b]	42.4±1.6[a]	57.6±1.6[a]	1.5±0.4[a]	1.7±0.1[a]	1.6±0.4[a]	16.8±0.5[a]	18.5±0.6[a]
SSIIIa-W931 triple wildtype	5	38.0±4.2[a]	62.9±0.8[a]	32.5±0.6[b]	67.6±0.6[b]	0.4±0.2[b]	1.0±0.0[b]	1.4±0.2[a]	15.4±1.4[b]	16.8±1.5[b]
l.s.d.		4.6	2.5	1.2	1.2	0.3	0.1	0.3	0.9	1.0
% of wild-type		89.0	89.5	130.7	85.3	359.8	161.0	118.2	109.3	110.1

Table 12. Characteristics of *ssIIa-ssIIIa* triple null grain relative to *ssIIa* triple null and *SSIIIa* wild-type grain

Genotype	No of lines	Seed weight (mg)	Starch content (%)	Amylose content (%)	Amylopectin content (%)	RS content (%)	β-glucan content (%)	Fructan content (%)	TDF content (%)	Total fibre content (%)
ssIIa triple null	1	34.8	50.3	43.8	56.2	3.6	1.7	2.8	-	19.6
ssIIIa-W931 triple null	1	39.4	61.4	35.5	64.5	1.3	1.6	1.0	16.8	17.8
ssIIa-ssIIIa double triple null	11	36.7±3.1[a]	53.1±3.8[b]	43.7±2.4[a]	56.3±2.4[a]	3.2±0.3[a]	2.2±0.5[a]	4.6±0.8[a]	21.5±0.9[a]	26.1±1.2[a]
SSIIa-SSIIIa double wildtype	5	39.8±4.2[a]	66.9±2.5[a]	28.7±0.5[b]	71.3±0.5[b]	0.4±0.2[b]	0.7±0.1[b]	1.2±0.1[b]	15.1±0.7[b]	16.2±0.7[b]
l.s.d.		3.9	4.0	2.4	2.4	0.3	0.4	0.8	1	1.3
% of wild-type		92.2	79.4	152.3	79.0	787.6	292.2	397.4	143.0	161.1
% of *ssIIIa* triple null		93.3	86.6	123.1	87.3	243.9	138.8	477.7	128.0	146.8
% of *ssIIa* triple null		105.6	105.7	99.7	100.3	89.1	127.2	166.8		133.5

Table 13. Per seed contents of grain components for *ssIIIa* triple null and *ssIIa-ssIIIa* double triple null

Genotype	No of lines	Seed weight (mg)	Starch content (mg)	Amylose content (mg)	Amylopectin content (mg)	RS content (mg)	β-glucan content (mg)	Fructan content (mg)	TDF content (mg)	Total fibre content (mg)
ssIIIa-Q865 triple null	5	34.2±4.2[a]	20.6	9.0	5.1	0.2	0.6	0.7	5.9	6.7
SSIIIa-Q865 triple wildtype	5	34.1±6.3[a]	22.3	7.4	4.9	0.08	0.4	0.5	5.8	6.3
% of wild-type		100.4	92.3	121.9	102.7	249.1	149.5	144.1	103.0	106.3
ssIIIa-W931 triple null	5	33.9±4.7[a]	19.1	8.1	4.7	0.3	0.6	0.6	5.7	6.3
SSIIIa-W931 triple wildtype	5	38.0±4.2[a]	23.9	7.8	5.2	0.1	0.4	0.5	5.9	6.4
% of wild-type		89.0	79.7	104.1	88.8	286.6	143.4	105.3	97.4	98.0
ssIIa triple null	1	34.8	17.5	7.7	4.3	0.6	0.6	0.9		6.8
ssIIIa-W931 triple null	1	39.4	24.2	8.6	5.5	0.3	0.6	0.4	6.6	7.0
ssIIa-ssIIIa double triple null	11	36.7±3.1[a]	19.5	8.5	4.8	0.6	0.8	1.7	7.9	9.6
SSIIa-SSIIIa double wildtype	5	39.8±4.1[a]	26.7	7.6	5.5	0.1	0.3	0.5	6.0	6.5

EXAMPLE 18. Further double triple null mutant lines

[0464] Another *ssIIa* triple null line (SS047, Examples 4 and 5, see Table 6) was identified with high fibre, including ß-glucan and fructan contents, and was therefore selected for further crosses to generate alternative double triple null mutant lines for high fibre wheat. In analogous fashion to the crossing program using SS028 as described above, crosses were carried out between SS047 and triple null *ssIIIa* plants. F1 seeds were obtained and were sown to produce F1 plants. Progeny plants and grain are identified which are double triple null mutant (*ssIIa-ssIIIa*), and are shown to have increased levels of grain components such as one or more of starch content, amylose content, RS, ß-glucan, fructan, TDF and total fibre contents. Amylose content in some progeny grain is about 60% of the starch content of the grain.

EXAMPLE 19. Field-grown *ssIIa-ssIIIa* null mutants having some wild-type alleles

[0465] Five to seven grams of F5 seeds of selected lines from the breeding populations (Example 16) were grown in single rows in the field at CSIRO Ginninderra Experiment Station. These included 11 *ssIIa-ssIIIa* double triple null mutant lines and 5 wild-type lines for both genes, 8 lines which were *ssIIa* triple null mutant with one or two homozygous null alleles of *ssIIIa* genes, and 7 lines containing *ssIIIa* triple null mutations and one or two homozygous null alleles for the *ssIIa* gene. The grain composition and grain yield are analysed as described above and compared to the double triple null mutant lines.

EXAMPLE 20. Grain properties of double triple mutants for *SSIIa* and *SSIIIa* genes

[0466] Grain samples from five double triple mutant lines for *SSIIa* and *SSIIIa* genes (WDTM-6, 12, 16, 23, and 25) i.e. triple null mutant *ssIIa* and triple null *ssIIIa*, two wild-type segregant lines (WDTM-27, 28) and two wild-type cultivars (Sunco and EGA Hume) grown in 2018 were analysed for several grain properties. The results showed that the grain from double triple mutants contained 10.3% to 13.4% moisture, had a hardness index value in the range 69 to 78, contained 13.6% to 15.0% protein and provided a 54.8% to 88.2% milling yield. While the grain of the wild-type lines and cultivars contained 9.8% to 11.1% moisture, had hardness index value at 71 to 84, contained 11.9% to 12.8% protein and provided 52.5% to 75.8% milling yield (Table 14). Statistical analyses showed that there

were no statistically significantly different in moisture content, grain hardness index and milling yield between grains from double triple mutants and that from wildtype lines and cultivars (Table 15). However, grains from double triple mutants contained statistically significantly more protein and wet gluten comparing to that from wildtype lines and cultivars (Table 15).

EXAMPLE 21. Noodle properties of double triple mutants for SSIIa and SSIIIa genes

[0467] Grain samples from the double triple mutant lines and the wild-type lines and cultivars were milled to produce flour and the flour used to make noodles, of the yellow alkaline noodle (YAN) type, to test the noodle properties. Each flour sample was mixed with water and a solution of sodium carbonate in the following proportions by weight: flour, 100; water, 32; and 10% (w/v) Na_2CO_3 aqueous solution, 1. YAN assessment was undertaken at small scale involving the formulation listed below with noodle crumb being mixed on a 10 g Mixograph mixer (National TMCO, Lincoln, NE, USA) and the noodle sheets developed using a custom small scale noodle sheeter based upon the Ohtake noodle machine (OHTAKE Noodle Machine MFG Co. Ltd., Tokyo, Japan). Tristimulus colour (CIE L*, a*, b*) was assessed on uncooked noodle sheets 30 min and 24 hours after sheeting using a Konica Minolta CR-400 Chroma Meter. The firmness of cooked and uncooked noodle strands were assessed with a Cooked Pasta Quality/Firmness Rig (A/LKBF) probe attached to a Stable Micro Systems TA.XT2 (Stable Micro Systems, Godalming, UK) universal testing instrument with a 50 N load cell. The TA.XT2 was used to measure the force necessary to simultaneously cut 5 noodle strands as per AACC Method 66.50.01 with noodle firmness defined as the maximum cutting force measured. Greater firmness values indicate a stronger and firmer noodle, which is desirable in certain markets.

[0468] The results showed that the YAN noodles made from the double triple mutant grain samples had Brightness (L*) values from 72 to 80 and Yellowness (b*) values from 27 to 33 for uncooked noodles at 30 minutes after cutting, and colour stability (ΔL^*) values from 5.4 to 8.5 at 24 hours after cutting (Table 16). The cutting force (g) values of double triple mutants were from 800 to 3200 for uncooked noodles and 700 to 2100 for cooked noodles (Table 16). The noodles from wild-type controls and cultivars had

Brightness (L*) values from 78 to 80 and Yellowness (b*) values from 29.5 to 38 for uncooked noodles at 30 minutes after cutting, and colour stability (ΔL*) values from 2.7 to 5.8 at 24 hours after cutting (Table 16). The cutting force (g) values of wildtype lines and cultivars were from 700 to 3400 for uncooked noodles and 800 to 2550 for cooked noodles (Table 16). Statistical analyses showed that there were no statistically significantly different in brightness (L*) values and Yellowness (b*) values for uncooked noodles at 30 minutes after cutting, as well as in cutting force for both uncooked and cooked noodles between noodles made from double triple mutants and wildtype controls and cultivars (Table 15). However, colour stability (ΔL*) values at 24 hours after cutting for noodles made from the double triple mutants were statistically significantly higher than that from grains from wildtype lines and cultivars (Table 15), which indicated that noodle brightness of double triple mutants significantly reduced their brightness values at 24 hours after cutting.

Table 14. Grain properties of double triple mutants for SSIIa and SSIIIa and their wild-type controls and cultivars

Line name	Genotype	Moisture Content (%) (NIR)	Hardness Index (SKCS)	Protein Content (%) (NIR)	Wet Gluten content (%)(NIR)	Mean mill yield (%)
WDTM-6	double triple mutant	12.9	69	15.0	33.9	83.4
WDTM-12	double triple mutant	10.3	69	14.5	32.5	79.9
WDTM-16	double triple mutant	13.4	77	14.2	32.6	88.2
WDTM-23	double triple mutant	10.6	74	14.3	30.9	70.0
WDTM-25	double triple mutant	10.6	78	13.6	28.6	54.8
WDTM-27	double wild-type	9.8	76	12.3	28.3	52.5
WDTM-28	double wild-type	10.6	81	12.8	29.7	75.8
Sunco	double wild-type	11.1	84	12.4	28.8	67.1
EGA Hume	double wild-type	10.7	71	11.9	29.5	57.3

Note: Double triple mutant: null mutations for all three genomes for each of SSIIa and SSIIIa genes. Double wild-type: wild-type for all three genomes for each of SSIIa and SSIIIa genes.

Table 15. Statistical analyses of grain and noodle properties of double triple mutants for SSIIa and SSIIIa and their wild-type controls and cultivars

Genotype	Moisture Content (%) (NIR)	Hardness Index (SKCS)	Protein Content (%) (NIR)	Wet Gluten content (%)(NIR)	mean mill yield (%)	Brightness (L*) 30 min uncooked	Yellowness (b*) 30 min uncooked	Colour stability (ΔL*) uncooked	Cutting force (g) cooked	Cutting force (g) uncooked
double triple mutant	11.56[a]	73.4 [a]	14.3 [a]	31.7 [a]	75.3 [a]	77 [a]	29.6 [a]	7.1 [a]	1570 [a]	1940 [a]
double wildtype	10.55 [a]	78 [a]	12.4 [b]	29.1 [b]	63.2 [a]	78.9 [a]	33.6 [a]	3.9 [b]	1337.5 [a]	2025 [a]
LSD ($p<0.05$)	1.8	7.8	0.7	2.5	19.2	3.8	5.2	2	1192.3	1804.1

The letter (a and b) besides of numbers are based on the LSD; mean values with the same letter are not statistically significantly different, and those with different letters ae statistically significantly different at $p<0.05$.

Table 16. Noodle properties of double triple mutants for SSIIa and SSIIIa and their wild-type controls and cultivars

Line name	Genotype	Brightness (L*) 30 min uncooked	Yellowness (b*) 30 min uncooked	Colour stability (ΔL*) uncooked	Cutting force (g) cooked	Cutting force (g) uncooked
WDTM-6	double triple mutant	72	33	7.8	2100	3200
WDTM-12	double triple mutant	77	30	8.5	2100	1000
WDTM-16	double triple mutant	77	31	6.2	950	1950
WDTM-23	double triple mutant	80	27	5.4	2000	2750
WDTM-25	double triple mutant	79	27	7.4	700	800
WDTM-27	double wild-type	79.5	36	2.7	2550	2700
WDTM-28	double wild-type	78	38	3.8	800	1300
Sunco	double wild-type	78	31	3.4	800	3400
EGA Hume	double wild-type	80	29.5	5.8	1200	700

Note: Double triple mutant: null mutations for all three genomes for each of SSIIa and SSIIIa genes.

Double wild-type: wildtype for all three genomes for each of SSIIa and SSIIIa genes.

General Discussion

Triple null mutation of ssIIIa genes maintained the seed weight and increased the RS content and dietary fibre content in wild-type SSIIa genetic background

[0469] Two *ssIIIa* triple null mutants were obtained by combining single null ssIIIa mutations in the A, B and D genomes. Comparing to the wild-type plants and grain, *ssIIIa* triple null plants produced grain of about the same weight but having a reduced starch content. However, the mutant grain contained more amylose, RS, ß-glucan, fructan, TDF and total fibre than the wild-type grain. These results indicated that the homozygous, null mutations of the wheat *SSIIIa* genes in each of the three genomes had little impact on the grain size but had significant, favourable changes on the grain composition by reducing the synthesis of amylopectin, and by increasing other dietary fibre contents including RS, ß-glucan and fructan. The results were also demonstrated when the fiber contents were calculated on a per caryopsis basis, except for the TDF and total fibre contents.

Triple null mutation of ssIIIa gene maintained the seed weight and increased the RS content and dietary fibre content in ssIIa triple null genetic background

[0470] The *ssIIa-ssIIIa* double triple null mutants were obtained by combining the three *ssIIIa* null mutations with the three *ssIIa* null mutations to see whether the high RS and high fibre wheat lines with commercially useful seed weight could be obtained. Compared to the *ssIIa* triple null mutant plants and grain, homozygous *ssIIa-ssIIIa* double triple null plants in some instances produced grain having increased average weight and starch content. Surprisingly, the *ssIIa-ssIIIa* double triple null mutant grain contained more ß-glucan, fructan and total fibre than the *ssIIa* triple null mutant grain, while the synthesis of amylose and amylopectin remained mostly unchanged, comparing the *ssIIa-ssIIIa* double triple null mutant grain with the wild-type grain. Three out of 11 *ssIIa-ssIIIa* double triple null mutant lines contained about the same RS content as the *ssIIa* triple null grain. These results indicated that the double triple null mutations had significant impacts on increasing the grain weight and improving the grain composition by increasing dietary fibre contents including ß-glucan, fructan and total fibre, while maintaining the RS content. These

results were also observed when the fibre contents were calculated on a per caryopsis basis.

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Claims

1. Wheat grain of the species *Triticum aestivum*, the grain comprising

   i) mutations in each of its *SSIIa* genes and each of its *SSIIIa* genes such that the grain is homozygous for a null mutation in its *SSIIa-A* gene, homozygous for a null mutation in its *SSIIa-B* gene, homozygous for a null mutation in its *SSIIa-D* gene, homozygous for a null mutation in its *SSIIIa-A* gene, homozygous for a null mutation in its *SSIIIa-B* gene and homozygous for a null mutation in its *SSIIIa-D* gene,

   ii) a total starch content comprising an amylose content and an amylopectin content,

   iii) a fructan content which is increased relative to wild-type wheat grain on a weight basis, preferably between 3% and 12% of the grain weight,

   iv) a β-glucan content,

   v) an arabinoxylan content,

   vi) a cellulose content,

the grain having a grain weight of between 25mg and 60mg, wherein the amylose content is between 45% and 70% on a weight basis of the total starch content of the grain as determined by iodine binding assay, wherein the amylopectin content on a weight basis is reduced relative to the wild-type wheat grain, wherein each of the β-glucan content, arabinoxylan content and cellulose content are increased relative to the wild-type wheat grain on a weight basis, whereby the sum of the fructan content, β-glucan content, arabinoxylan content and cellulose content is between 15% and 30% of the grain weight, wherein the wheat grain further comprises one or more or all of the characteristics selected from the group consisting of an increased total fibre content, an increased total dietary fibre (TDF) content, the fructan content is increased, the ß-glucan content is increased, the amylose content is increased and an increased starch content, each increase being relative to wheat grain which is homozygous for the null mutation in its *SSIIa-A* gene, homozygous for the null mutation in its *SSIIa-B* gene, homozygous for the null mutation in its *SSIIa-D* gene, homozygous wild-type for the *SSIIIa-A* gene, homozygous wild-type for the *SSIIIa-B* gene and homozygous wild-type for the *SSIIIa-D* gene.

2. The wheat grain of claim 1 which is further characterised by one or more or all of the features:

i) a starch content of between 30% and 70% of the grain weight,

ii) the amylose content is between 45% and 65% of the total starch content of the grain as determined by iodine binding assay,

iii) the starch content has a chain length distribution as determined by fluorescence-activated capillary electrophoresis (FACE) after debranching of the starch samples which is increased in the proportion of chain lengths of DP 7-10 and decreased in the proportion of chain lengths DP 11-24, relative to wild-type wheat starch,

iv) the fructan content comprises fructan of DP 3-12 such that at least 50% of the fructan content is of DP 3-12,

v) the fructan content is increased by between 2-fold and 10-fold relative to the wild-type wheat grain on a weight basis,

vi) the β-glucan content is increased by 1% or by 2% on an absolute basis, and/or is increased by between 2-fold and 7-fold relative to the wild-type wheat grain on a weight basis,

vii) the β-glucan content is between 1% and 4% of the grain weight,

viii) the arabinoxylan content is increased by between 1% and 5% on an absolute basis,

ix) the cellulose content is increased by between 1% and 5% on an absolute basis,

x) the grain has a germination rate which is between about 70% and about 100% relative to the wild-type wheat grain,

xi) the grain, when sown, gives rise to wheat plants which are male and female fertile.

3. The grain of claim 1 or claim 2, wherein the grain lacks one or more or all of SSIIa-A protein, SSIIa-B protein and SSIIa-D protein.

4. The grain of any one of claims 1 to 3, wherein the grain comprises one, two or three null mutations in *SSIIIa* genes which are premature translation stop codons.

5. The grain of any one of claims 1 to 4, wherein each null mutation is selected, independently, from the group consisting of a deletion mutation, an insertion mutation, a premature translation stop codon, a splice site mutation and a non-conservative amino acid substitution mutation, preferably wherein the grain comprises deletion mutations in each of two or three *SSIIa* genes.

6. The grain of any one of claims 1 to 5, which further comprises a loss of function mutation in an endogenous gene which encodes a starch synthesis polypeptide, or a chimeric polynucleotide which encodes an RNA which reduces the expression of the endogenous gene which encodes the starch synthesis polypeptide, said starch synthesis polypeptide being selected from the group consisting of SSI and SSIV, wherein said mutation is selected from the group consisting of a deletion mutation, an insertion mutation, a premature translation stop codon, a splice site mutation and a non-conservative amino acid substitution mutation.

7. The grain of any one of claims 1 to 6, which comprises one, two or three null mutations in SSIIIa genes which are deletion mutations.

8. The grain of any one claims 1 to 7 wherein at least one, more than one, or all of the mutations are i) introduced mutations, ii) were induced in a parental wheat plant or seed by mutagenesis with a mutagenic agent such as a chemical agent, biological agent or irradiation, or iii) were introduced in order to modify the plant genome.

9. The grain of any one of claims 1 to 8 having an amylose content of about 60% on a weight basis of the total starch content of the grain.

10. The grain of any one of claims 1 to 9 which is non-transgenic or is free of any exogenous nucleic acid that encodes an RNA which reduces expression of a *SSIIa* gene and/or a *SBEIIa* gene.

11. The grain of any one of claims 3 to 10, wherein the SSIIa or SSIIIa level and/or activity is determined by assaying the SSIIa or SSIIIa level and/or activity in developing endosperm, or by assaying the amount of SSIIa or SSIIIa protein in harvested grain by immunological or other means.

12. The grain of any one of claims 1 to 11, wherein the starch granules of the grain and/or the starch of the grain is characterised by one or more of properties selected from the group consisting of:

i) comprising at least 2% resistant starch;

ii) the starch characterised by a reduced glycaemic index (GI);

iii) the starch granules being distorted in shape;

iv) the starch granules having reduced birefringence when observed under polarized light;

v) the starch characterized by a reduced swelling volume;

vi) modified chain length distribution and/or branching frequency in the starch;

vii) the starch characterized by a reduced peak temperature of gelatinisation;

viii) the starch characterized by a reduced peak viscosity;

ix) reduced starch pasting temperature;

x) reduced peak molecular weight of amylose as determined by size exclusion chromatography;

xi) reduced starch crystallinity; and

xii) reduced proportion of A-type and/or B-type starch, and/or increased proportion of V-type crystalline starch;

each property being relative to wild-type wheat starch granules or wild-type wheat starch.

13. The grain of any one of claims 1 to 12 which is mature, harvested grain.

14. The grain of any one of claims 1 to 12 which is processed so that it is no longer capable of germinating, such as heat-treated grain, or kibbled, cracked, par-boiled, rolled, pearled, milled or ground grain.

15. The grain of any one of claims 1 to 12 which is comprised in a wheat plant.

16. A wheat plant which produces, or is obtained from, the grain of any one of claims 1 to 13.

17. The wheat plant of claim 16 which lacks one or more or all of SSIIa-A protein, SSIIa-B protein, SSIIa-D protein, SSIIIa-A protein, SSIIIa-B protein and SSIIIa-D protein.

18. The wheat plant of claim 16 or claim 17 which is male and female fertile.

19. Flour produced from the grain of any one of claims 1 to 14, which is preferably wholemeal, or wheat bran produced from the grain of any one of claims 1 to 14.

20. Wheat starch granules or wheat starch produced from the grain of any one of claims 1 to 14.

21. The wheat starch granules or wheat starch of claim 20 comprising 45%, preferably about 50%, about 55% or about 60% amylose, or between 45% and 70% amylose, each on a weight basis as a proportion of the total starch content of the starch granules or starch, the starch granules preferably comprising wheat GBSSI polypeptide, and wherein the starch granules and/or starch is characterised by one or more of:
    a. having no detectable SSIIa polypeptide or SSIIIa polypeptide, or both, as determined by an immunological means;
    b. comprising at least 2% resistant starch on a weight basis;
    c. the starch characterised by a reduced glycaemic index (GI);
    d. the starch granules being distorted in shape;
    e. the starch granules having reduced birefringence when observed under polarized light;
    f. the starch characterized by a reduced swelling volume;
    g. modified chain length distribution and/or branching frequency in the starch;
    h. the starch characterized by a reduced peak temperature of gelatinisation;
    i. the starch characterized by a reduced peak viscosity;
    j. reduced starch pasting temperature;
    k. reduced peak molecular weight of amylose as determined by size exclusion chromatography;

l. reduced starch crystallinity; and

m. reduced proportion of A-type and/or B-type starch, and/or increased proportion of V-type crystalline starch;

each property being relative to wild-type wheat starch granules or starch.

22. A food ingredient that comprises the grain of any one of claims 1 to 14, the flour, preferably the wholemeal, or wheat bran of claim 19, or the wheat starch granules or wheat starch of claim 20 or 21, preferably at a level of at least 10% on a dry weight basis.

23. The food ingredient of claim 22 wherein the food ingredient is kibbled, cracked, par-boiled, rolled, pearled, milled or ground grain or any combination of these.

24. A food product comprising a food ingredient at a level of at least 10% on a dry weight basis, wherein the food ingredient is wheat grain of any one of claims 1 to 14, the flour, preferably the wholemeal, or wheat bran of claim 19, or the wheat starch granules or wheat starch of claim 20 or 21.

25. A composition comprising the wheat grain of any one of claims 1 to 14, the flour, preferably the wholemeal, or wheat bran of claim 19, or the wheat starch granules or wheat starch of claim 20 or 21, at a level of at least 10% by weight, and wheat grain having a level of amylose lower than 45% (w/w) or flour, wholemeal, starch granules or starch obtained therefrom.

26. A process for producing wheat grain according to any one of claims 1 to 12, the process comprising harvesting wheat grain from a wheat plant according to any one of claims 16 to 18, and optionally processing the grain to produce a food ingredient according to claim 22 or claim 23.

27. A process for producing a wheat plant that produces grain according to any one of claims 1 to 13, the process comprising step (i) crossing two parental wheat plants each comprising a null mutation in each of one, two or three *SSIIa* genes selected from the group consisting of *SSIIa*-A, *SSIIa*-B and *SSIIa*-D and a null mutation in each of one, two or three *SSIIIa* genes selected from the group consisting of *SSIIIa*-

A, *SSIIIa*-B and *SSIIIa*-D, or of mutagenising a parental plant comprising said null mutations; and step (ii) screening plants or grain obtained from the cross or mutagenesis, or progeny plants or grain obtained therefrom, by analysing DNA, RNA, protein, starch granules or starch from the plants or grain, and step (iii) selecting a fertile wheat plant that has reduced SSIIa activity and reduced SSIIIa activity relative to at least one of the parental wheat plants of step (i).

28. A process for screening wheat grain or a wheat plant, the method comprising (i) determining the amount or activity of SSIIa or SSIIIa or both relative to the amount or activity in wild-type wheat grain or a wild-type wheat plant and selecting grain, or a plant which produces grain, according to any one of claims 1 to 13.

29. A process for producing a food comprising steps of (i) adding a food ingredient according to claim 22 or claim 23 to another food ingredient, and (ii) mixing the food ingredients, thereby producing the food.

30. The process of claim 29 which further comprises a step of processing grain according to any one of claim 1 to 14 to produce the food ingredient, prior to step (i), or a step of heating the mixed food ingredients from step (ii) at a temperature of at least 100°C for at least 10 minutes.

31. A process for improving one or more parameters of metabolic health, bowel health or cardiovascular health in a subject in need thereof, or of preventing or reducing the severity or incidence of a metabolic disease such as diabetes, bowel disease or cardiovascular disease, the method comprising providing to the subject the grain of any one of claim 1 to 14, the food product of claim 24 or the food or drink product produced by the process of claim 29 or claim 30.

32. The food product of claim 24 for use in improving one or more parameters of metabolic health, bowel health or cardiovascular health, or of preventing or reducing the severity or incidence of metabolic, bowel or cardiovascular disease in a subject.

33. A process for producing starch, comprising the steps of i) obtaining wheat grain according to any one of claims 1 to 14, and ii) extracting starch from the grain, thereby producing the starch.

34. A process of producing bins of wheat grain comprising:

a) reaping wheat stalks comprising wheat grain as defined in any one of claims 1 to 12;

b) threshing and/or winnowing the stalks to separate the grain from the chaff; and

c) sifting and/or sorting the grain separated in step b), and loading the sifted and/or sorted grain into bins, thereby producing bins of wheat grain.

FIGURE 1

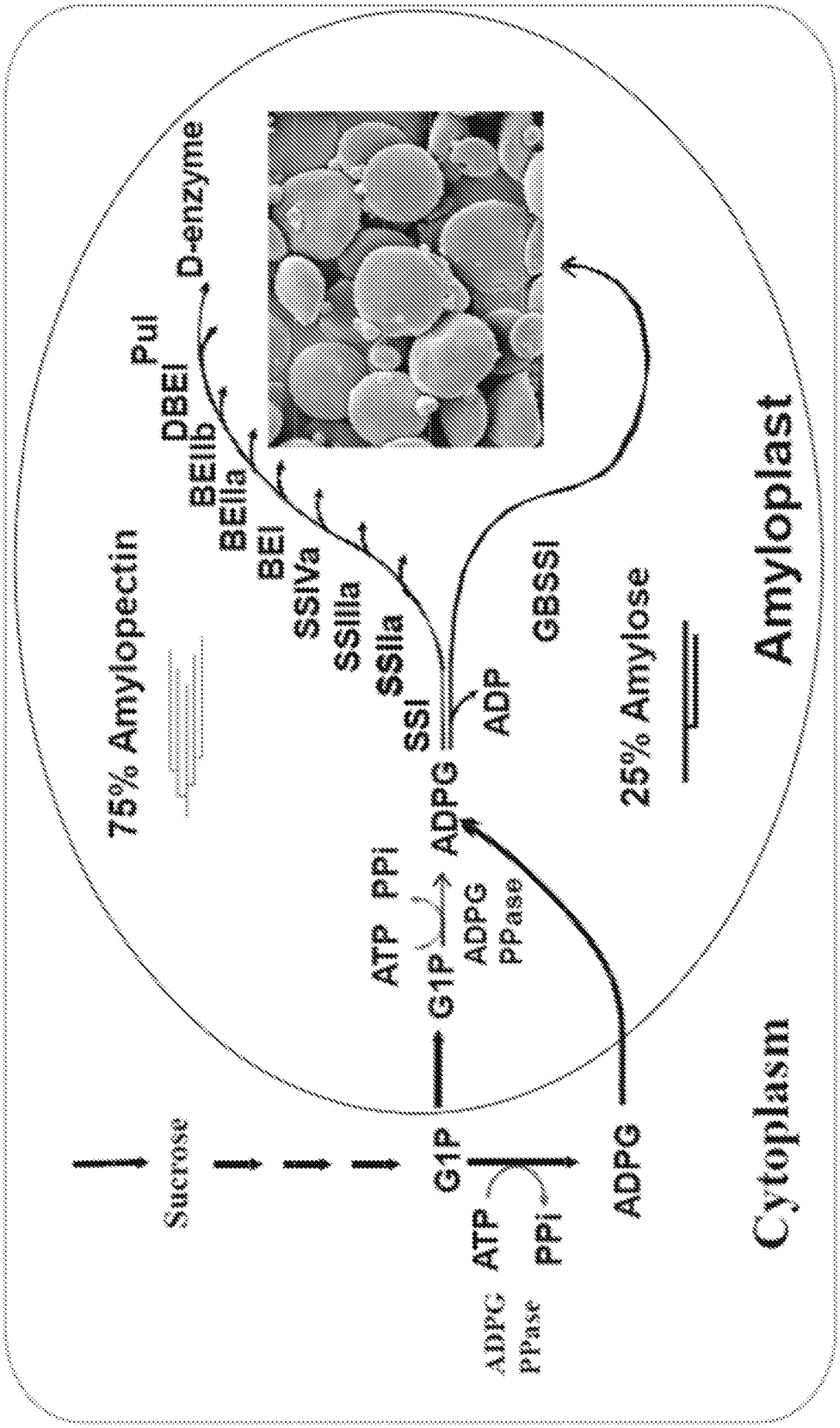

Sucrose
G1P
ATP
PPi
ADPG PPase
ADPG
Cytoplasm
Amyloplast
75% Amylopectin
25% Amylose
ATP PPi
G1P
ADPG PPase
ADPG
ADP
SSI
SSIIIa
SSIIa
SSIVa
BEI
BEIIa
BEIIb
DBEI
Pul
D-enzyme
GBSSI

FIGURE 2

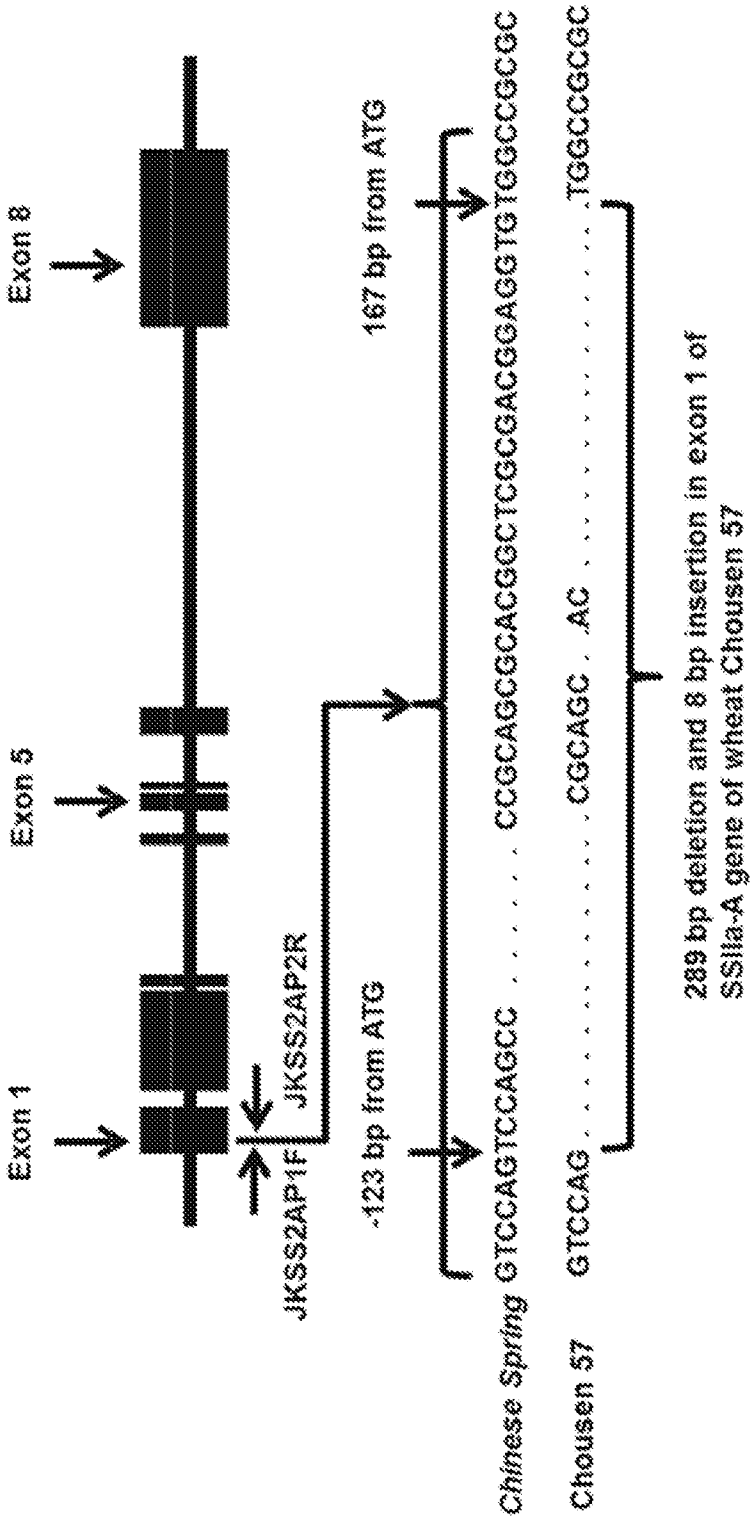

Exon 1
Exon 5
Exon 8
JKSS2AP1F
JKSS2AP2R
-123 bp from ATG
167 bp from ATG
Chinese Spring GTCCAGTCCAGCC . . . . . CCGCAGCGCACGGCTCGCGACGGAGGTGTGGCCGCGC
Chousen 57 GTCCAG . . . . . CGCAGC . AC . . . . . TGGCCGCGC
289 bp deletion and 8 bp insertion in exon 1 of
SSIIa-A gene of wheat Chousen 57

FIGURE 3

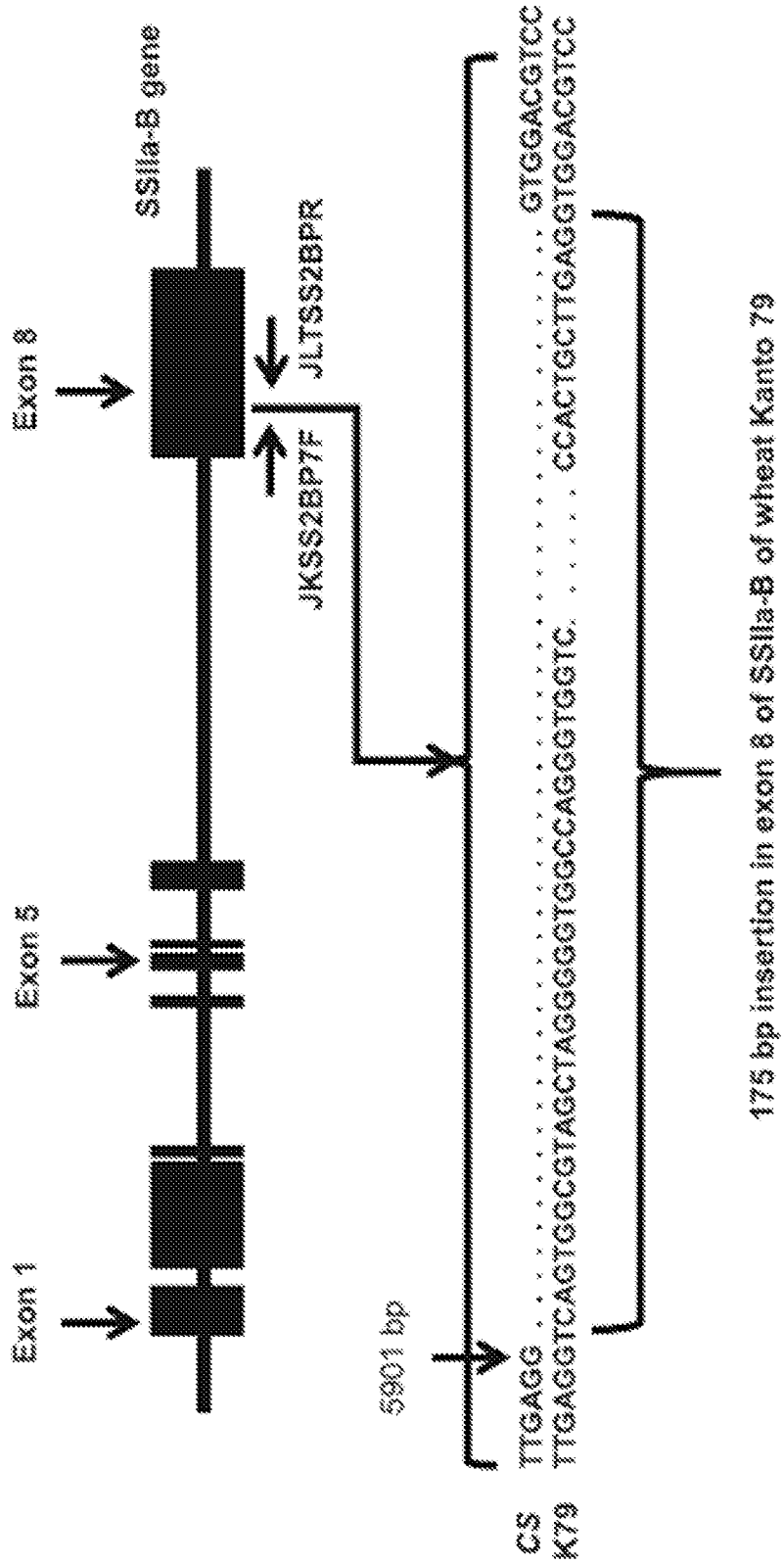

SSIIa-B gene
Exon 8
JLTSS2BPR
JKSS2BP7F
Exon 5
Exon 1
5901 bp
C5 TTGAGG . . . . . GTGGACGTCC
K79 TTGAGGTCAGTGGCGTAGCTAGGGGGTGGCCAGGGTGGTC. . . . . CCACTGCTTGAGGTGGACGTCC
175 bp insertion in exon 8 of SSIIa-B of wheat Kanto 79

FIGURE 4

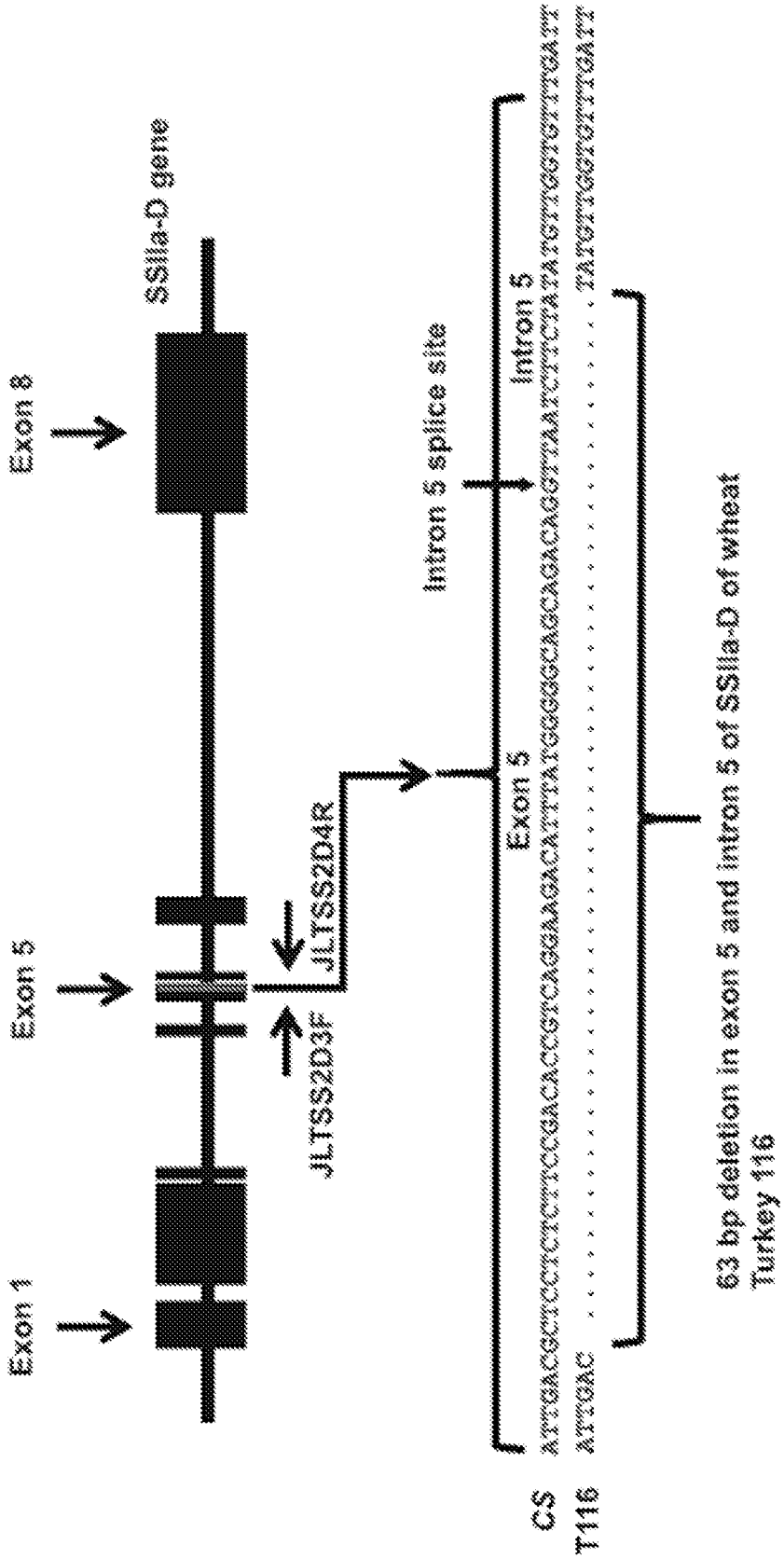

SSIIa-D gene
Exon 8
Exon 5
Exon 1
JLTSS2D3F
JLTSS2D4R
Intron 5 splice site
Intron 5
Exon 5
CS
T116
63 bp deletion in exon 5 and intron 5 of SSIIa-D of wheat
Turkey 116

FIGURE 5

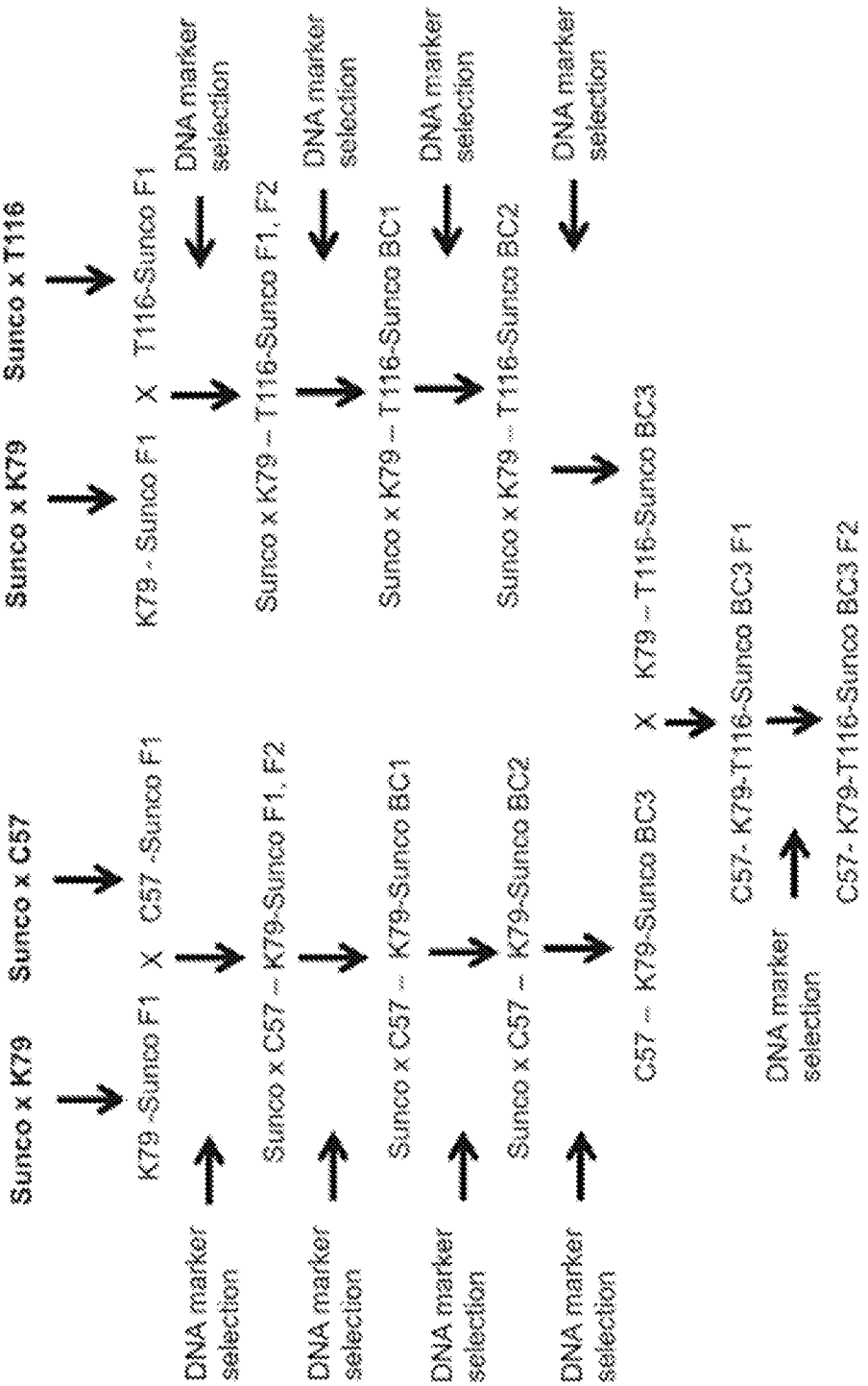

Sunco x K79
Sunco x C57
Sunco x K79
Sunco x T116
K79 -Sunco F1
X
C57 -Sunco F1
K79 - Sunco F1
X
T116-Sunco F1
DNA marker selection
DNA marker selection
DNA marker selection
Sunco x C57 – K79-Sunco F1, F2
Sunco x K79 – T116-Sunco F1, F2
DNA marker selection
DNA marker selection
Sunco x C57 – K79-Sunco BC1
Sunco x K79 – T116-Sunco BC1
DNA marker selection
DNA marker selection
Sunco x C57 – K79-Sunco BC2
Sunco x K79 – T116-Sunco BC2
DNA marker selection
DNA marker selection
C57 – K79-Sunco BC3
X
K79 – T116-Sunco BC3
C57- K79-T116-Sunco BC3 F1
DNA marker selection
C57- K79-T116-Sunco BC3 F2

FIGURE 6

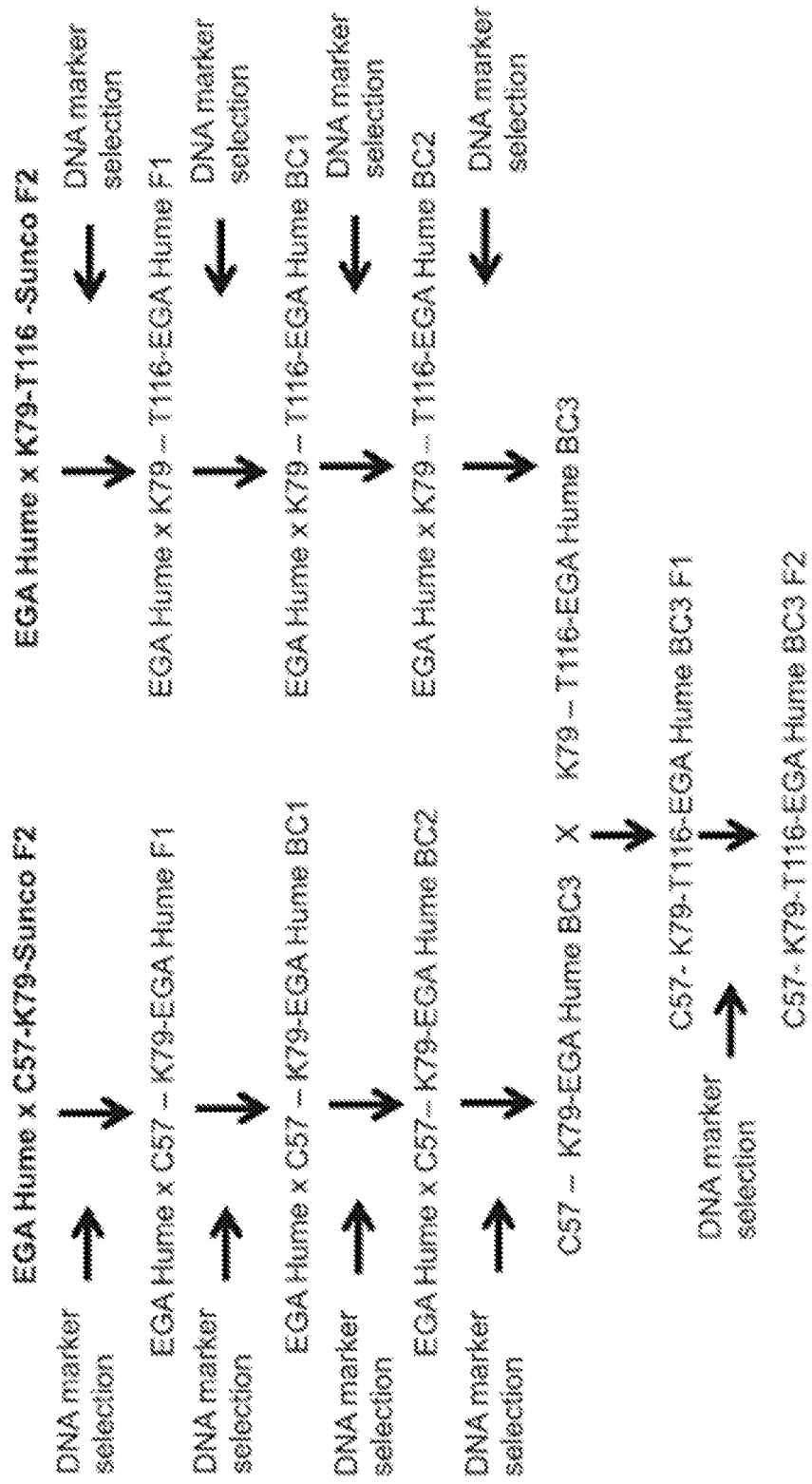

EGA Hume x C57-K79-Sunco F2
DNA marker selection
EGA Hume x C57 – K79-EGA Hume F1
DNA marker selection
EGA Hume x C57 – K79-EGA Hume BC1
DNA marker selection
EGA Hume x C57 – K79-EGA Hume BC2
DNA marker selection
C57 – K79-EGA Hume BC3
X
K79 – T116-EGA Hume BC3
C57- K79-T116-EGA Hume BC3 F1
DNA marker selection
C57- K79-T116-EGA Hume BC3 F2
EGA Hume x K79-T116 -Sunco F2
DNA marker selection
EGA Hume x K79 – T116-EGA Hume F1
DNA marker selection
EGA Hume x K79 – T116-EGA Hume BC1
DNA marker selection
EGA Hume x K79 – T116-EGA Hume BC2
DNA marker selection

FIGURE 7

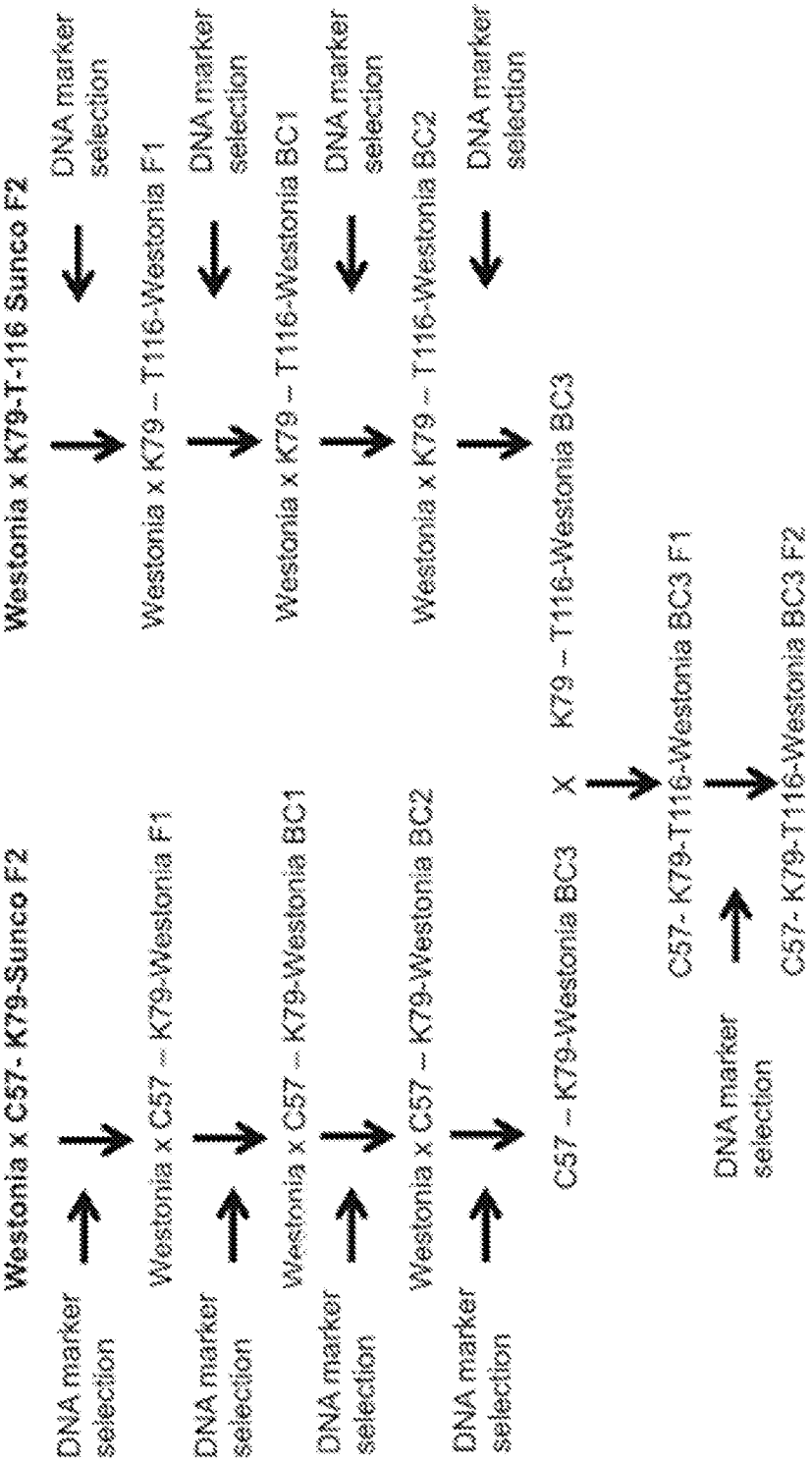

Westonia x C57- K79-Sunco F2
DNA marker selection
Westonia x C57 – K79-Westonia F1
DNA marker selection
Westonia x C57 – K79-Westonia BC1
DNA marker selection
Westonia x C57 – K79-Westonia BC2
DNA marker selection
C57 – K79-Westonia BC3
X
Westonia x K79-T-116 Sunco F2
DNA marker selection
Westonia x K79 – T116-Westonia F1
DNA marker selection
Westonia x K79 – T116-Westonia BC1
DNA marker selection
Westonia x K79 – T116-Westonia BC2
DNA marker selection
K79 – T116-Westonia BC3
C57- K79-T116-Westonia BC3 F1
DNA marker selection
C57- K79-T116-Westonia BC3 F2

FIGURE 8

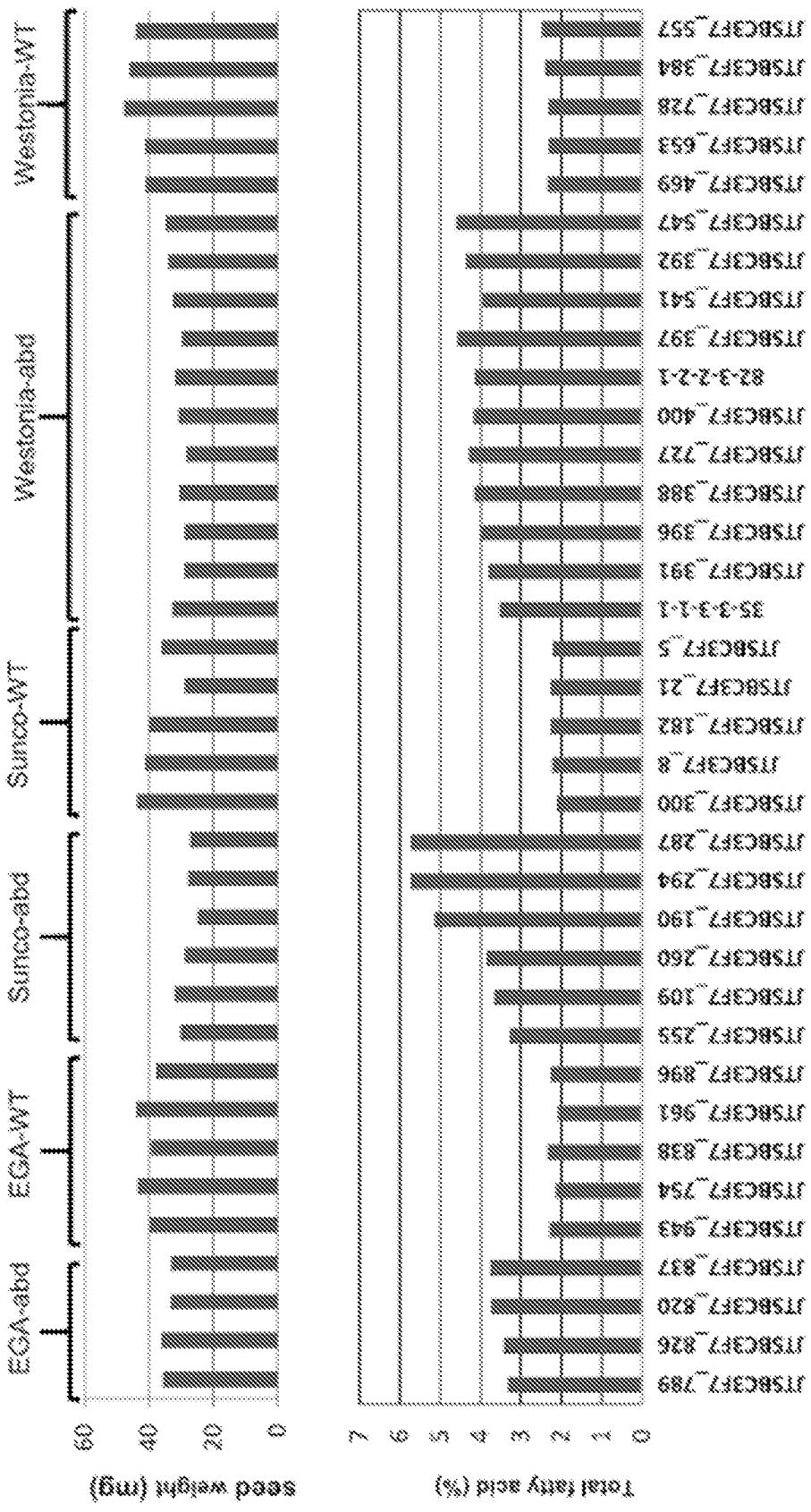

EGA-abd
EGA-WT
Sunco-abd
Sunco-WT
Westonia-abd
Westonia-WT
seed weight (mg)
60
40
20
0
Total fatty acid (%)
7
6
5
4
3
2
1
0
JTSBC3F7_789
JTSBC3F7_826
JTSBC3F7_820
JTSBC3F7_837
JTSBC3F7_943
JTSBC3F7_754
JTSBC3F7_838
JTSBC3F7_961
JTSBC3F7_896
JTSBC3F7_255
JTSBC3F7_109
JTSBC3F7_260
JTSBC3F7_190
JTSBC3F7_294
JTSBC3F7_287
JTSBC3F7_300
JTSBC3F7_8
JTSBC3F7_182
JTSBC3F7_21
JTSBC3F7_5
35-3-3-1-1
JTSBC3F7_391
JTSBC3F7_396
JTSBC3F7_388
JTSBC3F7_727
JTSBC3F7_400
82-3-2-2-1
JTSBC3F7_397
JTSBC3F7_541
JTSBC3F7_392
JTSBC3F7_547
JTSBC3F7_469
JTSBC3F7_653
JTSBC3F7_728
JTSBC3F7_384
JTSBC3F7_557

FIGURE 9

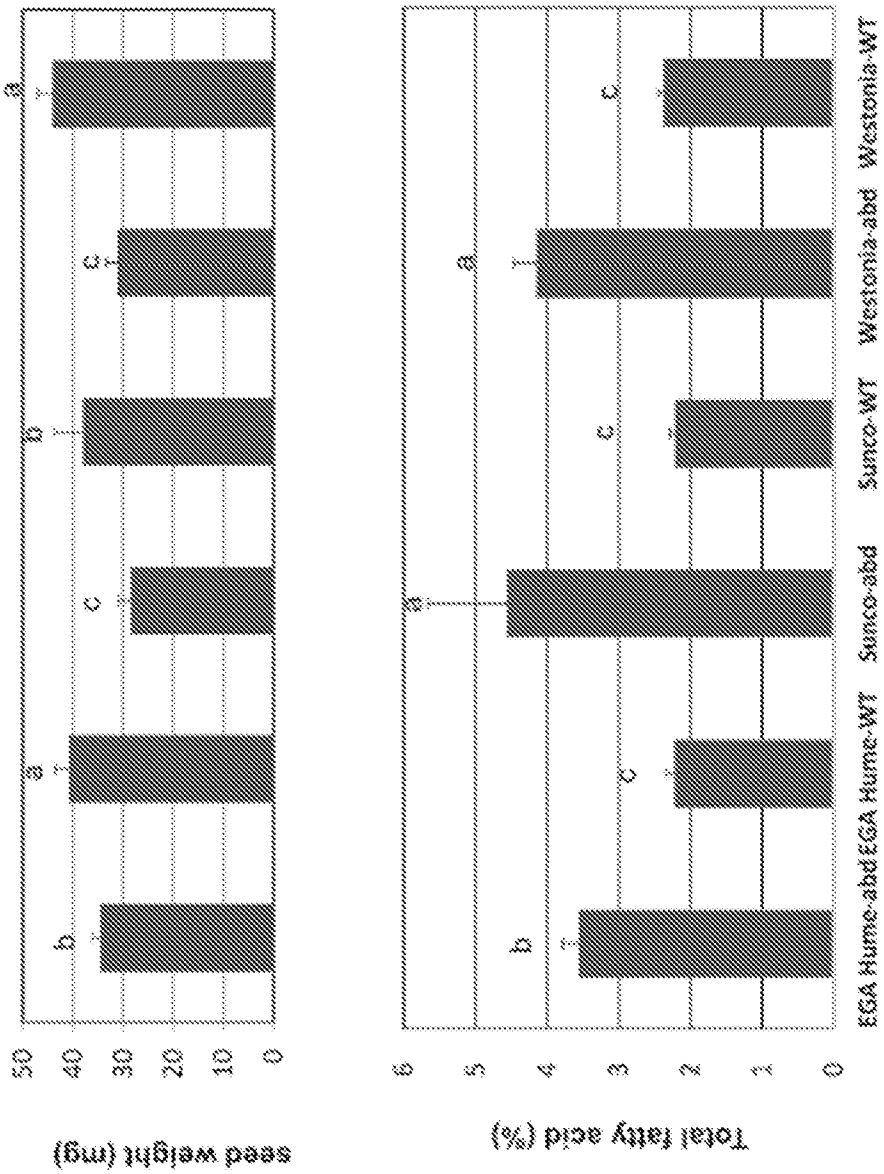

seed weight (mg)
50
40
30
20
10
0
a
b
c
Total fatty acid (%)
6
5
4
3
2
1
0
EGA Hume-abd
EGA Hume-WT
Sunco-abd
Sunco-WT
Westonia-abd
Westonia-WT

FIGURE 10

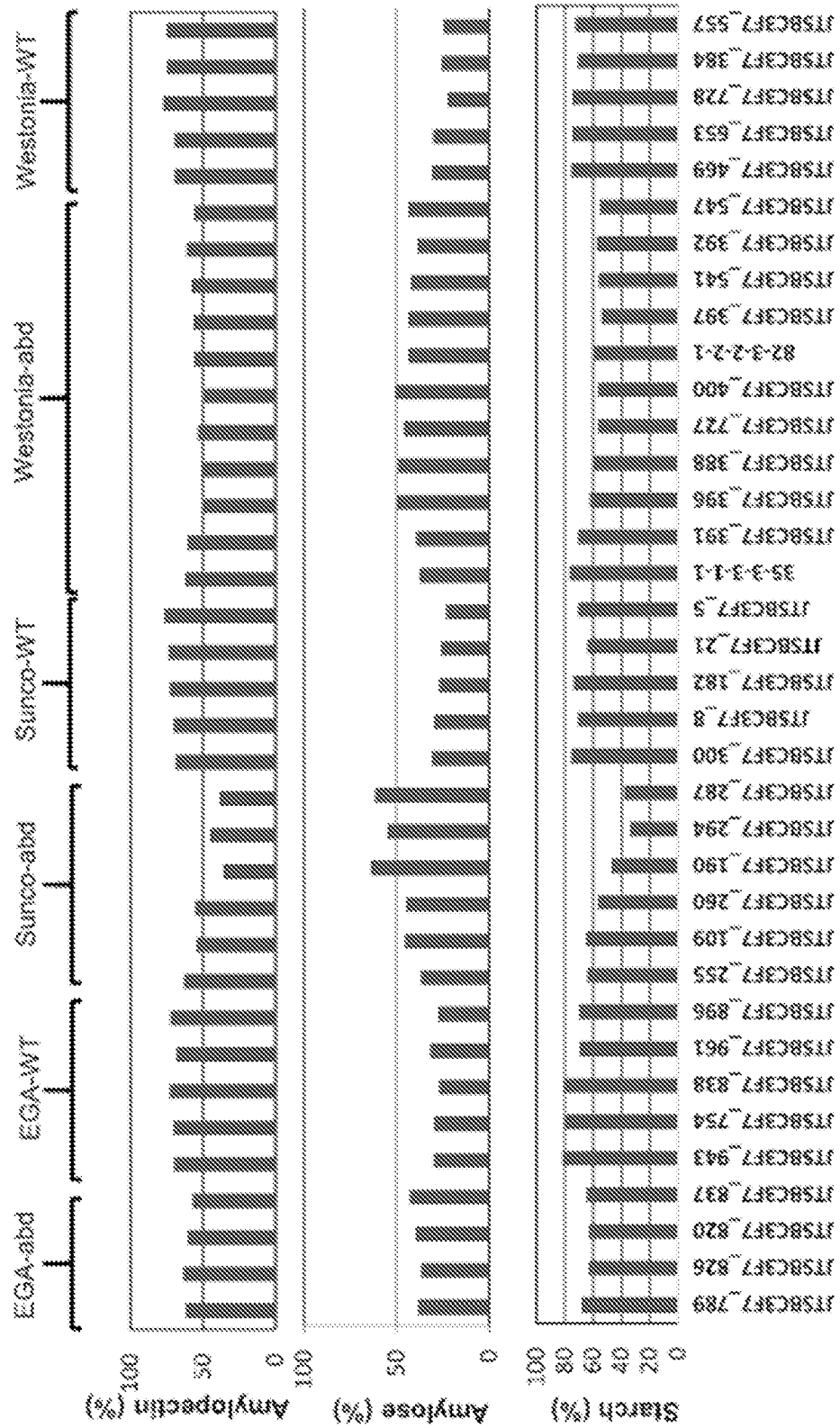

Starch (%)
Amylose (%)
Amylopectin (%)
EGA-abd
EGA-WT
Sunco-abd
Sunco-WT
Westonia-abd
Westonia-WT
JT58C3F7_789
JT58C3F7_826
JT58C3F7_820
JT58C3F7_837
JT58C3F7_943
JT58C3F7_754
JT58C3F7_838
JT58C3F7_961
JT58C3F7_896
JT58C3F7_255
JT58C3F7_109
JT58C3F7_280
JT58C3F7_190
JT58C3F7_294
JT58C3F7_287
JT58C3F7_300
JT58C3F7_8
JT58C3F7_182
JT58C3F7_21
JT58C3F7_5
33-3-3-1-1
JT58C3F7_391
JT58C3F7_396
JT58C3F7_388
JT58C3F7_727
JT58C3F7_400
82-3-2-2-1
JT58C3F7_397
JT58C3F7_341
JT58C3F7_392
JT58C3F7_347
JT58C3F7_469
JT58C3F7_653
JT58C3F7_728
JT58C3F7_384
JT58C3F7_357

FIGURE 11

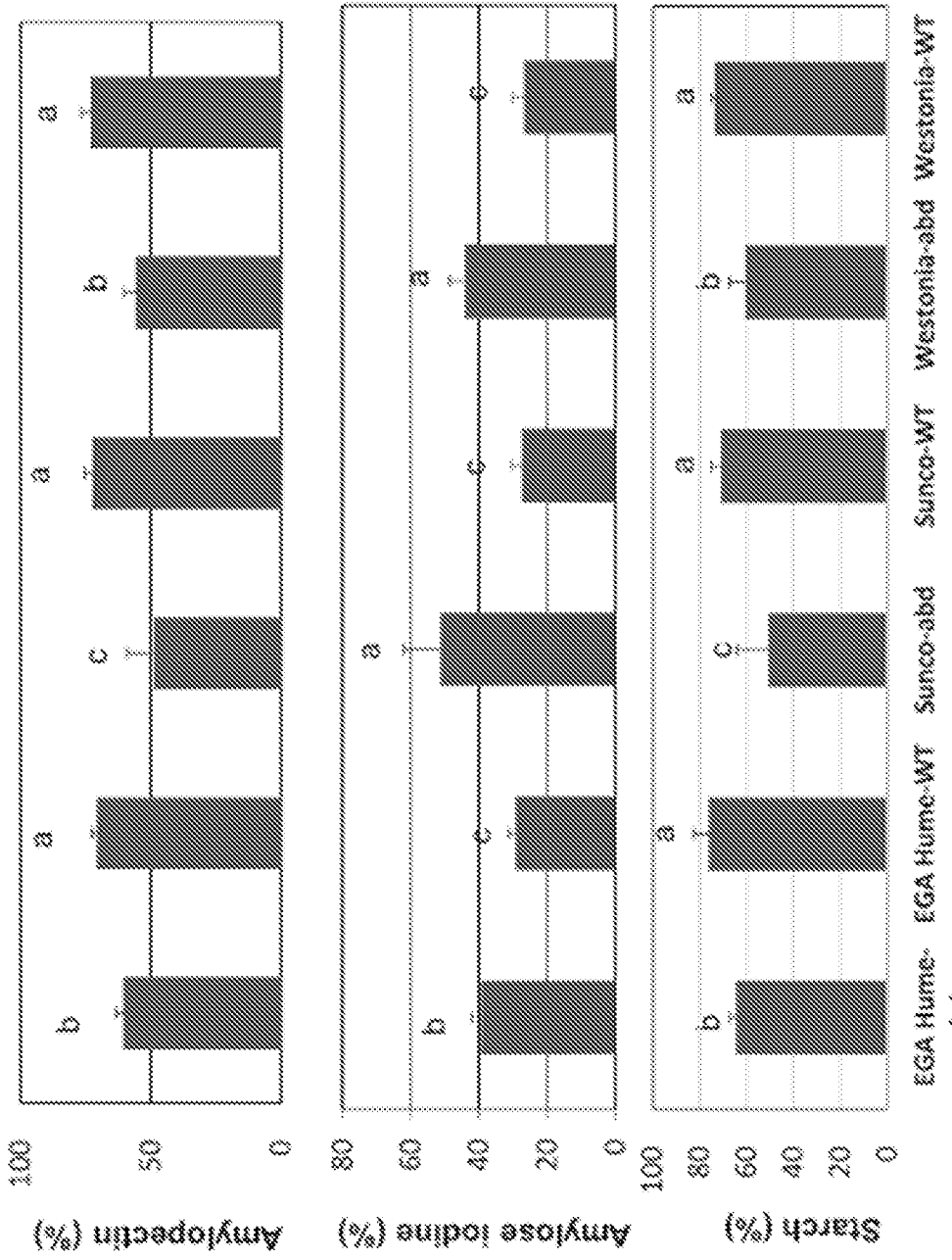

Amylopectin (%)
Amylose Iodine (%)
Starch (%)
EGA Hume-abd
EGA Hume-WT
Sunco-abd
Sunco-WT
Westonia-abd
Westonia-WT

FIGURE 12

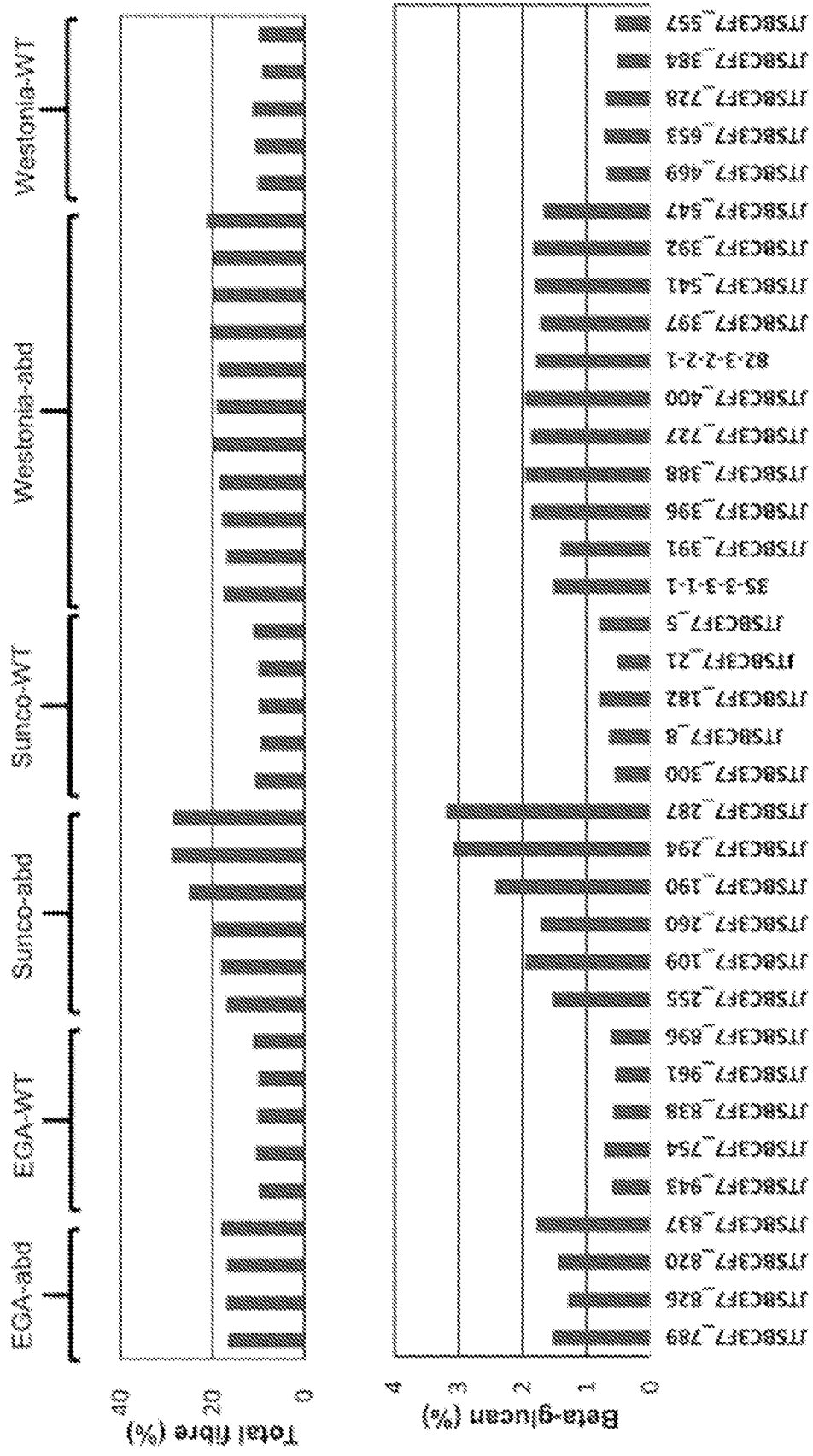

EGA-abd
EGA-WT
Sunco-abd
Sunco-WT
Westonia-abd
Westonia-WT
Total fibre (%)
40
20
0
Beta-glucan (%)
4
3
2
1
0
JTSBC3F7_789
JTSBC3F7_826
JTSBC3F7_820
JTSBC3F7_837
JTSBC3F7_943
JTSBC3F7_754
JTSBC3F7_838
JTSBC3F7_961
JTSBC3F7_896
JTSBC3F7_255
JTSBC3F7_109
JTSBC3F7_260
JTSBC3F7_190
JTSBC3F7_294
JTSBC3F7_287
JTSBC3F7_300
JTSBC3F7_8
JTSBC3F7_182
JTSBC3F7_21
JTSBC3F7_5
35-3-3-1-1
JTSBC3F7_391
JTSBC3F7_396
JTSBC3F7_388
JTSBC3F7_727
JTSBC3F7_400
82-3-3-2-1
JTSBC3F7_397
JTSBC3F7_541
JTSBC3F7_392
JTSBC3F7_547
JTSBC3F7_469
JTSBC3F7_653
JTSBC3F7_728
JTSBC3F7_384
JTSBC3F7_557

FIGURE 13

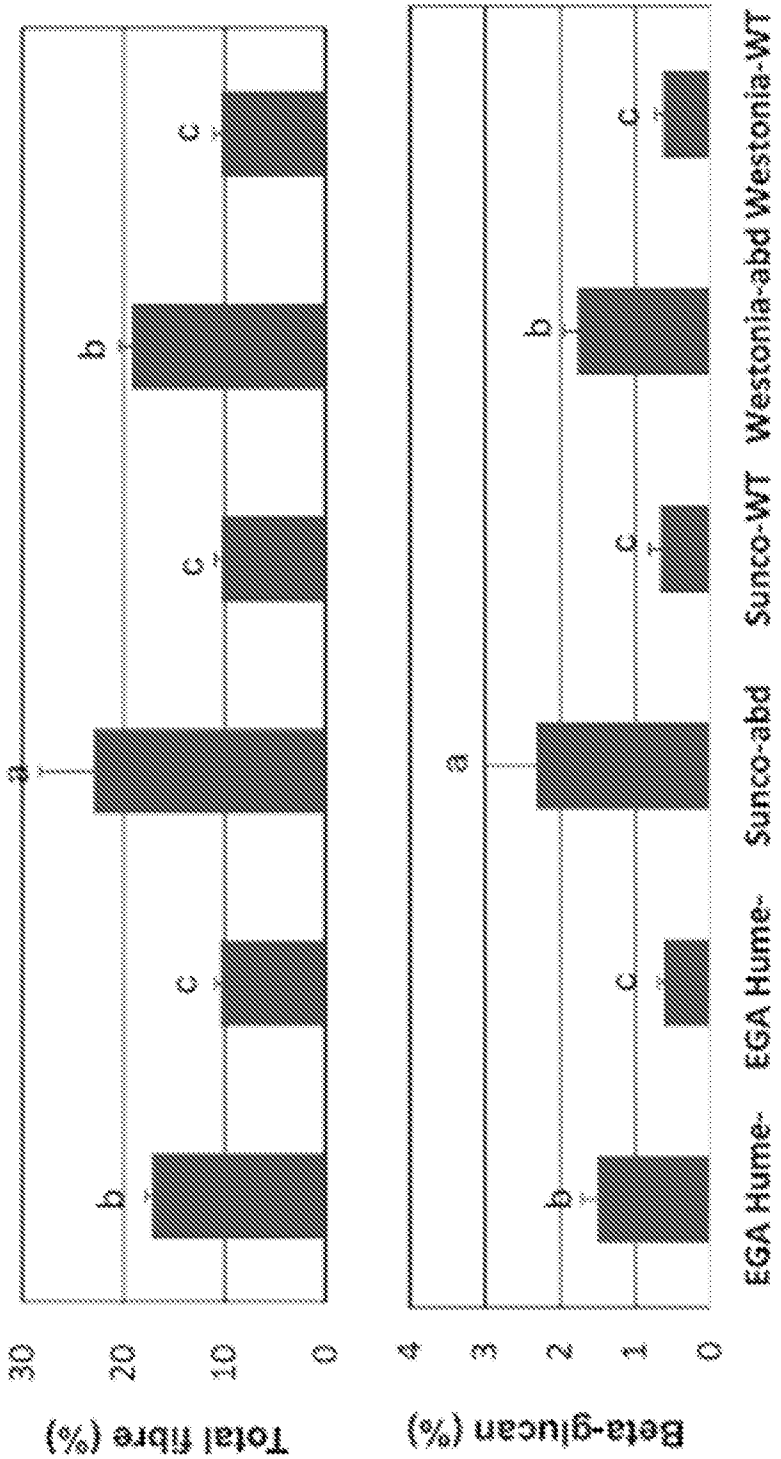

Total fibre (%)
30
20
10
0
a
b
c
b
c
Beta-glucan (%)
4
3
2
1
0
a
b
c
b
c
EGA Hume-abd
EGA Hume-WT
Sunco-abd
Sunco-WT
Westonia-abd
Westonia-WT

FIGURE 14

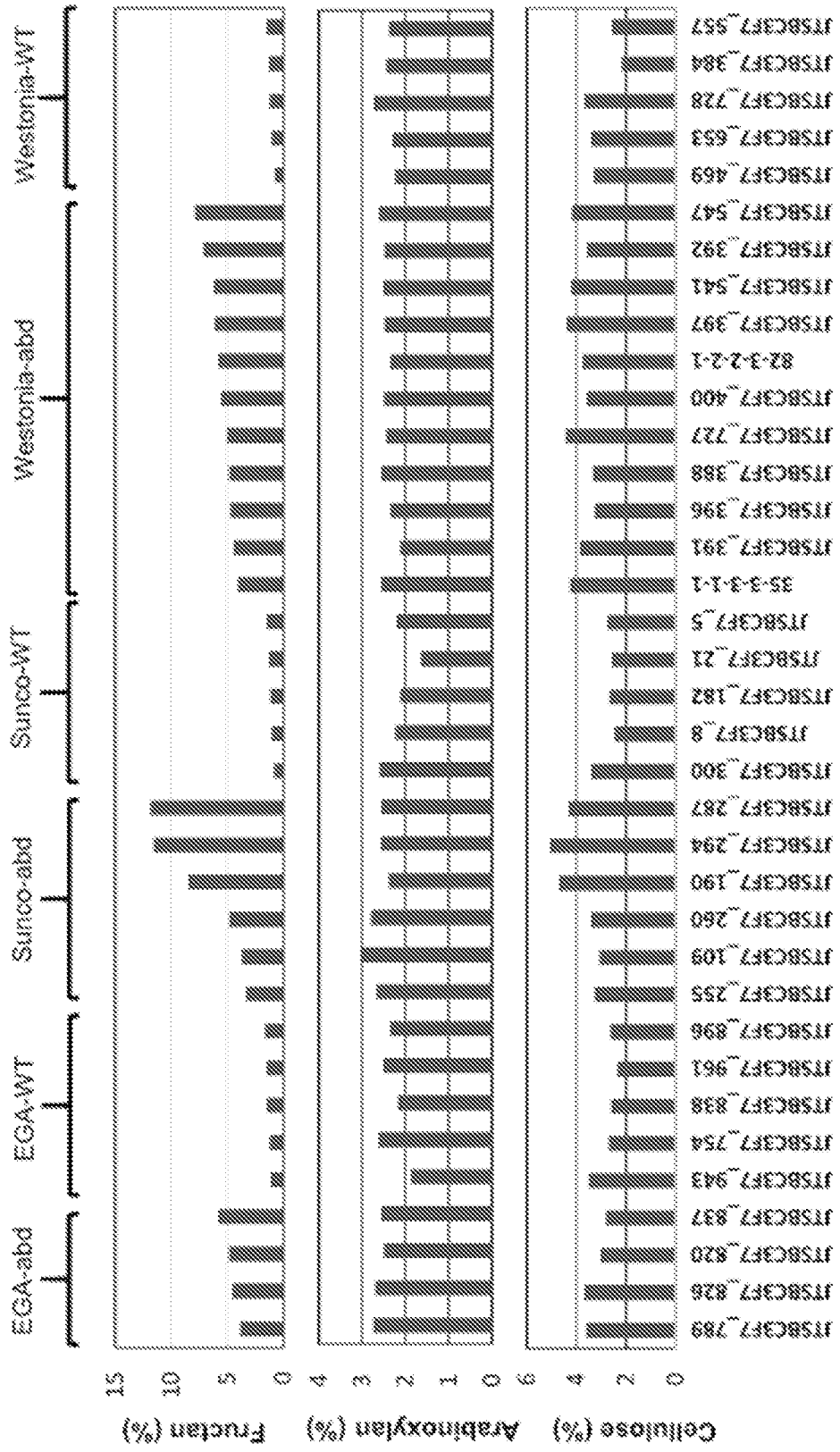

Cellulose (%)
Arabinoxylan (%)
Fructan (%)
0
2
4
6
0
1
2
3
4
0
5
10
15
EGA-abd
EGA-WT
Sunco-abd
Sunco-WT
Westonia-abd
Westonia-WT
JTSBC3F7_789
JTSBC3F7_826
JTSBC3F7_820
JTSBC3F7_837
JTSBC3F7_943
JTSBC3F7_754
JTSBC3F7_838
JTSBC3F7_961
JTSBC3F7_896
JTSBC3F7_255
JTSBC3F7_109
JTSBC3F7_260
JTSBC3F7_190
JTSBC3F7_294
JTSBC3F7_287
JTSBC3F7_300
JTSBC3F7_8
JTSBC3F7_182
JTSBC3F7_21
JTSBC3F7_5
35-3-3-1
JTSBC3F7_391
JTSBC3F7_396
JTSBC3F7_388
JTSBC3F7_727
JTSBC3F7_400
82-3-2-2-1
JTSBC3F7_397
JTSBC3F7_541
JTSBC3F7_392
JTSBC3F7_547
JTSBC3F7_469
JTSBC3F7_653
JTSBC3F7_728
JTSBC3F7_384
JTSBC3F7_557

FIGURE 15

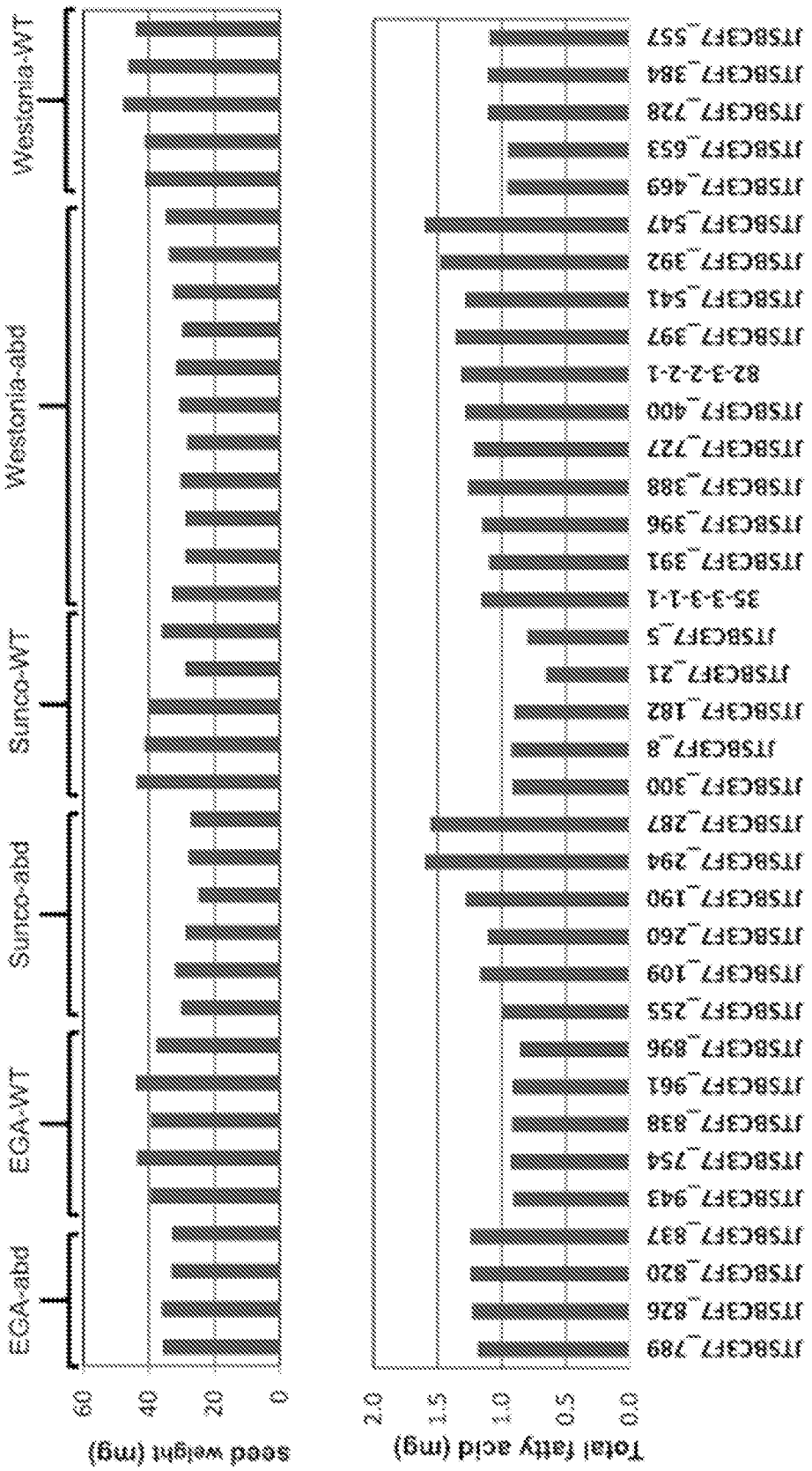

EGA-abd
EGA-WT
Sunco-abd
Sunco-WT
Westonia-abd
Westonia-WT
seed weight (mg)
60
40
20
0
Total fatty acid (mg)
2.0
1.5
1.0
0.5
0.0
JTSBC3F7_789
JTSBC3F7_826
JTSBC3F7_820
JTSBC3F7_837
JTSBC3F7_943
JTSBC3F7_754
JTSBC3F7_838
JTSBC3F7_961
JTSBC3F7_896
JTSBC3F7_255
JTSBC3F7_109
JTSBC3F7_260
JTSBC3F7_190
JTSBC3F7_294
JTSBC3F7_287
JTSBC3F7_300
JTSBC3F7_8
JTSBC3F7_182
JTSBC3F7_21
JTSBC3F7_5
35-3-3-1-1
JTSBC3F7_391
JTSBC3F7_396
JTSBC3F7_388
JTSBC3F7_727
JTSBC3F7_400
82-3-2-2-1
JTSBC3F7_397
JTSBC3F7_541
JTSBC3F7_392
JTSBC3F7_547
JTSBC3F7_469
JTSBC3F7_653
JTSBC3F7_728
JTSBC3F7_384
JTSBC3F7_557

FIGURE 16

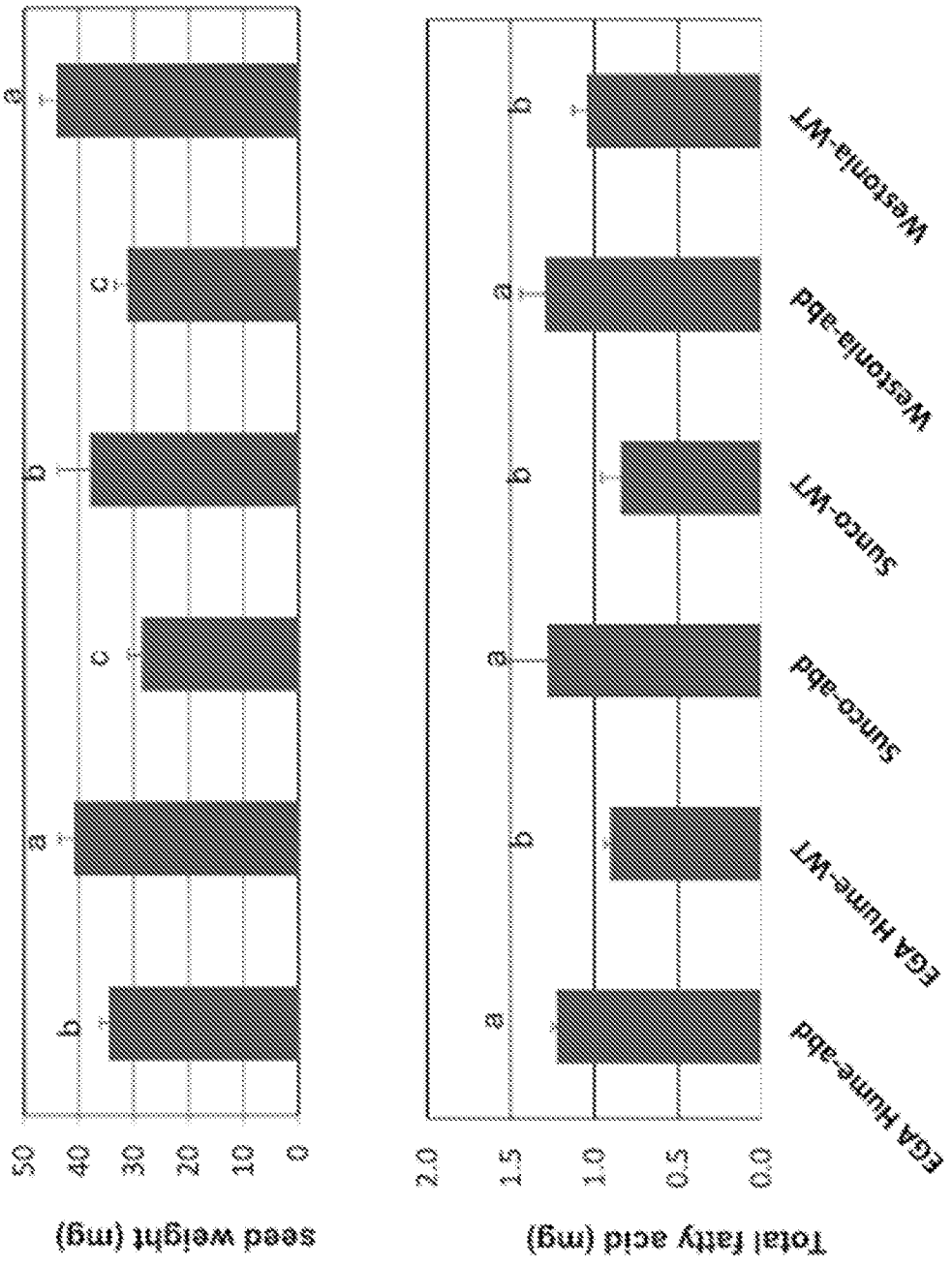

EGA Hume-abd
EGA Hume-WT
Sunco-abd
Sunco-WT
Westonia-abd
Westonia-WT
Total fatty acid (mg)
2.0
1.5
1.0
0.5
0.0
a
b
a
b
a
b
seed weight (mg)
50
40
30
20
10
0
a
b
c
a
b
c
a

FIGURE 17

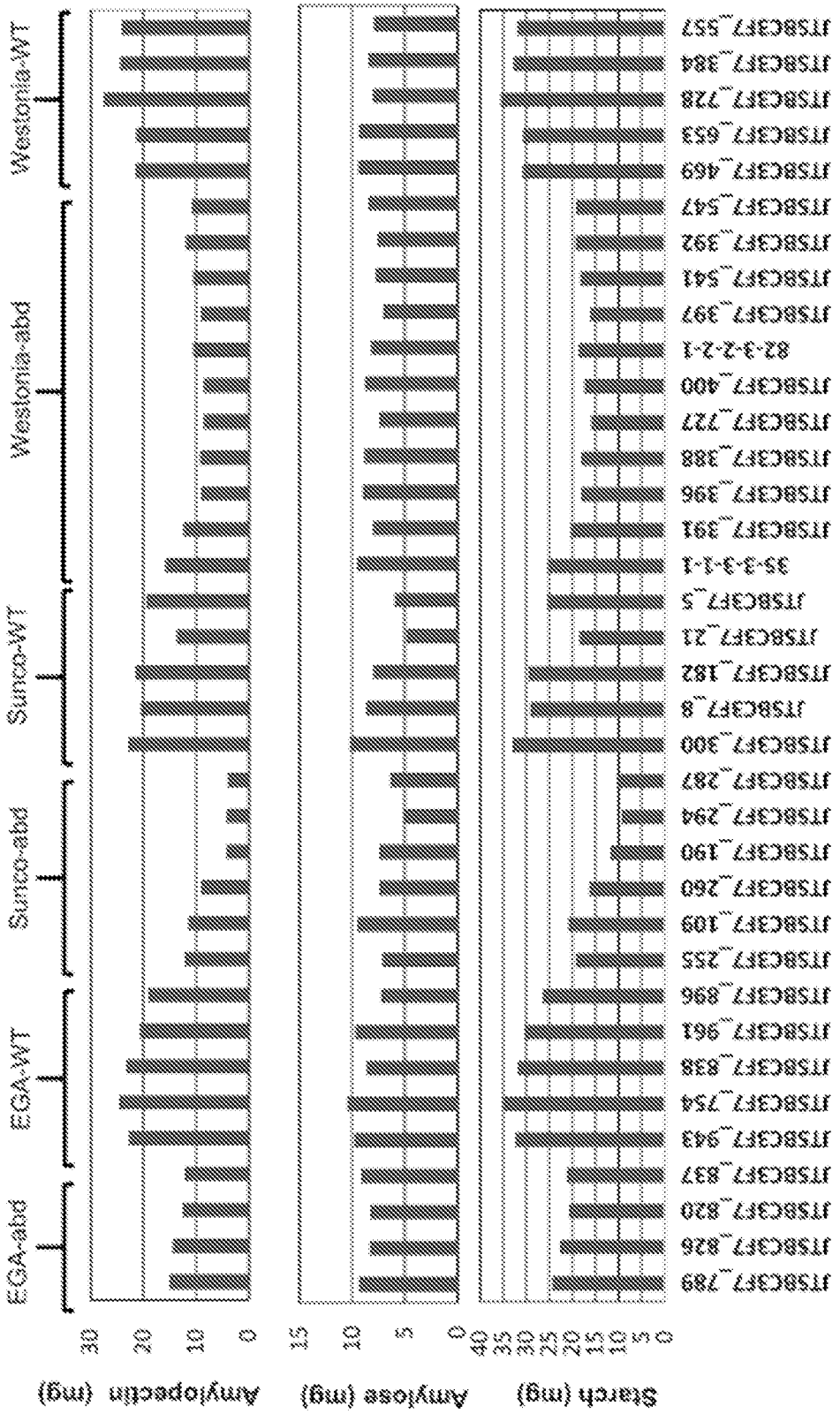

Starch (mg)
Amylose (mg)
Amylopectin (mg)
JT5BC3F7_789
JT5BC3F7_826
JT5BC3F7_820
JT5BC3F7_837
JT5BC3F7_943
JT5BC3F7_754
JT5BC3F7_838
JT5BC3F7_961
JT5BC3F7_896
JT5BC3F7_255
JT5BC3F7_109
JT5BC3F7_260
JT5BC3F7_190
JT5BC3F7_294
JT5BC3F7_287
JT5BC3F7_300
JT5BC3F7_8
JT5BC3F7_182
JT5BC3F7_21
JT5BC3F7_5
35-3-3-1-1
JT5BC3F7_391
JT5BC3F7_396
JT5BC3F7_388
JT5BC3F7_727
JT5BC3F7_400
82-3-2-2-1
JT5BC3F7_397
JT5BC3F7_541
JT5BC3F7_392
JT5BC3F7_547
JT5BC3F7_469
JT5BC3F7_653
JT5BC3F7_728
JT5BC3F7_384
JT5BC3F7_557
EGA-abd
EGA-WT
Sunco-abd
Sunco-WT
Westonia-abd
Westonia-WT

FIGURE 18

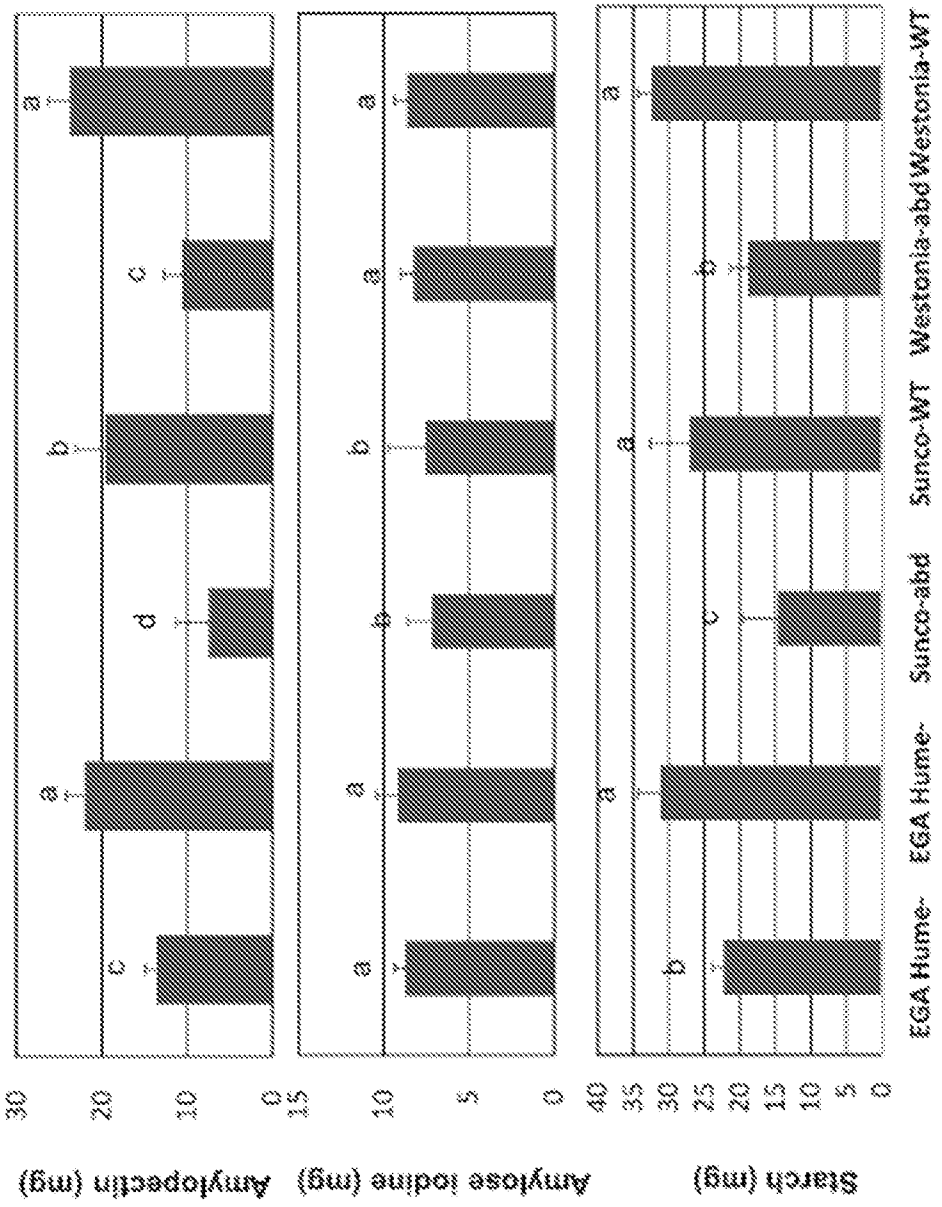

Amylopectin (mg)
Amylose iodine (mg)
Starch (mg)
EGA Hume-abd
EGA Hume-WT
Sunco-abd
Sunco-WT
Westonia-abd
Westonia-WT

FIGURE 19

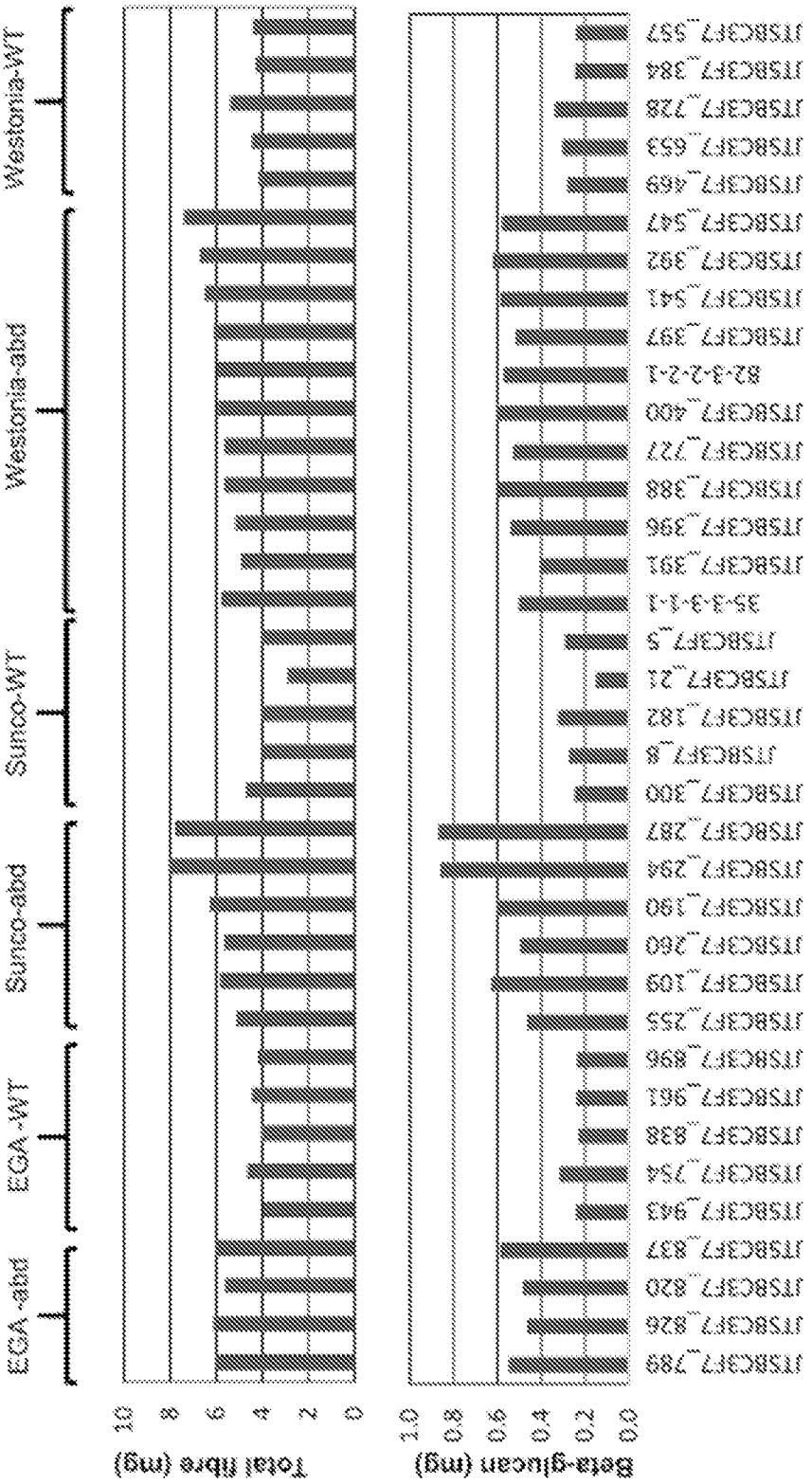

ECA -abd
EGA -WT
Sunco-abd
Sunco-WT
Westonia-abd
Westonia-WT
JTSBC3F7_789
JTSBC3F7_826
JTSBC3F7_820
JTSBC3F7_837
JTSBC3F7_943
JTSBC3F7_754
JTSBC3F7_838
JTSBC3F7_961
JTSBC3F7_896
JTSBC3F7_255
JTSBC3F7_109
JTSBC3F7_260
JTSBC3F7_190
JTSBC3F7_294
JTSBC3F7_287
JTSBC3F7_300
JTSBC3F7_8
JTSBC3F7_182
JTSBC3F7_21
JTSBC3F7_5
35-3-3-1-1
JTSBC3F7_391
JTSBC3F7_396
JTSBC3F7_388
JTSBC3F7_727
JTSBC3F7_400
82-3-2-2-1
JTSBC3F7_397
JTSBC3F7_541
JTSBC3F7_392
JTSBC3F7_547
JTSBC3F7_469
JTSBC3F7_653
JTSBC3F7_728
JTSBC3F7_384
JTSBC3F7_557
Beta-glucan (mg)
1.0
0.8
0.6
0.4
0.2
0.0
Total fibre (mg)
10
8
6
4
2
0

FIGURE 20

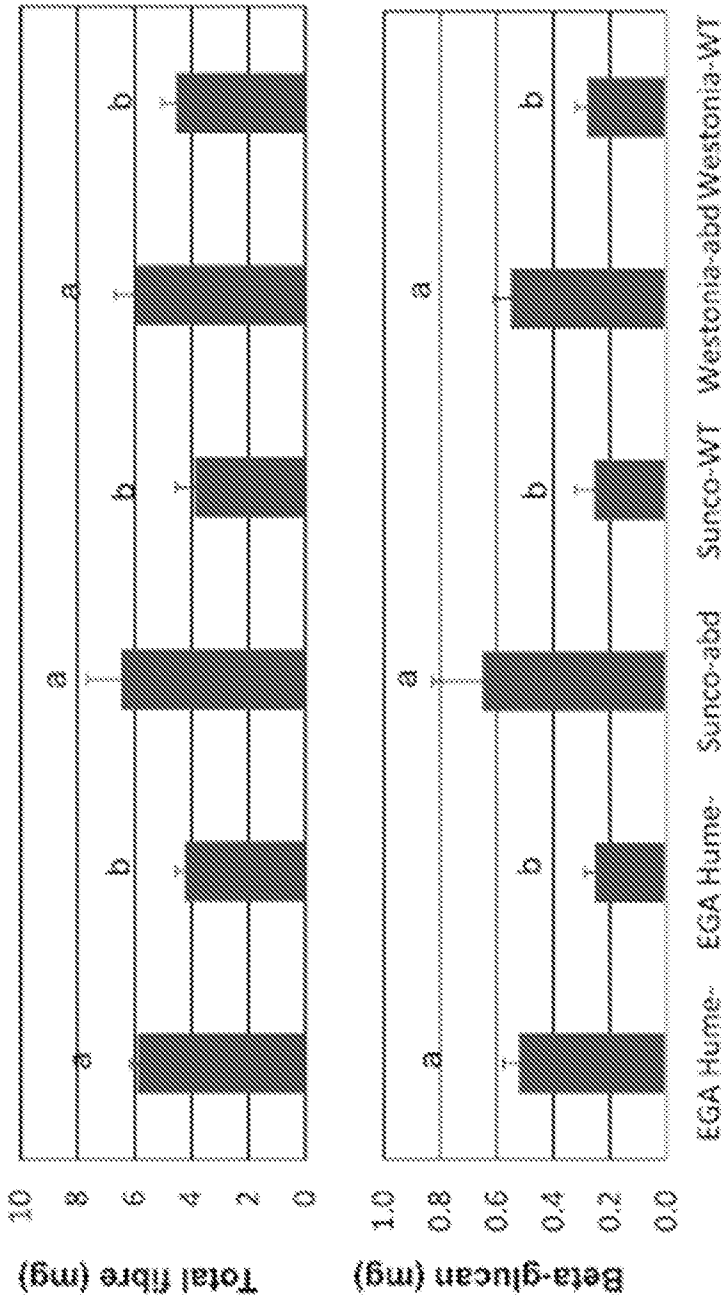

Total fibre (mg)
Beta-glucan (mg)
EGA Hume-abd
EGA Hume-WT
Sunco-abd
Sunco-WT
Westonia-abd
Westonia-WT

FIGURE 21

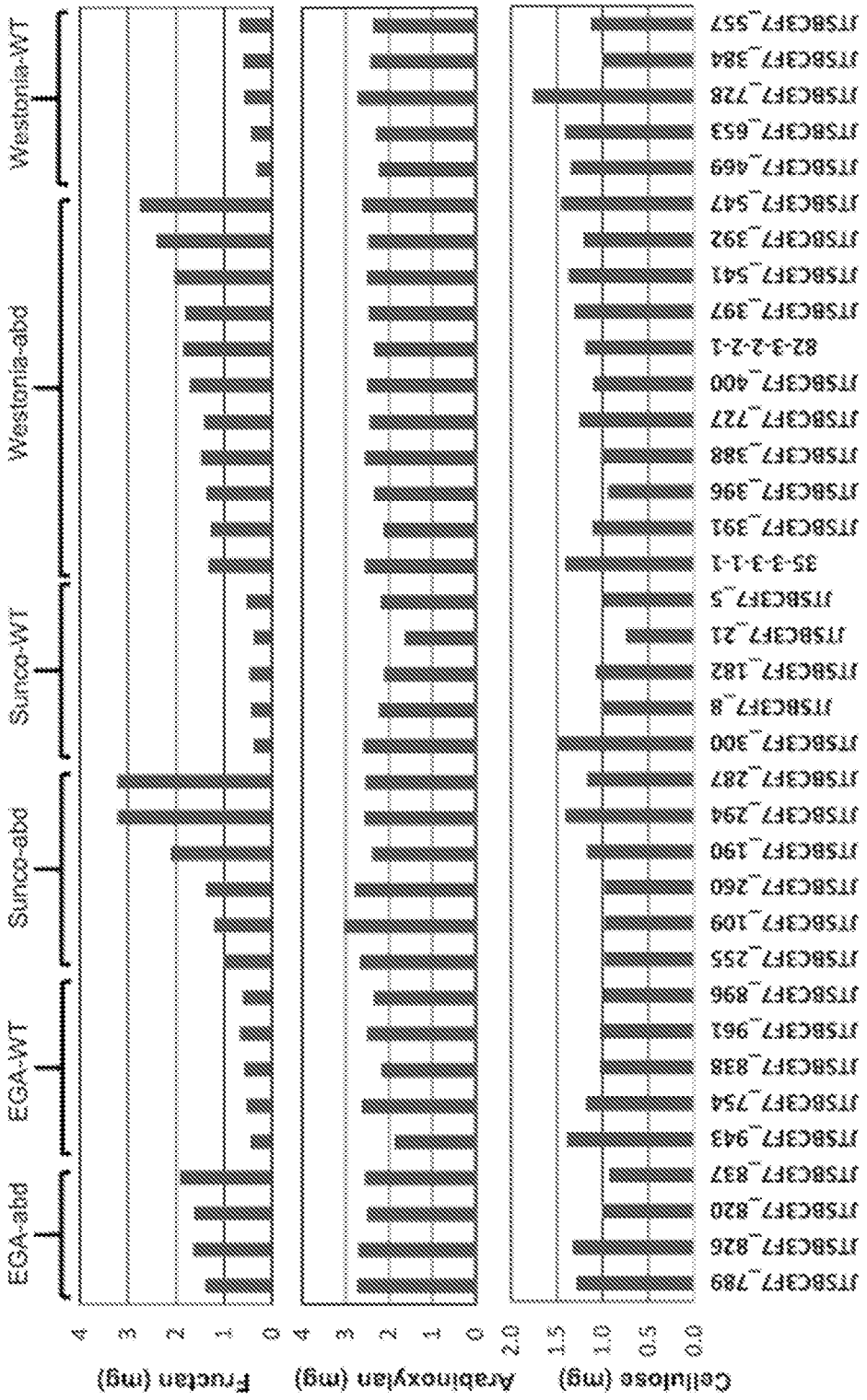

EGA-abd
EGA-WT
Sunco-abd
Sunco-WT
Westonia-abd
Westonia-WT
JTSBC3F7_789
JTSBC3F7_826
JTSBC3F7_830
JTSBC3F7_837
JTSBC3F7_943
JTSBC3F7_754
JTSBC3F7_838
JTSBC3F7_961
JTSBC3F7_896
JTSBC3F7_255
JTSBC3F7_109
JTSBC3F7_260
JTSBC3F7_190
JTSBC3F7_294
JTSBC3F7_287
JTSBC3F7_300
JTSBC3F7_8
JTSBC3F7_182
JTSBC3F7_21
JTSBC3F7_5
35-3-3-1-1
JTSBC3F7_391
JTSBC3F7_396
JTSBC3F7_388
JTSBC3F7_727
JTSBC3F7_400
82-3-2-2-1
JTSBC3F7_397
JTSBC3F7_541
JTSBC3F7_392
JTSBC3F7_547
JTSBC3F7_469
JTSBC3F7_653
JTSBC3F7_728
JTSBC3F7_384
JTSBC3F7_357
Cellulose (mg)
0.0
0.5
1.0
1.5
2.0
Arabinoxylan (mg)
0
1
2
3
4
Fructan (mg)
0
1
2
3
4

FIGURE 22

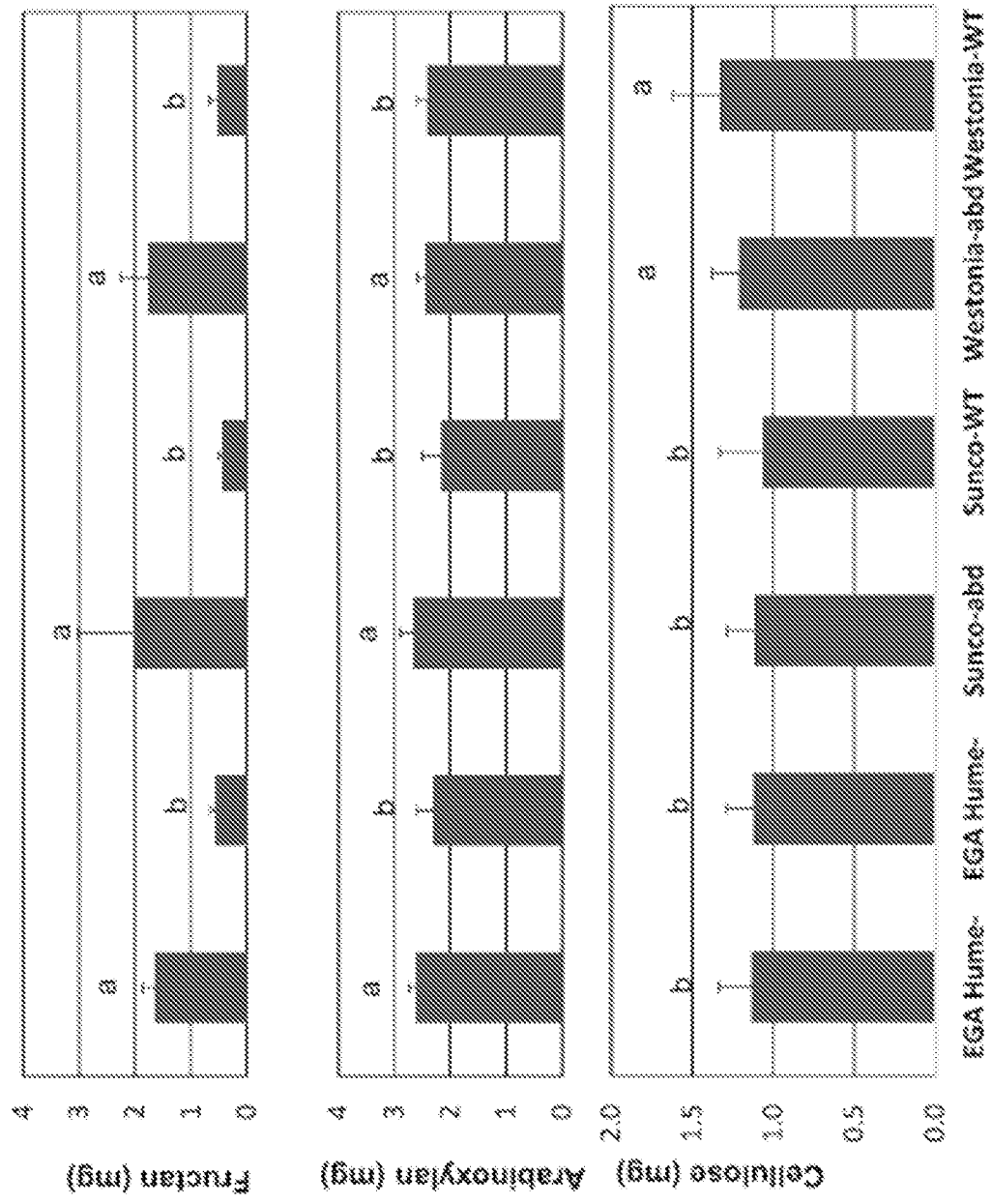

Fructan (mg)
Arabinoxylan (mg)
Cellulose (mg)
EGA Hume-abd
EGA Hume-WT
Sunco-abd
Sunco-WT
Westonia-abd
Westonia-WT

FIGURE 23

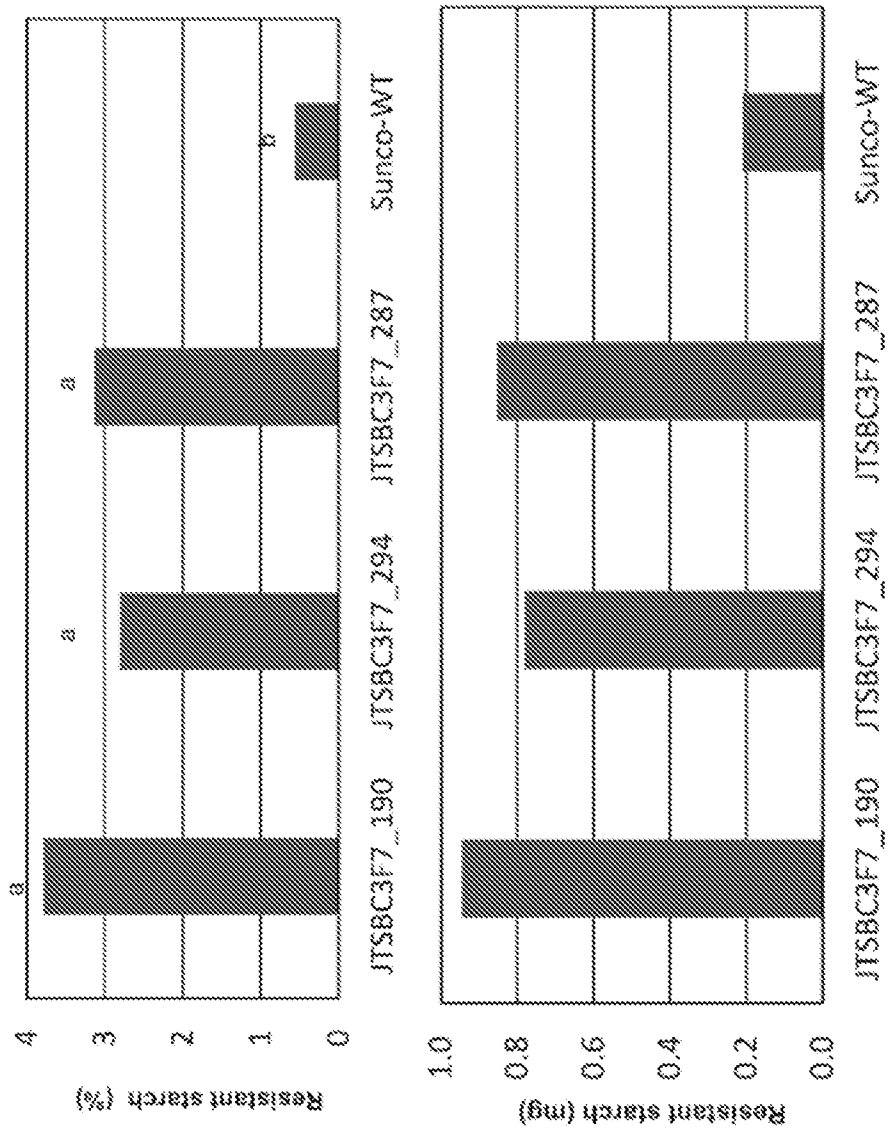

Sunco-abd
Sunco-WT
a
a
a
b
Resistant starch (%)
0
1
2
3
4
JTSBC3F7_190
JTSBC3F7_294
JTSBC3F7_287
Sunco-WT
Resistant starch (mg)
0.0
0.2
0.4
0.6
0.8
1.0
JTSBC3F7_190
JTSBC3F7_294
JTSBC3F7_287
Sunco-WT

FIGURE 24

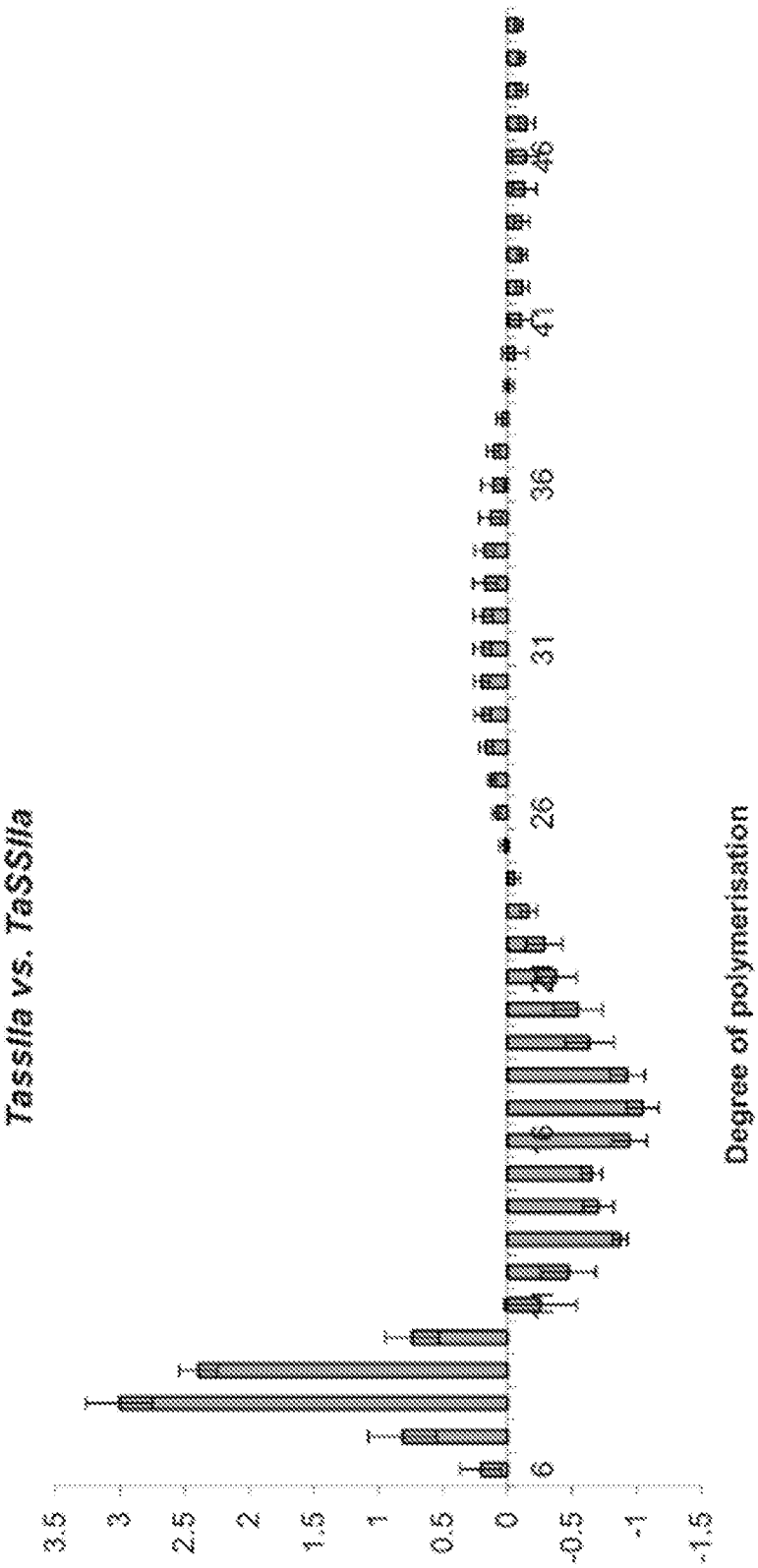

TassIIa vs. TaSSIIa
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
-1
-1.5
6
26
31
36
41
46
Degree of polymerisation

FIGURE 25

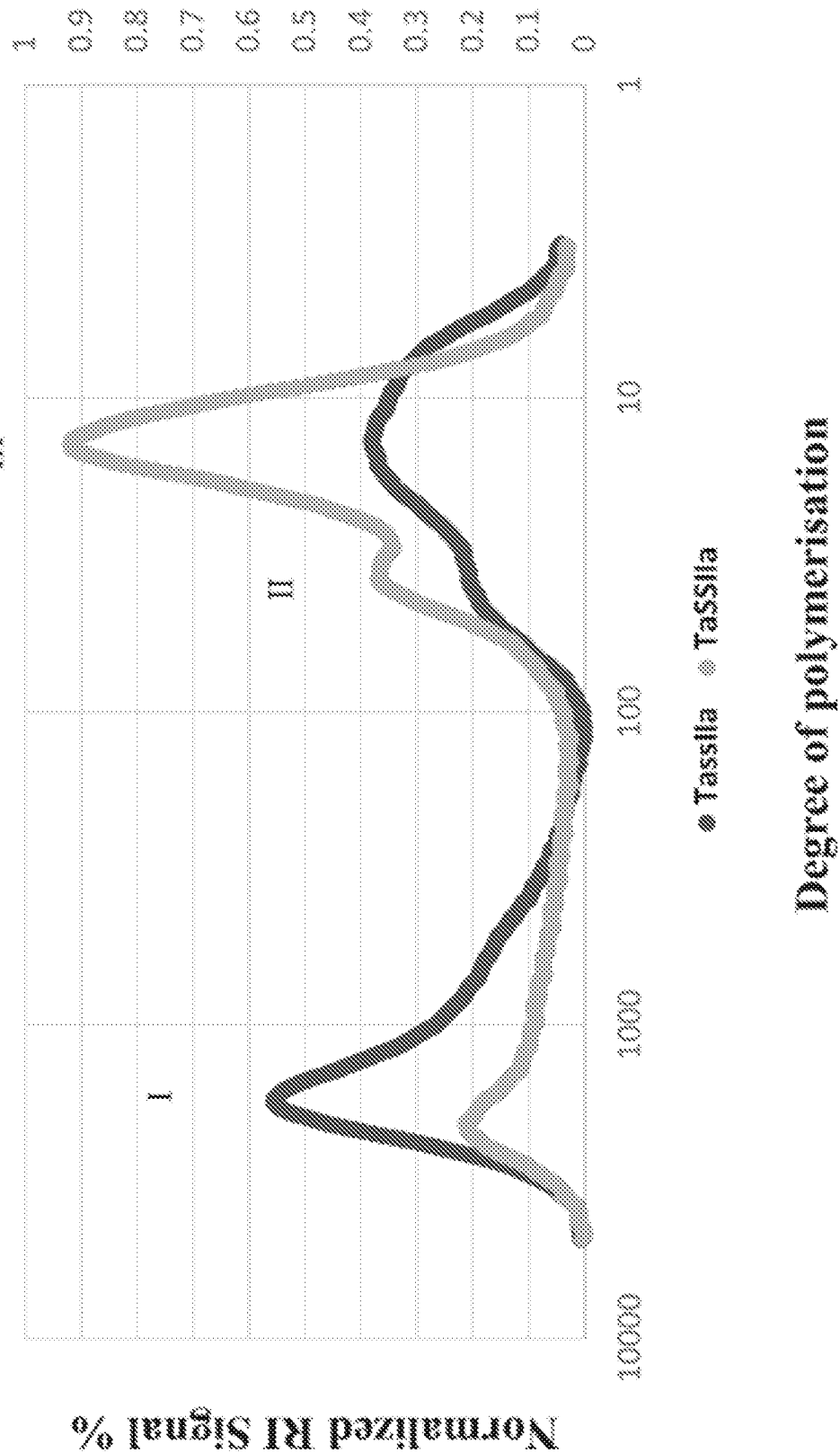

Normalized RI Signal %
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
I
II
III
1
10
100
1000
10000
Degree of polymerisation
TasSIIa
TaSSIIa

FIGURE 26

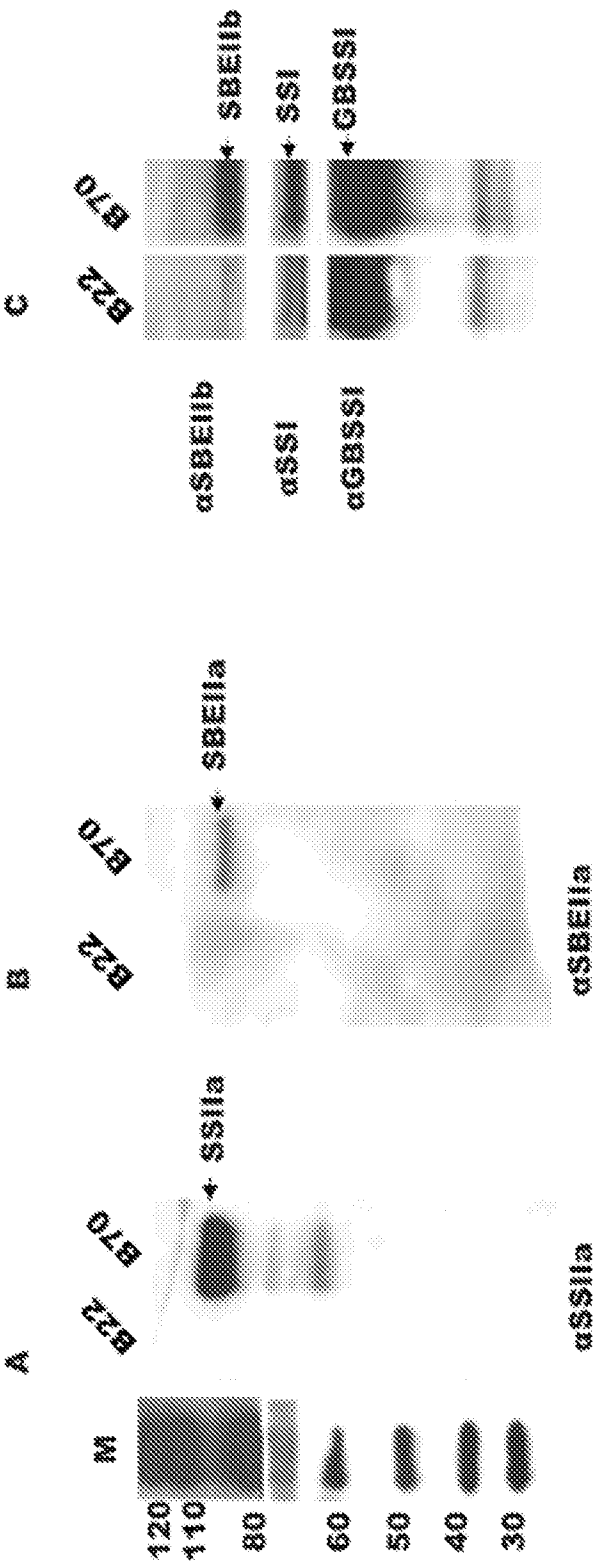

A
B
C
M
B22
B70
SSIIa
SBEIIa
SBEIIb
SSI
GBSSI
120
110
80
60
50
40
30
αSSIIa
αSBEIIa
αSBEIIb
αSSI
αGBSSI

FIGURE 27

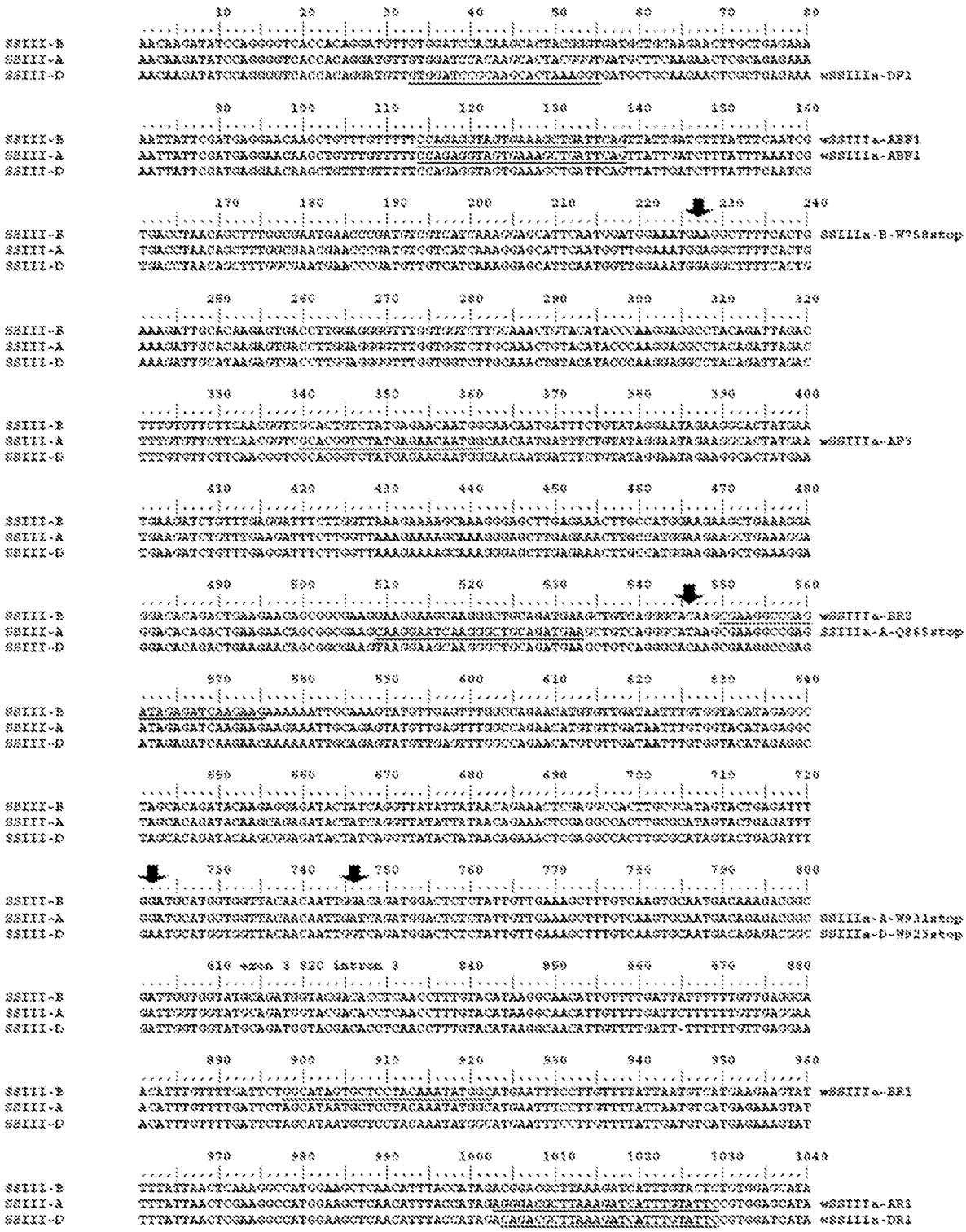
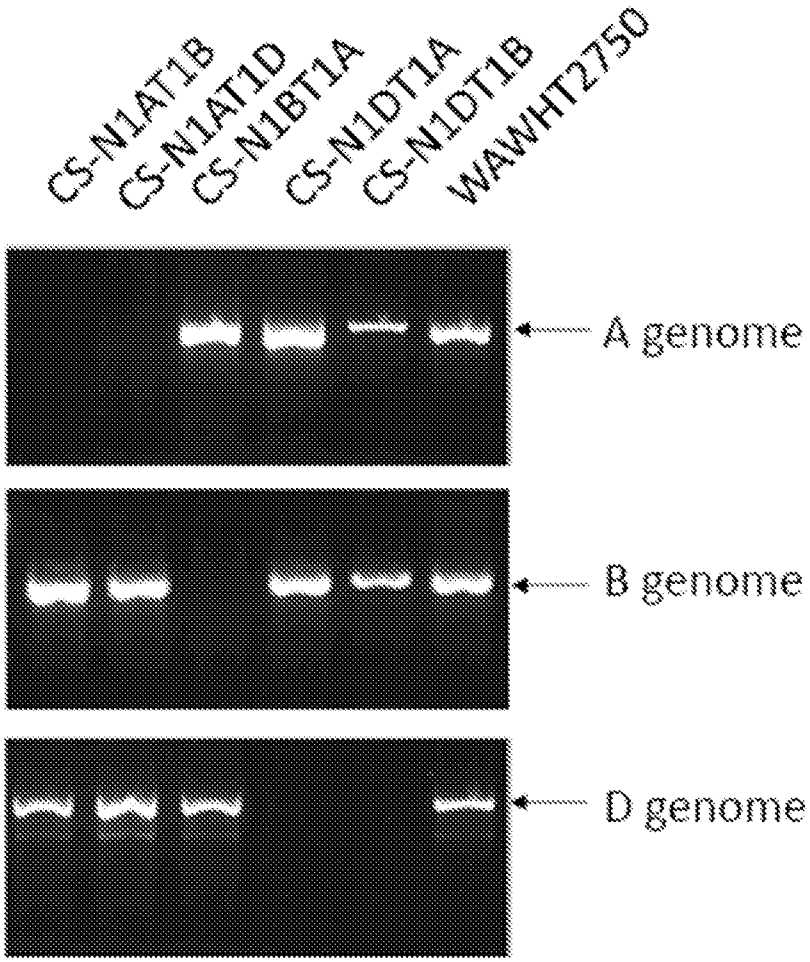


FIGURE 28


CS-N1AT1B
CS-N1AT1D
CS-N1BT1A
CS-N1DT1A
CS-N1DT1B
WAWHT2750
A genome
B genome
D genome

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2019/050008

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/52 (2006.01) A01H 1/06 (2006.01) A01H 5/00 (2018.01) C12N 9/10 (2006.01) A23L 29/212 (2016.01) A61K 31/718 (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)

PATENW: classification marks A23L29/212, A61K31/718
PATENW, CABA, BIOSIS, EMBASE, CAPLUS, FSTA & keywords: starch, starch synthase, SSIIa, SSIIIa, EC 2.4.1.21, wheat, amylose, null mutation, and like terms
Internal databases provided by IP Australia (including AUSPAT) and external database (ESPACENET, Google Scholar/Patents, ResearchGate): Inventor and applicant names

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 March 2019	19 March 2019
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Jessie Williams AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61262832865

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/AU2019/050008**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012/103594 A1 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 09 August 2012 [0006], [0069], [0070], [00384]; abstract; example 13	1-34
A	HOGG, A.C., et al., "Novel *ssIIa* alleles produce specific seed amylose levels in hexaploid wheat", Cereal chemistry. 2017, vol. 94, pages 1008-1015 in particular table III	
A	KONIK-ROSE, C., et al., "Effects of *starch synthase IIa* gene dosage on grain, protein and starch in endosperm of wheat", Theoretical and Applied Genetics. 2007, vol. 115, pages 1053-1065 in particular tables 1 and 2	
A	ZHANG, G., et al., "Double repression of soluble starch synthase genes *SSIIa* and *SSIIIa* in rice (*Oryza sativa* L.) uncovers interactive effects on the physicochemical properties of starch", Genome. 2011, vol. 54, pages 448-459	
A	YAMAMORI, M., et al., "Genetic elimination of a starch granule protein, SGP-1, of wheat generates an altered starch with apparent high amylose", Theoretical and Applied Genetics. 2000, vol. 101, pages 21-29	
A	WO 2017/008001 A1 (MONTANA STATE UNIVERSITY) 12 January 2017	
A	WO 2000/066745 A1 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION, et al.) 09 November 2000	
P,A	HAYASHI, M., et al., "Analyses of starch biosynthetic protein complexes and starch properties from developing mutant rice seeds with minimal starch synthase activities", BMC Plant Biology. 2018, vol. 18:59	
P,X	WO 2018/006126 A1 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 11 January 2018 abstract; claims 1-2, 6-7; para [0154]-[0163]	1-34
P,X	WO 2018/092155 A1 (LORVEN BIOLOGICS PVT. LTD.) 24 May 2018 page 12, lines 4-7; page 13, line 24 - page 14, line 24; table 1; claims 1-3	1-34

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2019/050008**

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Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
WO 2012/103594 A1	09 August 2012	WO 2012103594 A1	09 Aug 2012
		AU 2012212404 A1	09 May 2013
		AU 2012212404 B2	12 May 2016
		US 2014205709 A1	24 Jul 2014
		US 9826764 B2	28 Nov 2017
		US 2018077955 A1	22 Mar 2018
		US 10212959 B2	26 Feb 2019
WO 2017/008001 A1	12 January 2017	WO 2017008001 A1	12 Jan 2017
		AR 106285 A1	03 Jan 2018
		AU 2016291181 A1	18 Jan 2018
		BR 112018000131 A2	18 Sep 2018
		CA 2990240 A1	12 Jan 2017
		EP 3319999 A1	16 May 2018
		JP 2018518973 A	19 Jul 2018
		TW 201715957 A	16 May 2017
		US 2017006815 A1	12 Jan 2017
WO 2000/066745 A1	09 November 2000	WO 0066745 A1	09 Nov 2000
		AU 4092400 A	17 Nov 2000
		AU 780523 B2	24 Mar 2005
		CA 2365285 A1	09 Nov 2000
		EP 1179074 A1	13 Feb 2002
		US 7001771 B1	21 Feb 2006
		US 2006035379 A1	16 Feb 2006
		US 7700826 B2	20 Apr 2010
WO 2018/006126 A1	11 January 2018	WO 2018006126 A1	11 Jan 2018
		AU 2017292900 A1	17 Jan 2019

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2019/050008**

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Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
		CA 3029641 A1	11 Jan 2018
WO 2018/092155 A1	24 May 2018	WO 2018092155 A1	24 May 2018

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.