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CA 2529455 A1 2004/12/29

(21) 2 529 455

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2004/06/17
(87) Date publication PCT/PCT Publication Date: 2004/12/29
(85) Entrée phase nationale/National Entry: 2005/12/14
(86) N° demande PCT/PCT Application No.: GB 2004/002583
(87) N° publication PCT/PCT Publication No.: 2004/112468
(30) Priorité/Priority: 2003/06/17 (0313998.7) GB

(51) Cl.Int./Int.Cl. *A01H 5/00* (2006.01), *C12N 15/82* (2006.01), *C12N 15/29* (2006.01), *C07K 14/415* (2006.01), *A23L 1/0522* (2006.01)

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(54) Titre : INHIBITEUR DE DEXTRINASE LIMITE VEGETAL
(54) Title: PLANT LIMIT DEXTRINASE INHIBITOR

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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            10        20        30        40        50
Hordeum vulgare     ---MASD-HR RFVLSGAVLL SVLAVAAATL E--------- SVKDECQLGV
Oryza sativa        ---MASN-KV VFSVLLLAVV SVLAATATMA EYHHQDQVVY TPGPLCQPGM
Hordeum spontaneum  ---MAFK--Y QLLLSAAVML AILAATVT-- ---------- SFGDMCAPGD
Eleusine coracana   ---------- ---------- ---------- ---------- SVGTSCIPGM
Secale cereale      ---------- ---------- ---------- ---------- SVGGQCVPGL
Triticum durum      ---MACKSSC SLLLLAAVLL SVLAA--A-- ---------- SASGSCVPGV
Zea mays            MASSSSSHR RLILAAAVLL SVLAAASA-- ---------- SAGTSCVPGW
Tritcum aestivum    ---MASN-HR RFLLSGAVLL SVLAAVAA-L E--------- SVEDECQPGV

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Hordeum vulgare     DFPHNPLATC HTYVIKRVCG ---RGPSRPM LV-------- ------KERC
Oryza sativa        GYPMYPLPRC RALVKRQCVG ---RGTAAAA EQ-------- -----VRRDC
Hordeum spontaneum  ALPANPLRAC RTYVVSQIC- --HVGPRLST WD-------- -----MKRRC
Eleusine coracana   AIPHNPLDSC RWYVAKRACG ---VGPRLAT QE-------- -----MKARC
Secale cereale      AMPHNPLGAC RTYVVSQIC- --HVGPRLFT WD-------- -----MKRRC
Triticum durum      AFRTNLLPHC RDYVLQQTCG TFTPGSKLPE WMTSASIYSP GKPYLAKLYC
Zea mays            AIPHNPLPSC RWYVTSRTCG ---IGPRLPW PE-------- -----LKRRC
Tritcum aestivum    AFPHNALATC HTYVIKRVCG ---RGPSRPM LV-------- ------KERC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                           110       120       130       140       150
Hordeum vulgare     CRELAAVP-D HCRCEALRIL MDGVRTPE-- ----GRVVEG RLGDRRDCPR
Oryza sativa        CRQLAAVDDS WCRCEAISHM LGGIYRELG- ---APDVGHP MSEVFRGCRR
Hordeum spontaneum  CDELSAIP-A YCRCEALRII MDGTVTWQ-- ----GVFG-A YFKDMPNCPR
Eleusine coracana   CRQLEAIP-A YCRCEAVRIL MDGVVTP--- ---SGQHEGR LLQDLPGCPR
Secale cereale      CDELLAIP-A YCRCEALRIL MDGVVTQQ-- ----GVFEGG YLKDMPNCPR
Triticum durum      CQELAEIS-Q QCRCEALRYF IALPVPSQPV DPRSGNVGES GLIDLPGCPR
Zea mays            CRELADIP-A YCRCTALSIL MDGAIPPGP- ---DAQLEGR -LEDLPGCPR
Tritcum aestivum    CRELAVVP-D YCRCEALRVL MDGVRAEE-- ----GHVVEG RLGDRRDCPR

                    ....|....| ....|....| ....|....| ....|....| ...
                           160       170       180       190
Hordeum vulgare     EEQRAFAATL VTAAECNLSS VQAPGVRLVL LADG------ ---
Oryza sativa        GDLERAAASL PAFCNVDIPN GGG-GVCYWL ARSGY----- ---
Hordeum spontaneum  VMQTSYAANL VNPQECNLWT IHGSPSCPEL QPGYEVVL-- ---
Eleusine coracana   QVQRAFAPKL VTEVECNLAT IHGGPFCLSL LGAGE----- ---
Secale cereale      VTQRSYAATL VAPQECNLPT IHGSPYCPTL QAGY------ ---
Triticum durum      EMQWDFVRLL VAPGQCNLAT IHNVRYCPAV EQPLWI---- ---
Zea mays            EVQRGFAATL VTEAECNLAT ISGVAECPWI LGGGTMPSK- ---
Tritcum aestivum    EAQREFAATL VTAAECNLPT VS--GVGSTL GATGRWMTIE LPK
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(57) Abrégé/Abstract:
The invention provides a method of producing plants with altered number, size or composition of starch granules, including starch granules having a more uniform size or altered starch structure, the method comprising introducing into a plant a nucleotide

(21) 2 529 455

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(57) Abrégé(suite)/Abstract(continued):
sequence comprising a limit dextrinase inhibitor gene, or a fragment or variant, or sequence homologous to the gene sequence provided. Also provided are a nucleotide sequence and protein of the limit dextrinase inhibitor gene, and plant and plant parts produced by the method of the invention. Modified starch is also provided.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
29 December 2004 (29.12.2004)

PCT

(10) International Publication Number
WO 2004/112468 A1

(51) International Patent Classification[7]: A01H 5/00, C12N 15/82, 15/29, C07K 14/415, A23L 1/0522

(21) International Application Number: PCT/GB2004/002583

(22) International Filing Date: 17 June 2004 (17.06.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0313998.7 17 June 2003 (17.06.2003) GB

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(72) Inventors; and

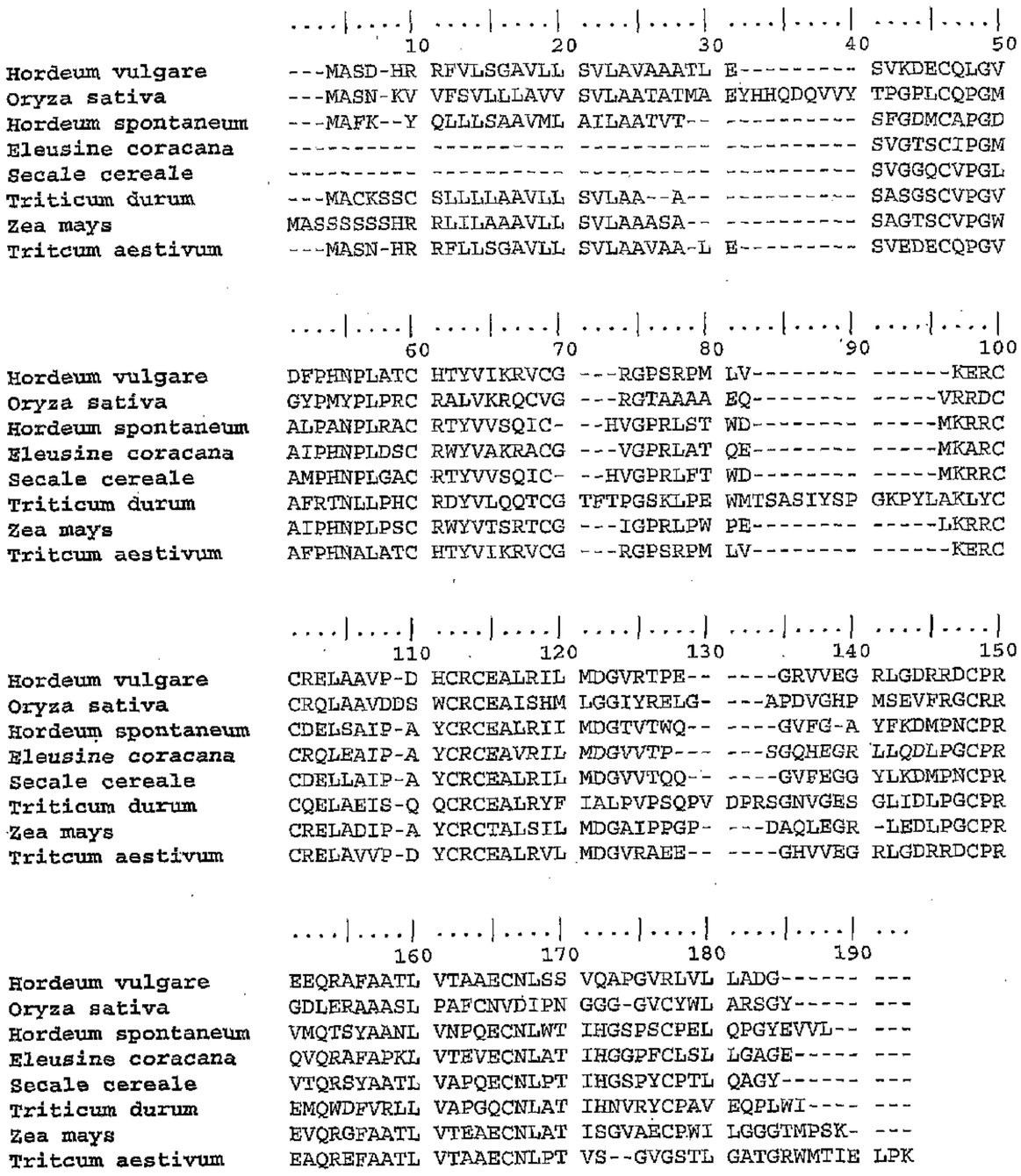
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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

[Continued on next page]

(54) Title: PLANT LIMIT DEXTRINASE INHIBITOR



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                                 ....|....| ....|....| ....|....| ....|....| ....|....|
                                         10         20         30         40         50
Hordeum vulgare                  ---MASD-HR RFVLSGAVLL SVLAVAAATL E--------- SVKDECQLGV
Oryza sativa                     ---MASN-KV VFSVLLLAVV SVLAATATMA EYHHQDQVVY TPGPLCQPGM
Hordeum spontaneum               ---MAFK--Y QLLLSAAVML AILAATVT-- ---------- SFGDMCAPGD
Eleusine coracana                ---------- ---------- ---------- ---------- SVGTSCIPGM
Secale cereale                   ---------- ---------- ---------- ---------- SVGGQCVPGL
Triticum durum                   ---MACKSSC SLLLLAAVLL SVLAA--A-- ---------- SASGSCVPGV
Zea mays                         MASSSSSSHR RLILAAAVLL SVLAAASA-- ---------- SAGTSCVPGW
Tritcum aestivum                 ---MASN-HR RFLLSGAVLL SVLAAVAA-L E--------- SVEDECQPGV

                                 ....|....| ....|....| ....|....| ....|....| ....|....|
                                        60         70         80         90        100
Hordeum vulgare                  DFPHNPLATC HTYVIKRVCG ---RGPSRPM LV-------- ------KERC
Oryza sativa                     GYPMYPLPRC RALVKRQCVG ---RGTAAAA EQ-------- -----VRRDC
Hordeum spontaneum               ALPANPLRAC RTYVVSQIC- --HVGPRLST WD-------- -----MKRRC
Eleusine coracana                AIPHNPLDSC RWYVAKRACG ---VGPRLAT QE-------- -----MKARC
Secale cereale                   AMPHNPLGAC RTYVVSQIC- --HVGPRLFT WD-------- -----MKRRC
Triticum durum                   AFRTNLLPHC RDYVLQQTCG TFTPGSKLPE WMTSASIYSP GKPYLAKLYC
Zea mays                         AIPHNPLPSC RWYVTSRTCG ---IGPRLPW PE-------- -----LKRRC
Tritcum aestivum                 AFPHNALATC HTYVIKRVCG ---RGPSRPM LV-------- ------KERC

                                 ....|....| ....|....| ....|....| ....|....| ....|....|
                                        110        120        130        140        150
Hordeum vulgare                  CRELAAVP-D HCRCEALRIL MDGVRTPE-- ----GRVVEG RLGDRRDCPR
Oryza sativa                     CRQLAAVDDS WCRCEAISHM LGGIYRELG- ---APDVGHP MSEVFRGCRR
Hordeum spontaneum               CDELSAIP-A YCRCEALRII MDGTVTWQ-- ----GVFG-A YFKDMPNCPR
Eleusine coracana                CRQLEAIP-A YCRCEAVRIL MDGVVTP--- ---SGQHEGR LLQDLPGCPR
Secale cereale                   CDELLAIP-A YCRCEALRIL MDGVVTQQ-- ----GVFEGG YLKDMPNCPR
Triticum durum                   CQELAEIS-Q QCRCEALRYF IALPVPSQPV DPRSGNVGES GLIDLPGCPR
Zea mays                         CRELADIP-A YCRCTALSIL MDGAIPPGP- ---DAQLEGR -LEDLPGCPR
Tritcum aestivum                 CRELAVVP-D YCRCEALRVL MDGVRAEE-- ----GHVVEG RLGDRRDCPR

                                 ....|....| ....|....| ....|....| ....|....| ...
                                        160        170        180        190
Hordeum vulgare                  EEQRAFAATL VTAAECNLSS VQAPGVRLVL LADG------ ---
Oryza sativa                     GDLERAAASL PAFCNVDIPN GGG-GVCYWL ARSGY----- ---
Hordeum spontaneum               VMQTSYAANL VNPQECNLWT IHGSPSCPEL QPGYEVVL-- ---
Eleusine coracana                QVQRAFAPKL VTEVECNLAT IHGGPFCLSL LGAGE----- ---
Secale cereale                   VTQRSYAATL VAPQECNLPT IHGSPYCPTL QAGY------ ---
Triticum durum                   EMQWDFVRLL VAPGQCNLAT IHNVRYCPAV EQPLWI---- ---
Zea mays                         EVQRGFAATL VTEAECNLAT ISGVAECPWI LGGGTMPSK- ---
Tritcum aestivum                 EAQREFAATL VTAAECNLPT VS--GVGSTL GATGRWMTIE LPK
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(57) Abstract: The invention provides a method of producing plants with altered number, size or composition of starch granules, including starch granules having a more uniform size or altered starch structure, the method comprising introducing into a plant a nucleotide sequence comprising a limit dextrinase inhibitor gene, or a fragment or variant, or sequence homologous to the gene sequence provided. Also provided are a nucleotide sequence and protein of the limit dextrinase inhibitor gene, and plant and plant parts produced by the method of the invention. Modified starch is also provided.

WO 2004/112468 A1

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(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

PLANT LIMIT DEXTRINASE INHIBITOR.

1 FIELD OF INVENTION

The present invention is based upon the identification of a protein, limit dextrinase inhibitor (LDI), which modifies starch metabolism in plants, especially the number, size and composition of starch granules in plants. In particular, the invention relates to plant limit dextrinase inhibitor nucleic acid molecules, plant limit dextrinase inhibitor gene products, antibodies to plant limit dextrinase inhibitor gene products, plant limit dextrinase inhibitor regulatory regions, vectors and expression vectors with plant limit dextrinase inhibitor genes, cells, plants and plant parts with plant limit dextrinase inhibitor genes, modified starch from such plants and the use of the foregoing to improve agronomically valuable plants.

2 BACKGROUND

Starch consists of two glucose polymers, essentially linear amylose and highly branched amylopectin, arranged into a three dimensional, semicrystalline structure – the starch granule. The starch granule consists of alternate semicrystalline and amorphous layers which contain different amounts of branched and unbranched polymer. Starch is the product of carbon fixation during photosynthesis in plants, and is the primary metabolic energy reserve stored in seeds and fruit. For example, up to 75% of the dry weight of grain in cereals is made up of starch. The importance of starch as a food source is reflected by the fact that two thirds of the world's food consumption (in terms of calories) is provided by the starch in grain crops such as wheat, rice and maize.

Starch is the product of photosynthesis, and is analogous to the storage compound glycogen found in bacteria, fungi and animals. It is produced in the chloroplasts or amyloplasts of plant cells, these being the plastids of photosynthetic cells and non-photosynthetic cells, respectively. The biochemical pathway leading to the production of starch in leaves has been well characterised, and considerable progress has also been made in elucidating the pathway of starch biosynthesis in storage tissues.

The biosynthesis of starch molecules is dependent on a complex interaction of numerous enzymes, including several essential enzymes such as ADP-Glucose pyrophosphorylase, a series of starch synthases which use ADP glucose as a substrate for forming chains of glucose linked by alpha-1-4 linkages, and a series of starch branching

enzymes that link sections of polymers with alpha-1-6 linkages to generate branched structures (Smith et al., 1995, Plant Physiology, 107:673-677). Further modification of the starch by yet other enzymes, i.e. debranching enzymes (isoamylases or limit dextrinases) or disproportionating enzymes, can be specific to certain species.

The fine structure of starch is a complex mixture of D-glucose polymers that consist essentially of linear chains (amylose) and branched chains (amylopectin) glucans. Typically, amylose makes up between 10 and 25% of plant starch, but varies significantly among species. Amylose is composed of linear D-glucose chains typically 250-670 glucose units in length (Tester, 1997, in: Starch Structure and Functionality, Frazier et al., eds., Royal Society of Chemistry, Cambridge, UK). The linear regions of amylopectin are composed of low molecular weight and high molecular weight chains, with the low ranging from 5 to 30 glucose units and the high molecular weight chains from 30 to 100 or more. The amylose/amylopectin ratio and the distribution of low and high molecular weight D-glucose chains can affect starch granule properties such as gelatinization temperature, retrogradation, and viscosity (Blanshard, 1987). The characteristics of the fine structure of starch mentioned above have been examined at length and are well known in the art of starch chemistry.

It is known that starch granule size and amylose percentage change during kernel development in maize and during tobacco leaf development (Boyer et al., 1976, Cereal Chem 53:327-337). In their classic study Boyer et al. concluded the amylose percentage of starch decreases with decreasing granule size in later stages of maize kernel development.

Starch is the most significant form of carbon reserve in plants in terms of the amount made and the universality of its distribution among different plant species. Starch is also highly significant to man. Firstly, it forms a major component of animal diets, supplying man and his domestic animals with a large portion of their carbohydrate intake. Secondly, purified starch is used industrially in the production of paper, textiles, plastics and adhesives, as well as providing the raw material for some bio-reactors. Starches from different species have preferred uses. On a world scale, starch producing crops are agriculturally and economically by far the most important, and these crops include wheat, barley, maize, rice and potatoes. Typically, starch is mixed with water and cooked to form a thickening agent or gel. Of central importance are the temperature at which the starch cooks, the viscosity that the agent or gel reaches, and the stability of the gel viscosity over time. The physical properties of unmodified starch limit its usefulness in many applications. As a result,

considerable effort and expenditure is allocated to chemically modify starch (i.e. cross-linking and substitution) in order to overcome the numerous limitations of unmodified starch and to expand industrial usefulness. Modified starches can be used in foods, paper, textiles, and adhesives.

The size and uniformity of the starch granule present in the harvested organ of a plant will affect the processing efficiency of the crop, the quality of a processed product and the profitability of the process.

Each species produces starch granules with a range of sizes. Potato starch, for example, comprises granules of all sizes within a certain range. By contrast, wheat or barley starch is composed of granules which may be either large (A type) or small (B type). The production of starch comprising granules of a more uniform size would reduce the need for, and cost of, post harvest processing. Such starch would have more uniform gelling properties. In wheat or barley the elimination of the B granules would improve starch extractability. Furthermore, it has recently been discovered that the proportion of B granules influences water absorption and hence the water content of dough, an important quality in breadmaking.The starch debranching enzyme limit dextrinase (LD) (EC 3.2.1.41) (also known as R-enzyme, pullulanase, α -dextrin 6-glucanohydrolase) hydrolyses specifically α 1-6 glucosidic links in branched dextrins. In cereals, LD is primarily synthesised in the aleurone during germination. Gene expression is stimulated by embryo-derived gibberellins, and the enzyme is exported to the endosperm (Manners and Yellowlees, 1971; Manners and Yellowlees 1973; Hardie, 1975; (Schroeder & MacGregor, 1998). Here, LD helps to break down starch during germination, or during the malting of barley, by releasing dextrins from amylopectin which can be further hydrolysed by α- and β-amylase to provide sugars to be used as a carbon source for the germinating embryo. LD is also synthesised and active in the embryo and endosperm during early grain development (up to four weeks post anthesis), albeit at much lower levels; about one tenth of that found during germination (Lenoir et al., 1984; Sissons et al., 1993; (Burton *et al.*, 1999); (Kristensen *et al.*, 1999)). The timing and location of LD activity during grain development suggests that LD may also play a role in starch formation.

A model of amylopectin synthesis, the glucan trimming model, is proposed to function through the coordinated action of starch synthases, starch branching enzyme, which

introduces the α 1-6 linkages, and starch debranching enzymes. Debranching enzyme is thought to remove outer-branched chains from the growing molecule but cannot access inner branches (Ball et al., 1996; Myers et al., 2000). An alternative model for amylopectin synthesis has been proposed whereby it has been argued that the starch degrading enzymes are not required for amylopectin synthesis, but serve to prevent the accumulation of non-amylopectin phytoglycogen-like polymers (Zeeman et al 1998). Furthermore, the roles of the two classes of debranching enzymes, LD and isoamylase, has been a matter of some debate. Barley isoamylase is also expressed in the endosperm during grain development (Sun et al., 1999). In the absence of debranching activity, a very highly branched form of starch, known as phytoglycogen is formed, as is seen in the SUGARY mutants of maize and rice. Both the maize (James et al., 1995) and rice (Nakamura et al., 1996a) *SU1* genes were found to encode isoamylase, however the *su1* mutations in maize and rice are pleiotropic, both have reduced LD activity (Nakamura et al. 1996b, 1997). Isoamylase mutants of maize (Creech, 1968) and barley (Burton et al., 2002) have changes in the number and structure of starch granules. Recently a null mutation in maize LD (*zpu1*) has been described, in which apparently normal starch quality and quantity accumulates in the endosperm. However, the *zpu1/su1* double maize mutant, having reduced activity of isoamylse and pullulanase showed reduced starch levels compared to *su1* alone, arguing for a compensatory role of LD in the *su1* mutant for amylopectin biosynthesis (Dinges et al., 2003). In addition it was observed that there was an increase in the number of starch granules in the double mutant and a decrease in the average size of the granules (Dinges et al., 2003).

Although the biochemical pathway leading to the production of starch in leaves and storage organs has been extensively studied, the processes involved in the initiation and control of granule size are not understood. There is therefore an interest in, and a need for, a method of modifying the number and/or size of starch granules in plants which has not been met by the prior art.

During germination, LD is found in a "free" and an inactive "bound" form, which can be released and activated by treatment with reducing agents, which may activate proteases (Longstaff and Bryce, 1993). It is thought that bound LD may be a limiting factor in the conversion of starch to sugars during malting (Sissons et al., 1995) and hence contribute to the efficiency of the conversion of starch to alcohol in brewing. Bound LD is thought to be

complexed with limit dextrinase inhibitor (LDI), two heat stable low molecular weight (approximately 12.5 kD) proteins of differing pI (7.2 and 6.7) (Macri *et al.*, 1993); (MacGregor *et al.*, 1994). Peptide sequencing of these LDIs showed them both to have the same amino acid sequence, and to be identical to the deduced sequence of a barley cDNA thought to encode an α-amylase/trypsin inhibitor, with a proposed structure consisting of four α-helices joined by loops with four intramolecular disulphide bonds and one free cysteine. The isoforms of the inhibitors were found to differ through a glutathione residue bound to the free thiol group of the low pI form, and cysteine to the high pI form and were found to be specific in their inhibitory effect for LD, not inhibiting α-amylase or trypsin (MacGregor *et al.*, 2000). The nascent protein is predicted to possess a cleaved N-terminal signalling peptide of 24 amino acids, and a mature length of 123 amino acids. Modification of this inhibitor activity may alter the malting quality of barley, but to date this has not been demonstrated (MacGregor *et al.*, 2000). There is, furthermore, no indication or suggestion in the prior art that any limit dextrinase inhibitor gene can be used to alter the number and/or size of starch granules in plants.

3 SUMMARY OF THE INVENTION

In a first aspect of the invention, there is provided a method for producing plants with starch granule modification comprising introducing into a plant a nucleotide sequence comprising a limit dextrinase inhibitor gene as shown in SEQ ID No.1, or a fragment or variant thereof, or a sequence having at least 40% identity thereto.

Preferably the starch granule modification is one or more of the following: altered number and/or size of starch granules or granules of a more uniform size, or altered structure or composition of starch.

In a second aspect of the invention, there is provided a method for producing plants with altered ability to degrade starch comprising introducing into a plant a nucleotide sequence comprising a limit dextrinase inhibitor gene as shown in SEQ ID No.1, or a fragment or variant thereof, or a sequence having at least 40% identity thereto.

In the present application, a limit dextrinase inhibitor gene is defined as:

(i) a nucleotide sequence encoding a limit dextrinase inhibitor protein; or

(ii) a nucleotide sequence encoding a protein which is substantially homologous to a limit dextrinase inhibitor protein, the sequence having at least 60% identity thereto; or

(iii) a nucleotide sequence encoding a polypeptide comprising an amino acid sequence which is substantially homologous to a limit dextrinase inhibitor protein, the sequence having at least 60% identity thereto; or

(iv) a nucleotide sequence which hybridises under stringent conditions to a sequence of (i), (ii) or (iii) or its complement.

The nucleotide sequence may be an isolated nucleotide sequence.

Preferably the limit dextrinase inhibitor gene is a plant gene. Suitable genes include the *Hordeum vulgare* sequence given in SEQ ID No. 3; the *Triticum aestivum* sequence given in SEQ ID No. 5; the *Hordeum spontaneum* sequence given in SEQ ID No. 7; the *Oryza sativa* sequence given in SEQ ID No. 9; the *Triticum durum* sequence given in SEQ ID No. 11 or the *Zea mays* sequence given in SEQ ID No. 13. Particularly preferred nucleotide sequences comprising limit dextrinase inhibitor genes include the barley limit dextrinase inhibitor sequence shown in SEQ ID No. 1. Suitably a fragment or variant of any one of the above sequences or a sequence substantially homologous thereto may also be used in the present invention. Further nucleotide sequences comprising limit dextrinase inhibitor genes may be identified by sequence homology, for example by designing degenerate PCR primers using known limit dextrinase inhibitor genes as described in the Examples herein.

Nucleotide sequences comprising limit dextrinase inhibitor genes may also be identified within database collections of nucleic acid or protein sequences by, for example performing a BLAST with a known limit dextrinase inhibitor sequence in order to recover homologous sequences. Preferred nucleotide sequences according to this embodiment of the invention include the barley limit dextrinase inhibitor sequence shown in SEQ ID No. 1.

A nucleotide sequence comprising a limit dextrinase inhibitor gene, as defined above, may additionally comprise regulatory elements controlling its expression. The regulatory elements may be homologous or heterologous to the nucleotide sequence. The nucleotide sequence comprising a limit dextrinase inhibitor gene, and/or the regulatory elements, may be native or foreign to the plant into which it is introduced.

The nucleotide sequences of the invention may be DNA, RNA or any other option.

The present invention provides a method for producing plants with an altered number, size or composition of starch granules. Starch degradation may be altered by augmenting or disrupting the expression of the endogenous gene or genes involved in starch degradation, particularly debranching enzymes. Enzyme activity may be up-regulated or down-regulated.

Over expression of the introduced nucleotide sequence comprising a limit dextrinase inhibitor gene, *i.e.* increasing the copy number of the introduced sequence such that more limit dextrinase inhibitor protein is produced will lead to there being less limit dextrinase activity and less starch degradative capacity. Decreasing the amount of limit dextrinase inhibitor, which may be achieved, for example, by antisense down regulation, co-suppression (*e.g.* by introduction of partial sense sequences), or double stranded RNA technology (also known as duplex technology), all techniques well known in the art, will lead to there being more limit dextrinase activity and more starch degradative capacity

As far as antisense nucleic acid is concerned, introducing the coding region in the reverse orientation to that found in nature can result in the down-regulation of the gene and hence the production of less or none of the gene product. The RNA transcribed from antisense DNA is capable of binding to, and destroying the function of, a sense RNA of the sequence normally found in the cell, thereby disrupting function. Examples of suitable antisense DNA's are the antisense DNA's of the sense sequence shown in SEQ ID No. 1. Double stranded RNA technology also acts via the formation of stable double stranded RNA molecules.

The invention also encompasses plants produced by the method of the invention, and propagating material of said plants such as seeds and tubers. The present invention also provides a plant cell from a plant produced by the method of the invention. In each case, the plant, propagating material or plant cell contains therein a nucleotide sequence encoding a limit dextrinase inhibitor according to the invention. The invention further provides starch of said plants.

In a further aspect of the invention there are provided novel nucleotide sequences comprising limit dextrinase inhibitor genes, or fragments thereof, useful in the method of the invention. These sequences include the barley sequence shown in SEQ ID NO 1, or part thereof, or a homologous sequence having at least 60% identity thereto.

In a further aspect of the invention is provided limit dextrinase inhibitor gene products. The invention includes a protein comprising an amino acid sequence as shown in SEQ ID No.2, a fragment thereof, or an amino acid sequence having at least 99% identity thereto.

The degree to which the number and/or size of the starch granules of the plant is affected will depend at least upon the nature of the nucleotide sequence introduced into the

plant, and the amount present. By altering these variables, a person skilled in the art can regulate the degree to which starch granule number and/or size is altered according to the desired end result.

Preferably the sequence is under the control of a promoter, which promoter preferably directs expression in the starch storage tissue or is a constitutive promoter which is expressed in a starch storage tissue. A suitable promoter in potato would be the promoter of the patatin gene, for example. A suitable promoter in cereals would be an endosperm specific promoter such as the promoter of the wheat high molecular weight glutenin (HMWG) gene, or the maize ubiquitin promoter, for example.

The nucleotide sequences of the invention are preferably in the form of a vector. Such vectors form an additional aspect of the invention. The vector may be, for example, a plasmid, cosmid or phage. Vectors will frequently include one or more selectable markers to enable selection of cells that have been transfected or transformed and to enable the selection of cells harbouring vectors incorporating heterologous DNA. Alternatively the selectable marker gene may be in a different vector to be used simultaneously with a vector containing the gene of interest.

Examples of suitable marker genes include antibiotic resistance genes such as those conferring resistance to kanamycin, G418 and hygromycin (*npt-II, hyg*-B); herbicide resistance genes such as those conferring resistance to phosphinothricin and sulphonamide based herbicides (*bar* and *sul* respectively; EP-A-242246, EP-A- 0369637) and screenable markers such as beta-glucuronidase (GB2197653), luciferase and green fluorescent protein. The marker gene is preferably controlled by a second promoter which allows expression in cells other than the seed, thus allowing selection of cells or tissue containing the marker at any stage of development of the plant. Preferred second promoters are the promoter of the nopaline synthase gene of *Agrobacterium* and the promoter derived from the gene which encodes the 35S subunit of cauliflower mosaic virus (CaMV) coat protein. In cereals, the promoters of the rice actin gene and the maize ubiquitin gene are preferred. However, any other suitable second promoter may be used.

The present invention is applicable to all plants which produce or store starch. Examples of such plants are cereals such as maize, wheat, rice, sorghum, barley; fruit producing species such as banana, apple, tomato or pear; root crops such as cassava, potato,

yam or turnip; oilseed crops such as rape seed, canola, sunflower, oil palm, coconut, linseed or groundnut; meal crops such as soya bean or pea; and any other suitable species.

In a preferred embodiment of the present invention, the method comprises the additional step of growing the plant, and harvesting the starch therefrom. In order to harvest the starch, it is preferred that the plant is grown until it begins to yield fruit, seed or tubers which may then be removed. In a further preferred embodiment, the propagating material from the plant may be removed, for example the seeds. The plant part can be an organ such as a stem, root, leaf, or reproductive body. Alternatively, the plant part may be a modified organ such as a tuber, or the plant part is a tissue such as endosperm.

The present invention also provides a plant cell harbouring a suitable sequence, such as a sequence disclosed in the present invention, under the control of a suitable promoter as described above.

In a still further aspect the invention provides the use of a nucleotide sequence comprising a limit dextrinase inhibitor gene to produce plants with altered number, size or composition of starch granules.

Preferred features of each aspect of the invention are as for each other aspect *mutatis mutandis*.

3.1 SEQUENCE IDENTIFIERS

The present invention will now be illustrated by way of non-limiting examples, with reference to the sequence identifiers and Figures, in which:

SEQ ID NO 1 shows the nucleotide and derived amino acid sequence for the isolated *Hordeum vulgare* cDNA clone.

SEQ ID NO 2 shows the derived amino acid sequence for the isolated *Hordeum vulgare* cDNA clone.

SEQ ID NO 3 shows the nucleotide and derived amino acid sequence for the *Hordeum vulgare* cDNA clone for an alpha-amylase/trypsin inhibitor Genbank accession number X13443.

SEQ ID NO 4 shows the derived amino acid sequence for the *Hordeum vulgare* cDNA clone for an alpha-amylase/trypsin inhibitor Genbank accession number X13443.

SEQ ID NO 5 shows the nucleotide and derived amino acid sequence for the *Triticum aestivum* cDNA for the PUP88 protein Genbank accession number X99982.

SEQ ID NO 6 shows the derived amino acid sequence for the *Triticum aestivum* cDNA for the PUP88 protein Genbank accession number X99982.

SEQ ID NO 7 shows the nucleotide and derived amino acid sequence for the *Hordeum sponteneum* cDNA for the Itr1 gene for BTI-Cme2.2 protein Genbank accession number AJ222975.

SEQ ID NO 8 shows the derived amino acid sequence for the *Hordeum sponteneum* cDNA for the Itr1 gene for BTI-Cme2.2 protein Genbank accession number AJ222975.

SEQ ID NO 9 shows the nucleotide and derived amino acid sequence for the *Oryza sativa* cDNA for the putative hageman factor inhibitor protein Genbank accession number AP005197.

SEQ ID NO 10 shows the derived amino acid sequence for the *Oryza sativa* cDNA for the putative hageman factor inhibitor protein Genbank accession number AP005197.

SEQ ID NO 11 shows the nucleotide and derived amino acid sequence for the *Triticum durum* cDNA for the alpha amylase inhibitor protein Genbank accession number X61032.

SEQ ID NO 12 shows the derived amino acid sequence for the *Triticum durum* cDNA for the alpha amylase inhibitor protein Genbank accession number X61032.

SEQ ID NO 13 shows the nucleotide and derived amino acid sequence for the *Zea mays* cDNA for the Hageman factor inhibitor protein Genbank accession number X54064.

SEQ ID NO 14 shows the derived amino acid sequence for the *Zea mays* cDNA for the Hageman factor inhibitor protein Genbank accession number X54064.

SEQ ID NO 15 shows the amino acid sequence for the *Eleusine coracana* alpha amylase/trypsin inhibitor protein accession number WILAI.

SEQ ID NO 16 shows the amino acid sequence for the *Secale cereale* trypsin inhibitor protein accession number S29002.

SEQ ID NO 17 shows the nucleotide sequence for a PCR primer Inhib5.

SEQ ID NO 18 shows the nucleotide sequence for a PCR primer Inhib6.

SEQ ID NO 19 shows the nucleotide sequence for a PCR primer OCSF.

SEQ ID NO 20 shows the nucleotide sequence for a PCR primer OCS-R.

SEQ ID NO 21 shows the nucleotide sequence for a PCR primer BarI.

SEQ ID NO 22 shows the nucleotide sequence for a PCR primer BarII.

SEQ ID NO 23 shows the nucleotide sequence for a PCR primer OCSII.

SEQ ID NO 24 shows the nucleotide sequence for a PCR primer TUBF.

SEQ ID NO 25 shows the nucleotide sequence for a PCR primer TUBR.

4. BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows an alignment of limit dextrinase inhibitor amino acid sequences.

Figure 2 shows the transformation vector pYSUanti. pYSUanti consists of the maize ubiquitin (*Ubi-1*) promoter, the *LDI* gene (LDI) in antisense direction and the *OCS* terminator (OCS). I: primer site Inhib-6, O: primer site OCS-II.

Figure 3 shows PCR of genomic DNA from T_0 transformed plants of different lines transformed with pYSUanti. **(A)** primers Inhib-6 + OCS-II for the *LDI* gene in antisense direction (817 bp). **(B)** primers BAR-I + BAR-II for the *bar* gene (534 bp). **(C)** primers TUB-F+ TUB-R for the *tubulin* gene (217 bp). **U1-7:** independent transgenic lines; **wt**: wildtype barley plant; λ: λ/*Hind* III molecular weight marker; **-ve**: negative control of PCR.

Figure 4 shows the analysis of six single segregating T_1 seeds of transgenic line U3.

Figure 5 shows an immunoblot of LDI extracts of mature transgenic and wildtype grain.

Figure 6 shows the limit dextrinase inhibitor activity of mature wildtype and homozygous transgenic grain. The control (100%) represents the amount of LD used for each assay. LDI extracts corresponding to 10 μg protein were mixed with LD and assayed for LD activity. **wt**: wildtype; **U3**: homozygous T_2 generation transgenic line U3; **U4**: homozygous T_2 generation transgenic line U4. Each value represents the mean ± SE of three replicate experiments.

Figure 7 shows environmental scanning electron micrographs of the outer endosperm region of cross-sections of four single barley grains.

Figure 8 shows the separation of total α-glucan by Sepharose CL2B chromatography. **(A)** wildtype and homozygous T_2 generation transgenic line U3. **(B)** wildtype and homozygous T_2 generation transgenic line U4. Samples of total α-glucans were prepared from mature endosperms of wildtype and transgenic barley grains as described in experimental procedures. After chromatography, the fractions were stained with Iodine solution and the absorbance at 595nm measured. The absorbance is expressed as percentages of maximum absorbance. Each value represents the mean ± SE of three replicate experiments. Where absent, the error bars are smaller than the symbols.

Figure 9 shows the nucleotide and amino acid percentage identities of the sequences hereof.

5. DETAILED DESCRIPTION OF THE INVENTION

The invention relates to a family of plant limit dextrinase inhibitor genes. In various embodiments, the invention provides plant limit dextrinase inhibitor nucleic acid molecules; plant limit dextrinase inhibitor regulatory regions; plant limit dextrinase inhibitor promoters; and vectors incorporating sequences encoding plant limit dextrinase inhibitor nucleic acid molecules of the invention. Also provided are plant limit dextrinase inhibitor gene products, including, but not limited to, transcriptional products such as mRNAs, antisense and ribozyme molecules, and translational products such as the plant limit dextrinase inhibitor protein, polypeptides, peptides and fusion proteins related thereto; genetically engineered host cells that contain any of the foregoing nucleic acid molecules and/or coding sequences or compliments, variants, or fragments thereof operatively associated with a regulatory element that directs the expression of the gene and/or coding sequences in the host cell; genetically-engineered plants derived from host cells; modified starch and starch granules produced by genetically-engineered host cells and plants; and the use of the foregoing to improve agronomically valuable plants.

In the context of the present invention, "plant limit dextrinase inhibitor protein" includes any limit dextrinase inhibitor protein which is capable of changing starch granule production in a plant. By definition, the plant limit dextrinase inhibitor protein will be of plant origin. Preferred fragments of plant limit dextrinase inhibitor proteins are those which retain the ability to change starch granule synthesis.

For purposes of clarity, and not by way of limitation, the invention is described in the subsections below in terms of (a) plant limit dextrinase inhibitor nucleic acid molecules; (b) plant limit dextrinase inhibitor gene products; (c) transgenic plants that ectopically express plant limit dextrinase inhibitor protein; (d); transgenic plants in which endogenous plant limit dextrinase inhibitor protein expression is suppressed; (e) starch characterized by altered structure and physical properties produced by the methods of the invention.

5.1 PLANT LIMIT DEXTRINASE INHIBITOR NUCLEIC ACIDS

The nucleic acid molecules of the invention may be DNA, RNA and comprises the nucleotide sequences of a plant limit dextrinase inhibitor gene, or fragments or variants thereof. A polynucleotide is intended to include DNA molecules (*e.g.*, cDNA, genomic

DNA), RNA molecules (*e.g.*, hnRNA, pre-mRNA, mRNA, double-stranded RNA), and DNA or RNA analogs generated using nucleotide analogues. The polynucleotide can be single-stranded or double-stranded.

The nucleic acid molecules are characterized by their homology to known limit dextrinase inhibitor genes, such as those from barley, wheat, maize, rice, ragi, rye and durum wheat.

The present invention provides:

(i) an isolated nucleotide sequence of SEQ. ID. No. 1, or a sequence having at least 80% identity thereto;

(ii) an isolated nucleotide sequence that is more than 66%, 66%, 47% and 50% identical to SEQ. ID. Nos. 5, 7, 9 and 13 respectively, or a fragment or variant thereof; or

(iii) an isolated nucleotide sequence encoding a polypeptide comprising an amino acid sequence that is more than 82%, 80%, 68%, 35%, 55%, 68% and 81% identical to SEQ. ID. Nos. 2, 6, 8, 10, 14, 15 and 16 respectively, or a fragment or variant thereof; or

(iv) a nucleotide sequence which hybridises under stringent conditions to a sequence of (i), (ii) or (iii), or its complement.

As used herein the phrase nucleic acid sequence refers to the sequence of a nucleic acid molecule.

A preferred nucleic acid molecule of this embodiment is one that encodes the amino acid sequence of SEQ ID NO: 2, or a fragment or variant thereof. In a most preferred embodiment, the nucleic acid molecule comprises the nucleotide sequence shown in SEQ ID NO: 1, or a fragment or variant thereof, or a sequence substantially similar to SEQ ID NO: 1. The percentage identity to SEQ. ID. No. 1 or 2 may be as high as 99%.

The variants may be an allelic variant. Allelic variants are multiple forms of a particular gene or protein encoded by a particular gene. Fragments of a plant limit dextrinase inhibitor gene may include regulatory elements of the gene such as promoters, enhancers, transcription factor binding sites, and/or segments of a coding sequence for example, a conserved domain, exon, or transit peptide.

In a preferred embodiment, the isolated nucleic acid molecules of the invention are comprised of full length sequences in that they encode an entire plant limit dextrinase

inhibitor protein as it would occur in nature. Examples of such sequences include SEQ ID NOs: 1 and 3. The corresponding amino acid sequences of full length limit dextrinase inhibitor proteins are SEQ ID NOs: 2 and 4.

In alternative embodiments, the nucleic acid molecules of the invention comprise a nucleotide sequence of SEQ ID NOs: 1, 3, 5, 7, 9, 11 or 13.

The nucleic acid molecules and their variants can be identified by several approaches including but not limited to analysis of sequence similarity and hybridization assays.

In the context of the present invention the term "substantially homologous," "substantially identical," or "substantial similarity," when used herein with respect to sequences of nucleic acid molecules, means that the sequence has either at least 40% sequence identity with the reference sequence, preferably 50% sequence identity, more preferably at least 60%, 70%, 80%, 90% and most preferably at least 95% sequence identity with said sequences, in some cases the sequence identity may be 98% or more preferably 99%, or above, or the term means that the nucleic acid molecule is capable of hybridizing to the complement of the nucleic acid molecule having the reference sequence under stringent conditions.

Where homology is determined on the basis of percentage identity between the two sequences, the homologous sequences are those which have at least 45% sequence identity, preferably 50% sequence identity, more preferably at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% sequence identity with said sequences. In some cases the sequence identity may be 98% or more preferably 99%, or above.

"% identity", as known in the art, is a measure of the relationship between two polynucleotides or two polypeptides, as determined by comparing their sequences. In general, the two sequences to be compared are aligned to give a maximum correlation between the sequences. The alignment of the two sequences is examined and the number of positions giving an exact amino acid or nucleotide correspondence between the two sequences determined, divided by the total length of the alignment and multiplied by 100 to give a % identity figure. This % identity figure may be determined over the whole length of the sequences to be compared, which is particularly suitable for sequences of the same or very similar length and which are highly homologous, or over shorter defined lengths, which is more suitable for sequences of unequal length or which have a lower level of homology.

For example, sequences can be aligned with the software clustalw under Unix which generates a file with a ".aln" extension, this file can then be imported into the Bioedit program (Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95-98) which opens the .aln file. In the Bioedit window, one can choose individual sequences (two at a time) and alignthem. This method allows for comparison of entire sequences.

Methods for comparing the identity of two or more sequences are well known in the art. Thus, for instance, programs available in the Wisconsin Sequence Analysis Package, version 9.1 (Devereux J *et al*, Nucleic Acids Res. 12:387-395, 1984, available from Genetics Computer Group, Madison, Wisconsin, USA). The determination of percent identity between two sequences can be accomplished using a mathematical algorithm. For example, the programs BESTFIT and GAP, may be used to determine the % identity between two polynucleotides and the % identity between two polypeptide sequences. BESTFIT uses the "local homology" algorithm of Smith and Waterman (Advances in Applied Mathematics, 2:482-489, 1981) and finds the best single region of similarity between two sequences. BESTFIT is more suited to comparing two polynucleotide or two polypeptide sequences which are dissimilar in length, the program assuming that the shorter sequence represents a portion of the longer. In comparison, GAP aligns two sequences finding a "maximum similarity" according to the algorithm of Neddleman and Wunsch (J. Mol. Biol. 48:443-354, 1970). GAP is more suited to comparing sequences which are approximately the same length and an alignment is expected over the entire length. Preferably the parameters "Gap Weight" and "Length Weight" used in each program are 50 and 3 for polynucleotides and 12 and 4 for polypeptides, respectively. Preferably % identities and similarities are determined when the two sequences being compared are optimally aligned.

Other programs for determining identity and/or similarity between sequences are also known in the art, for instance the BLAST family of programs (Karlin & Altschul, 1990, *Proc. Natl. Acad. Sci. USA* 87:2264-2268, modified as in Karlin & Altschul, 1993, *Proc. Natl. Acad. Sci. USA* 90:5873-5877, available from the National Center for Biotechnology Information (NCB), Bethesda, Maryland, USA and accessible through the home page of the NCBI at www.ncbi.nlm.nih.gov). These programs exemplify a preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, et al., 1990, *J. Mol. Biol.* 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score =

50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., 1997, *Nucleic Acids Res.* 25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules (*Id.*). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. *See* http://www.ncbi.nlm.nih.gov. Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, 1988, *CABIOS* 4:11-17. Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

Another non-limiting example of a program for determining identity and/or similarity between sequences known in the art is FASTA (Pearson W.R. and Lipman D.J., Proc. Nat. Acac. Sci., USA, 85:2444-2448, 1988, available as part of the Wisconsin Sequence Analysis Package). Preferably the BLOSUM62 amino acid substitution matrix (Henikoff S. and Henikoff J.G., Proc. Nat. Acad. Sci., USA, 89:10915-10919, 1992) is used in polypeptide sequence comparisons including where nucleotide sequences are first translated into amino acid sequences before comparison.

Yet another non-limiting example of a program known in the art for determining identity and/or similarity between amino acid sequences is SeqWeb Software (a web-based interface to the GCG Wisconsin Package: Gap program) which is utilized with the default algorithm and parameter settings of the program: blosum62, gap weight 8, length weight 2.

The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, typically exact matches are counted.

Preferably the program BESTFIT is used to determine the % identity of a query polynucleotide or a polypeptide sequence with respect to a polynucleotide or a polypeptide sequence of the present invention, the query and the reference sequence being optimally aligned and the parameters of the program set at the default value.

Alternatively, variants and fragments of the nucleic acid molecules of the invention can be identified by hybridization to SEQ ID NOs: 1, 3, 5, 7, 9, 11 or 13. In the context of the present invention "stringent conditions" are defined as those given in Martin *et al* (EMBO J 4:1625-1630 (1985)) and Davies *et al* (Methods in Molecular Biology Vol 28: Protocols for

nucleic acid analysis by non-radioactive probes, Isaac, P.G. (ed) pp 9-15, Humana Press Inc., Totowa N.J, USA)). The conditions under which hybridization and/or washing can be carried out can range from 42°C to 68°C and the washing buffer can comprise from 0.1 x SSC, 0.5 % SDS to 6 x SSC, 0.5 % SDS. Typically, hybridization can be carried out overnight at 65°C (high stringency conditions), 60°C (medium stringency conditions), or 55°C (low stringency conditions). The filters can be washed for 2 x 15 minutes with 0.1 x SSC, 0.5 % SDS at 65°C (high stringency washing). The filters were washed for 2 x 15 minutes with 0.1 x SSC, 0.5 % SDS at 63°C (medium stringency washing). For low stringency washing, the filters were washed at 60°C for 2 x 15 minutes at 2 x SSC, 0.5% SDS.

In instances wherein the nucleic acid molecules are oligonucleotides ("oligos"), highly stringent conditions may refer, *e.g.*, to washing in 6xSSC / 0.05% sodium pyrophosphate at 37°C (for 14-base oligos), 48°C (for 17-base oligos), 55°C (for 20-base oligos), and 60°C (for 23-base oligos). These nucleic acid molecules may act as plant limit dextrinase inhibitor gene antisense molecules, useful, for example, in plant limit dextrinase inhibitor gene regulation and/or as antisense primers in amplification reactions of plant limit dextrinase inhibitor gene and/or nucleic acid molecules. Further, such nucleic acid molecules may be used as part of ribozyme and/or triple helix sequences, also useful for plant limit dextrinase inhibitor gene regulation. Still further, such molecules may be used as components in probing methods whereby the presence of a plant limit dextrinase inhibitor allele may be detected.

In one embodiment, a nucleic acid molecule of the invention may be used to identify other plant limit dextrinase inhibitor genes by identifying homologues. This procedure may be performed using standard techniques known in the art, for example screening of a cDNA library by probing; amplification of candidate nucleic acid molecules; complementation analysis, and yeast two-hybrid system (Fields and Song Nature 340 245-246 (1989); Green and Hannah Plant Cell 10 1295-1306 (1998)).

The invention also includes nucleic acid molecules, preferably DNA molecules, that are amplified using the polymerase chain reaction and that encode a gene product functionally equivalent to a plant limit dextrinase inhibitor gene product.

In another embodiment of the invention, nucleic acid molecules which hybridize under stringent conditions to the nucleic acid molecules comprising a plant limit dextrinase inhibitor gene and its complement are used in altering starch synthesis in a plant. Such nucleic acid molecules may hybridize to any part of a plant limit dextrinase inhibitor gene, including the regulatory elements. Preferred nucleic acid molecules are those which hybridize under stringent conditions to a nucleic acid molecule comprising the nucleotide sequence encoding the amino

acid sequence of SEQ ID NO: 2, 15 or 16 and/or a nucleotide sequence of any one of SEQ ID NOs: 1, 3, 5, 7, 9, 11 or 13, or their complement sequences. Preferably the nucleic acid molecule which hybridizes under stringent conditions to a nucleic acid molecule comprising the sequence of a plant limit dextrinase inhibitor gene or its complement are complementary to the nucleic acid molecule to which they hybridize.

In another embodiment of the invention, nucleic acid molecules which hybridize under stringent conditions to the nucleic acid molecules of SEQ ID NOs: 1, 3, 5, 7, 9, 11 or 13 hybridize over the full length of the sequences of the nucleic acid molecules are provided.

Alternatively, nucleic acid molecules of the invention or their expression products may be used in screening for agents which alter the activity of a plant limit dextrinase inhibitor protein of a plant. Such a screen will typically comprise contacting a putative agent with a nucleic acid molecule of the invention or expression product thereof and monitoring the reaction there between. The reaction may be monitored by expression of a reporter gene operably linked to a nucleic acid molecule of the invention, or by binding assays which will be known to persons skilled in the art.

Fragments of a plant limit dextrinase inhibitor nucleic acid molecule of the invention preferably comprise or consist of at least 40 continuous or consecutive nucleotides of the plant limit dextrinase inhibitor nucleic acid molecule of the invention, more preferably at least 60 nucleotides, at least 80 nucleotides, or most preferably at least 100 or 150 nucleotides in length. Fragments of a plant limit dextrinase inhibitor nucleic acid molecule of the invention encompassed by the invention may include elements involved in regulating expression of the gene or may encode functional plant limit dextrinase inhibitor proteins. Fragments of the nucleic acid molecules of the invention encompasses fragments of SEQ ID NOs: 1, 3, 5, 7, 9, 11 or 13, as well as fragments of the variants of those sequences identified as defined above by percent homology or hybridization.

Further, a plant limit dextrinase inhibitor nucleic acid molecule of the invention can comprise two or more of any above-described sequences, or variants thereof, linked together to form a larger subsequence.

In certain embodiments, the plant limit dextrinase inhibitor nucleic acid molecules and polypeptides do not include sequences consisting of those sequences known in the art. For example, in one embodiment, the plant limit dextrinase inhibitor nucleic acid molecules do not include EST sequences.

An isolated nucleic acid molecule encoding a variant protein can be created by introducing one or more nucleotide substitutions, additions or deletions into the plant limit

dextrinase inhibitor nucleic acid molecule, such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations can be introduced by standard techniques, such as, ethyl methane sulfonate, X-rays, gamma rays, T-DNA mutagenesis, site-directed mutagenesis, or PCR-mediated mutagenesis. Briefly, PCR primers are designed that delete the trinucleotide codon of the amino acid to be changed and replace it with the trinucleotide codon of the amino acid to be included. This primer is used in the PCR amplification of DNA encoding the protein of interest. This fragment is then isolated and inserted into the full length cDNA encoding the protein of interest and expressed recombinantly.

An isolated nucleic acid molecule encoding a variant protein can be created by any of the methods described in section 5.1. Either conservative or non-conservative amino acid substitutions can be made at one or more amino acid residues. Both conservative and non-conservative substitutions can be made. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids can be divided into four families: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) nonpolar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. In similar fashion, the amino acid repertoire can be grouped as (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine histidine, (3) aliphatic = glycine, alanine, valine, leucine, isoleucine, serine, threonine, with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic = phenylalanine, tyrosine, tryptophan; (5) amide = asparagine, glutamine; and (6) sulfur -containing = cysteine and methionine. (*See*, for example, Biochemistry, 4th ed., Ed. by L. Stryer, WH Freeman and Co.: 1995).

Alternatively, mutations can be introduced randomly along all or part of the coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity to identify mutants that retain activity. Following mutagenesis, the encoded protein can be expressed recombinantly and the activity of the protein can be determined.

The invention also encompasses (a) DNA vectors that contain any of the foregoing nucleic acids and/or coding sequences (i.e. fragments and variants) and/or their complements (*i.e.*, antisense molecules); (b) DNA expression vectors that contain any of the foregoing nucleic acids and/or coding sequences operatively associated with a regulatory region that directs the expression of the nucleic acids and/or coding sequences; and (c) genetically engineered host cells that contain any of the foregoing nucleic acids and/or coding sequences operatively associated with a regulatory region that directs the expression of the gene and/or coding sequences in the host cell. As used herein, regulatory regions include, but are not limited to, inducible and non-

inducible genetic elements known to those skilled in the art that drive and regulate expression of a nucleic acid. The nucleic acid molecules of the invention may be under the control of a promoter, enhancer, operator, cis-acting sequences, or trans-acting factors, or other regulatory sequence. The nucleic acid molecules encoding regulatory regions of the invention may also be functional fragments of a promoter or enhancer. The nucleic acid molecule encoding a regulatory region is preferably one which will target expression to desired cells, tissues, or developmental stages.

Examples of highly suitable nucleic acid molecules encoding regulatory regions are endosperm specific promoters, such as that of the high molecular weight glutenin (HMWG) gene of wheat, prolamin or ITR1, or other suitable promoters available to the skilled person such as gliadin, branching enzyme, ADPG pyrophosphorylase, patatin, starch synthase, and actin, for example.

Other suitable promoters include the stem organ specific promoter gSPO-A, the seed specific promoters Napin, KTI 1, 2, & 3, beta-conglycinin, beta-phaseolin, heliathin, phytohemaglutinin, legumin, zein, lectin, leghemoglobin c3, ABI3, PvAlf, SH-EP, EP-C1, 2S1, EM 1, and ROM2.

Constitutive promoters, such as CaMV promoters, including CaMV 35S and CaMV 19S may also be suitable. Other examples of constitutive promoters include Actin 1, Ubiquitin 1, and HMG2.

In addition, the regulatory region of the invention may be one which is environmental factor-regulated such as promoters that respond to heat, cold, mechanical stress, light, ultra-violet light, drought, salt and pathogen attack. The regulatory region of the invention may also be one which is a hormone-regulated promoter that induces gene expression in response to phytohormones at different stages of plant growth. Useful inducible promoters include, but are not limited to, the promoters of ribulose bisphosphate carboxylase (RUBISCO) genes, chlorophyll a/b binding protein (CAB) genes, heat shock genes, the defense responsive genes (*e.g.*, phenylalanine ammonia lyase genes), wound induced genes (*e.g.*, hydroxyproline rich cell wall protein genes), chemically-inducible genes (*e.g.*, nitrate reductase genes, gluconase genes, chitinase genes, PR-1 genes *etc.*), dark-inducible genes (*e.g.*, asparagine synthetase gene as described by U.S. Patent 5,256,558), and developmental-stage specific genes (*e.g.*, Shoot Meristemless gene, ABI3 promoter and the 2S1 and Em 1 promoters for seed development (Devic et al.,1996, Plant Journal 9(2):205-215), and the kin1 and cor6.6 promoters for seed development (Wang et al., 1995, Plant Molecular Biology, 28(4):619-634). Examples of other inducible promoters and developmental-stage specific promoters can be found in Datla et al., in

particular in Table 1 of that publication (Datla et al., 1997, Biotechnology annual review 3:269-296).

A vector of the invention may also contain a sequence encoding a transit peptide which can be fused in-frame such that it is expressed as a fusion protein.

Methods which are well known to those skilled in the art can be used to construct vectors and/or expression vectors containing plant limit dextrinase inhibitor protein coding sequences and appropriate transcriptional/translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination. See, for example, the techniques described in Sambrook et al., 1989, and Ausubel et al., 1989. Alternatively, RNA capable of encoding plant limit dextrinase inhibitor protein sequences may be chemically synthesized using, for example, synthesizers. See, for example, the techniques described in Gait, 1984, *Oligonucleotide Synthesis*, IRL Press, Oxford. In a preferred embodiment of the invention, the techniques described in section 6, example 6, and illustrated in figure 6 are used to construct a vector.

A variety of host-expression vector systems may be utilized to express the plant limit dextrinase inhibitor gene products of the invention. Such host-expression systems represent vehicles by which the plant limit dextrinase inhibitor gene products of interest may be produced and subsequently recovered and/or purified from the culture or plant (using purification methods well known to those skilled in the art), but also represent cells which may, when transformed or transfected with the appropriate nucleic acid molecules, exhibit the plant limit dextrinase inhibitor protein of the invention *in situ*. These include but are not limited to microorganisms such as bacteria (*e.g.*, *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing plant limit dextrinase inhibitor protein coding sequences; yeast (*e.g.*, *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing the plant limit dextrinase inhibitor protein coding sequences; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the plant limit dextrinase inhibitor protein coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV); plant cell systems transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing plant limit dextrinase inhibitor protein coding sequences; or mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter; the cytomegalovirus

promoter/enhancer; etc.). In a preferred embodiment of the invention, an expression vector comprising a plant limit dextrinase inhibitor nucleic acid molecule operably linked to at least one suitable regulatory sequence is incorporated into a plant by one of the methods described in this section, section 5.3, 5.4 and 5.5 or in examples 5, 6, 7, and 8.

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the plant limit dextrinase inhibitor protein being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of antibodies or to screen peptide libraries, for example, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther et al., 1983, *EMBO J.* 2:1791), in which the plant limit dextrinase inhibitor coding sequence may be ligated individually into the vector in frame with the *lac Z* coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, *Nucleic Acids Res.* 13:3101-9; Van Heeke & Schuster, 1989, *J. Biol. Chem.* 264:5503-9); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene protein can be released from the GST moiety.

In one such embodiment of a bacterial system, full length cDNA nucleic acid molecules are appended with in-frame Bam HI sites at the amino terminus and Eco RI sites at the carboxyl terminus using standard PCR methodologies (Innis et al., 1990, *supra*) and ligated into the pGEX-2TK vector (Pharmacia, Uppsala, Sweden). The resulting cDNA construct contains a kinase recognition site at the amino terminus for radioactive labelling and glutathione S-transferase sequences at the carboxyl terminus for affinity purification (Nilsson, et al., 1985, *EMBO J.* 4:1075; Zabeau and Stanley, 1982, *EMBO J.* 1: 1217).

The recombinant constructs of the present invention may include a selectable marker for propagation of the construct. For example, a construct to be propagated in bacteria preferably contains an antibiotic resistance gene, such as one that confers resistance to kanamycin, tetracycline, streptomycin, or chloramphenicol. Examples of other suitable marker genes include antibiotic resistance genes such as those conferring resistance to G4 18 and hygromycin (*npt-II, hyg*-B); herbicide resistance genes such as those conferring resistance to phosphinothricin and sulfonamide based herbicides (*bar* and *sulI* respectively; EP-A-242246, EP-A- 0369637) and screenable markers such as beta-glucoronidase (GB2 197653), luciferase and green fluorescent

protein. Suitable vectors for propagating the construct include, but are not limited to, plasmids, cosmids, bacteriophages or viruses.

The marker gene is preferably controlled by a second promoter which allows expression in cells other than the seed, thus allowing selection of cells or tissue containing the marker at any stage of development of the plant. Preferred second promoters are the promoter of nopaline synthase gene of *Agrobacterium* and the promoter derived from the gene which encodes the 35S subunit of cauliflower mosaic virus (CaMV) coat protein. However, any other suitable second promoter may be used.

The nucleic acid molecule encoding a plant limit dextrinase inhibitor protein may be native or foreign to the plant into which it is introduced. One of the effects of introducing a nucleic acid molecule encoding a plant limit dextrinase inhibitor gene into a plant is to increase the amount of plant limit dextrinase inhibitor protein present by increasing the copy number of the nucleic acid molecule. Foreign plant limit dextrinase inhibitor nucleic acid molecules may in addition have different temporal and/or spatial specificity for plastid division and starch granule synthesis compared to the native plant limit dextrinase inhibitor protein of the plant, and so may be useful in altering when and where or what type of starch is produced. Regulatory elements of the plant limit dextrinase inhibitor genes may also be used in altering plastid division and starch granule synthesis in a plant, for example by replacing the native regulatory elements in the plant or providing additional control mechanisms. The regulatory regions of the invention may confer expression of a plant limit dextrinase inhibitor gene product in a chemically-inducible, dark-inducible, developmentally regulated, developmental-stage specific, wound-induced, environmental factor-regulated, organ-specific, cell-specific, tissue-specific, or constitutive manner. Alternatively, the expression conferred by a regulatory region may encompass more than one type of expression selected from the group consisting of chemically-inducible, dark-inducible, developmentally regulated, developmental-stage specific, wound-induced, environmental factor-regulated, organ-specific, cell-specific, tissue-specific, and constitutive.

Further, any of the nucleic acid molecules and/or polypeptides and proteins described herein, can be used as markers for qualitative trait loci in breeding programs for crop plants. To this end, the nucleic acid molecules, including, but not limited to, full length plant limit dextrinase inhibitor genes coding sequences, and/or partial sequences, can be used in hybridization and/or DNA amplification assays to identify the endogenous plant limit dextrinase inhibitor genes, plant limit dextrinase inhibitor gene mutant alleles and/or plant limit dextrinase inhibitor gene expression products in cultivars as compared to wild-type plants. They can also be used as markers for linkage analysis of qualitative trait loci. It is also possible that the plant

limit dextrinase inhibitor genes may encode a product responsible for a qualitative trait that is desirable in a crop breeding program. Alternatively, the plant limit dextrinase inhibitor protein and/or peptides can be used as diagnostic reagents in immunoassays to detect expression of the plant limit dextrinase inhibitor genes in cultivars and wild-type plants.

Genetically-engineered plants containing constructs comprising the plant limit dextrinase inhibitor nucleic acid and a reporter gene can be generated using the methods described herein for each plant limit dextrinase inhibitor nucleic acid gene variant, to screen for loss-of-function variants induced by mutations, including but not limited to, deletions, point mutations, rearrangements, translocation, etc. The constructs can encode for fusion proteins comprising a plant limit dextrinase inhibitor protein fused to a protein product encoded by a reporter gene. Alternatively, the constructs can encode for a plant limit dextrinase inhibitor protein and a reporter gene product that are not fused. The constructs may be transformed into the homozygous recessive plant limit dextrinase inhibitor gene mutant background, and the restorative phenotype examined, i.e. quantity and quality of starch, as a complementation test to confirm the functionality of the variants isolated.

5.2 PLANT LIMIT DEXTRINASE INHIBITOR GENE PRODUCTS

The invention encompasses the polypeptides of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 15 or 16, or sequences that are at least 70% identical thereto, as described above. Plant limit dextrinase inhibitor proteins, polypeptides and peptide fragments, variants, allelic variants, mutated, truncated or deleted forms of plant limit dextrinase inhibitor proteins and/or plant limit dextrinase inhibitor fusion proteins can be prepared for a variety of uses, including, but not limited to, the generation of antibodies, as reagents in assays, the identification of other cellular gene products involved in plastid division and/or starch granule synthesis, etc.

Plant limit dextrinase inhibitor translational products include, but are not limited to those proteins and polypeptides encoded by the sequences of the plant limit dextrinase inhibitor nucleic acid molecules of the invention. The invention encompasses proteins that are functionally equivalent to the plant limit dextrinase inhibitor gene products of the invention.

The primary use of the plant limit dextrinase inhibitor gene products of the invention is to alter starch synthesis via changing plastid division

The present invention also provides variants of the polypeptides of the invention. Such variants have an altered amino acid sequence which can function as either agonists (mimetics) or as antagonists. Variants can be generated by mutagenesis, *e.g.*, discrete point mutation or truncation. An agonist can retain substantially the same, or a subset, of the biological activities

of the naturally occurring form of the protein. An antagonist of a protein can inhibit one or more of the activities of the naturally occurring form of the protein by, for example, deleting one or more of the receiver domains. Thus, specific biological effects can be elicited by addition of a variant of limited function.

Modification of the structure of the subject polypeptides can be for such purposes as enhancing efficacy, stability, or post-translational modifications (*e.g.*, to alter the phosphorylation pattern of the protein). Such modified peptides, when designed to retain at least one activity of the naturally-occurring form of the protein, or to produce specific antagonists thereof, are considered functional equivalents of the polypeptides. Such modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition.

For example, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (*i.e.* isosteric and/or isoelectric mutations) will not have a major effect on the biological activity of the resulting molecule.

Whether a change in the amino acid sequence of a peptide results in a functional homolog (*e.g.*, functional in the sense that the resulting polypeptide mimics or antagonizes the wild-type form) can be readily determined by assessing the ability of the variant peptide to produce a response in cells in a fashion similar to the wild-type protein, or competitively inhibit such a response. Polypeptides in which more than one replacement has taken place can readily be tested in the same manner.

In a preferred embodiment, a mutant polypeptide that is a variant of a polypeptide of the invention can be assayed for: (1) the ability to complement limit dextrinase inhibitor function in a plant system in which the native limit dextrinase inhibitor genes have been knocked out; (2) the ability to inhibit limit dextrinase; or (3) the ability to alter starch granule synthesis.

The invention encompasses functionally equivalent mutant plant limit dextrinase inhibitor proteins and polypeptides. The invention also encompasses mutant plant limit dextrinase inhibitor proteins and polypeptides that are not functionally equivalent to the gene products. Such a mutant plant limit dextrinase inhibitor protein or polypeptide may contain one or more deletions, additions or substitutions of plant limit dextrinase inhibitor amino acid residues within the amino acid sequence encoded by any one of the plant limit dextrinase inhibitor nucleic acid molecules described above in Section 5.1, and which result in loss of one or more functions of the plant limit dextrinase inhibitor protein, thus producing a plant limit

dextrinase inhibitor gene product not functionally equivalent to the wild-type plant limit dextrinase inhibitor protein.

Plant limit dextrinase inhibitor proteins and polypeptides bearing mutations can be made to plant limit dextrinase inhibitor DNA (using techniques discussed above as well as those well known to one of skill in the art) and the resulting mutant plant limit dextrinase inhibitor proteins tested for activity. Mutants can be isolated that display increased function, (*e.g.*, resulting in improved root formation), or decreased function (*e.g.*, resulting in suboptimal root function). In particular, mutated plant limit dextrinase inhibitor proteins in which any exons are deleted or mutated are within the scope of the invention. Additionally, peptides corresponding to one or more exons of the plant limit dextrinase inhibitor protein, a truncated or deleted plant limit dextrinase inhibitor protein are also within the scope of the invention. Fusion proteins in which the full length plant limit dextrinase inhibitor protein or a plant limit dextrinase inhibitor polypeptide or peptide fused to an unrelated protein are also within the scope of the invention and can be designed on the basis of the plant limit dextrinase inhibitor nucleotide and plant limit dextrinase inhibitor amino acid sequences disclosed herein.

While the plant limit dextrinase inhibitor polypeptides and peptides can be chemically synthesized (*e.g.*, see Creighton, 1983, *Proteins: Structures and Molecular Principles*, W.H. Freeman & Co., NY) large polypeptides derived from plant limit dextrinase inhibitor genes and the full length plant limit dextrinase inhibitor gene may advantageously be produced by recombinant DNA technology using techniques well known to those skilled in the art for expressing nucleic acid molecules.

Nucleotides encoding fusion proteins may include, but are not limited to, nucleotides encoding full length plant limit dextrinase inhibitor proteins, truncated plant limit dextrinase inhibitor proteins, or peptide fragments of plant limit dextrinase inhibitor proteins fused to an unrelated protein or peptide, such as for example, an enzyme, fluorescent protein, or luminescent protein that can be used as a marker or an epitope that facilitates affinity-based purificaiton. Alternatively, the fusion protein can further comprise a heterologous protein such as a transit peptide or fluorescence protein.

In an embodiment of the invention, the percent identity between two polypeptides of the invention is at least 25%. In a preferred embodiment of the invention, the percent identity between two polypeptides of the invention is at least 30%. In another embodiment, the percent identity between two polypeptides of the invention is at least 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, or at least 98%. Determining whether two sequences are substantially similar

may be carried out using any methodologies known to one skilled in the art, preferably using computer assisted analysis as described in section 5.1.

Further, it may be desirable to include additional DNA sequences in the protein expression constructs. Examples of additional DNA sequences include, but are not limited to, those encoding: a 3' untranslated region; a transcription termination and polyadenylation signal; an intron; a signal peptide (which facilitates the secretion of the protein); or a transit peptide (which targets the protein to a particular cellular compartment such as the nucleus, chloroplast, mitochondria or vacuole). The nucleic acid molecules of the invention will preferably comprise a nucleic acid molecule encoding a transit peptide, to ensure delivery of any expressed protein to the plastid. Preferably the transit peptide will be selective for plastids such as amyloplasts or chloroplasts, and can be native to the nucleic acid molecule of the invention or derived from known plastid sequences, such as those from the small subunit of the ribulose bisphosphate carboxylase enzyme (ssu of rubisco) from pea, maize or sunflower for example. The transit peptide comprising amino acid residues 1-198 of SEQ ID NO: 4 is an example of a transit peptide native to the polypeptide of the invention. Where an agonist or antagonist which modulates activity of the plant limit dextrinase inhibitor protein is a polypeptide, the polypeptide itself must be appropriately targeted to the plastids, for example by the presence of a plastid targeting signal at the N terminal end of the protein (Castro Silva Filho *et al* Plant Mol Biol 30 769-780 (1996) or by protein-protein interaction (Schenke PC *et al*, Plant Physiol 122 235-241 (2000) and Schenke *et al* PNAS 98(2) 765-770 (2001). The transit peptides of the invention are used to target transportation of plant limit dextrinase inhibitor proteins as well as agonists or antagonists thereof to plastids, the sites of starch synthesis, thus altering the plastid division and starch synthesis processes and resulting starch characteristics.

The plant limit dextrinase inhibitor proteins and transit peptides associated with the plant limit dextrinase inhibitor genes of the present invention have a number of important agricultural uses. The transit peptides associated with the plant limit dextrinase inhibitor genes of the invention may be used, for example, in transportation of desired heterologous gene products to a plastid in cells of a root, a root modified through evolution, tuber, stem, a stem modified through evolution, seed, and/or endosperm of transgenic plants transformed with such constructs.

The invention encompasses methods of screening for agents (i.e., proteins, small molecules, peptides) capable of altering the activity of a plant limit dextrinase inhibitor protein in a plant. Variants of a protein of the invention which function as either agonists (mimetics) or as antagonists can be identified by screening combinatorial libraries of mutants, *e.g.*, truncation

mutants, of the protein of the invention for agonist or antagonist activity. In one embodiment, a variegated library of variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into nucleic acid molecules such that a degenerate set of potential protein sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (*e.g.*, for phage display). There are a variety of methods which can be used to produce libraries of potential variants of the polypeptides of the invention from a degenerate oligonucleotide sequence. Methods for synthesizing degenerate oligonucleotides are known in the art (*see, e.g.,* Narang, 1983, *Tetrahedron* 39:3; Itakura et al., 1984, *Annu. Rev. Biochem.* 53:323; Itakura et al., 1984, *Science* 198:1056; Ike et al., 1983, *Nucleic Acid Res.*11:477).

In addition, libraries of fragments of the coding sequence of a polypeptide of the invention can be used to generate a variegated population of polypeptides for screening and subsequent selection of variants. For example, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of the coding sequence of interest with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double stranded DNA, renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes N-terminal and internal fragments of various sizes of the protein of interest.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. The most widely used techniques, which are amenable to high through-put analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify variants of a protein of the invention (Arkin and Yourvan, 1992, *Proc. Natl. Acad. Sci. USA* 89:7811-7815; Delgrave et al., 1993, *Protein Engineering* 6(3):327-331).

An isolated polypeptide of the invention, or a fragment thereof, can be used as an immunogen to generate antibodies using standard techniques for polyclonal and monoclonal

antibody preparation. The full-length polypeptide or protein can be used or, alternatively, the invention provides antigenic peptide fragments for use as immunogens. In one embodiment, the antigenic peptide of a protein of the invention or fragments or immunogenic fragments of a protein of the invention comprise at least 8 (preferably 10, 15, 20, 30 or 35) consecutive amino acid residues of the amino acid sequence of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 15 or 16 and encompasses an epitope of the protein such that an antibody raised against the peptide forms a specific immune complex with the protein.

Exemplary amino acid sequences of the polypeptides of the invention can be used to generate antibodies against plant limit dextrinase inhibitor genes. In one embodiment, the immunogenic polypeptide is conjugated to keyhole limpet hemocyanin ("KLH") and injected into rabbits. Rabbit IgG polyclonal antibodies can be purified, for example, on a peptide affinity column. The antibodies can then be used to bind to and identify the polypeptides of the invention that have been extracted and separated via gel electrophoresis or other means.

One aspect of the invention pertains to isolated plant limit dextrinase inhibitor polypeptides of the invention, variants thereof, as well as variants suitable for use as immunogens to raise antibodies directed against a plant limit dextrinase inhibitor polypeptide of the invention. In one embodiment, the native polypeptide can be isolated, using standard protein purification techniques, from cells or tissues expressing a plant limit dextrinase inhibitor polypeptide. In a preferred embodiment, plant limit dextrinase inhibitor polypeptides of the invention are produced from expression vectors by recombinant DNA techniques. In another preferred embodiment, a polypeptide of the invention is synthesized chemically using standard peptide synthesis techniques.

An isolated or purified protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the protein is derived, or substantially free of chemical precursors or other chemicals when chemically synthesized. The language "substantially free" indicates protein preparations in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. Thus, protein that is substantially free of cellular material includes protein preparations having less than 5%, 10% or 20% (by dry weight) of a contaminating protein. Similarly, when an isolated plant limit dextrinase inhibitor polypeptide of the invention is recombinantly produced, it is substantially free of culture medium. When the plant limit dextrinase inhibitor polypeptide is produced by chemical synthesis, it is preferably substantially free of chemical precursors or other chemicals.

Biologically active portions of a polypeptide of the invention include polypeptides comprising amino acid sequences identical to or derived from the amino acid sequence of the protein, such that the variants sequences comprise conservative substitutions or truncations (*e.g.*, amino acid sequences comprising fewer amino acids than those shown in any of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 15 or 16 but which maintain a high degree of homology to the remaining amino acid sequence). Typically, biologically active portions comprise a domain or motif with at least one activity of the corresponding protein. Domains or motifs include, but are not limited to, a biologically active portion of a protein of the invention can be a polypeptide which is, for example, at least 10, 25, 50, or 100 amino acids in length. Polypeptides of the invention can comprise, for example, a glycosylation domain or site for complexing with polypeptides or other proteins involved in plastid division.

5.3 PRODUCTION OF TRANSGENIC PLANTS AND PLANT CELLS

The invention also encompasses transgenic or genetically-engineered plants, and progeny thereof, transformed using the sequences of the invention hereof. As used herein, a transgenic or genetically-engineered plant refers to a plant and a portion of its progeny which comprises a nucleic acid molecule which is not native to the initial parent plant. The introduced nucleic acid molecule may originate from the same species e.g., if the desired result is over-expression of the endogenous gene, or from a different species. A transgenic or genetically-engineered plant may be easily identified by a person skilled in the art by comparing the genetic material from a non-transformed plant, and a plant produced by a method of the present invention for example, a transgenic plant may comprise multiple copies of plant limit dextrinase inhibitor genes, and/or foreign nucleic acid molecules. Transgenic plants are readily distinguishable from non-transgenic plants by standard techniques. For example a PCR test may be used to demonstrate the presence or absence of introduced genetic material. Transgenic plants may also be distinguished from non-transgenic plants at the DNA level by Southern blot or at the RNA level by Northern blot or at the protein level by western blot, by measurement of enzyme activity or by starch composition or properties.

The nucleic acids of the invention may be introduced into a cell by any suitable means. Preferred means include use of a disarmed Ti-plasmid vector carried by Agrobacterium according to procedures known in the art, for example as described in EP-A-01 16718 and EP-A-0270822. Agrobacterium mediated transformation methods are now available for monocots, for example as described in EP 0672752 and WO00/63398. Alternatively, the nucleic acid may be introduced directly into plant cells using a particle gun. A further method would be

to transform a plant protoplast, which involves first removing the cell wall and introducing the nucleic acid molecule and then reforming the cell wall. The transformed cell can then be grown into a plant.

In an embodiment of the present invention, *Agrobacterium* is employed to introduce the gene constructs into plants. Such transformations preferably use binary *Agrobacterium* T-DNA vectors (Bevan, 1984, *Nuc. Acid Res.* 12:8711-21), and the co-cultivation procedure (Horsch et al., 1985, *Science* 227:1229-31). Generally, the *Agrobacterium* transformation system is used to engineer dicotyledonous plants (Bevan et al., 1982, *Ann. Rev. Genet.* 16:357-84; Rogers et al., 1986, *Methods Enzymol.* 118:627-41). The *Agrobacterium* transformation system may also be used to transform, as well as transfer, DNA to monocotyledonous plants and plant cells (*see* Hernalsteen et al., 1984, *EMBO J.* 3:3039-41; Hooykass-Van Slogteren et al., 1984, *Nature* 311:763-4; Grimsley et al., 1987, *Nature* 325:1677-79; Boulton et al., 1989, *Plant Mol. Biol.* 12:31-40.; Gould et al., 1991, *Plant Physiol.* 95:426-34).

Various alternative methods for introducing recombinant nucleic acid constructs into plants and plant cells may also be utilized. These other methods are particularly useful where the target is a monocotyledonous plant or plant cell. Alternative gene transfer and transformation methods include, but are not limited to, protoplast transformation through calcium-, polyethylene glycol (PEG)- or electroporation-mediated uptake of naked DNA (*see* Paszkowski et al., 1984, *EMBO J.* 3:2717-22; Potrykus et al., 1985, *Mol. Gen. Genet.* 199:169-177; Fromm et al., 1985, *Proc. Natl. Acad. Sci. USA* 82:5824-8; Shimamoto, 1989, *Nature* 338:274-6), and electroporation of plant tissues (D'Halluin et al., 1992, *Plant Cell* 4:1495-1505). Additional methods for plant cell transformation include microinjection, silicon carbide mediated DNA uptake (Kaeppler et al., 1990, *Plant Cell Reporter* 9:415-8), and microprojectile bombardment (Klein et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:4305-9; Gordon-Kamm et al., 1990, *Plant Cell* 2:603-18).

According to the present invention, desired plants and plant cells may be obtained by engineering the gene constructs described herein into a variety of plant cell types, including, but not limited to, protoplasts, tissue culture cells, tissue and organ explants, pollen, embryos as well as whole plants. In an embodiment of the present invention, the engineered plant material is selected or screened for transformants (*i.e.*, those that have incorporated or integrated the introduced gene construct or constructs) following the approaches and methods described below. An isolated transformant may then be regenerated into a plant. Alternatively, the engineered plant material may be regenerated into a plant, or plantlet, before subjecting the derived plant, or plantlet, to selection or screening for the marker gene traits. Procedures for regenerating plants

from plant cells, tissues or organs, either before or after selecting or screening for marker gene or genes, are well known to those skilled in the art.

A transformed plant cell, callus, tissue or plant may be identified and isolated by selecting or screening the engineered plant material for traits encoded by the marker genes present on the transforming DNA. For instance, selection may be performed by growing the engineered plant material on media containing inhibitory amounts of the antibiotic or herbicide to which the transforming marker gene construct confers resistance. Further, transformed plants and plant cells may also be identified by screening for the activities of any visible marker genes (*e.g.*, the ß-glucuronidase, luciferase, green fluorescent protein, B or C1 anythocyanin genes) that may be present on the recombinant nucleic acid constructs of the present invention. Such selection and screening methodologies are well known to those skilled in the art.

5.4 TRANSGENIC PLANTS THAT ECTOPICALLY EXPRESS PLANT LIMIT DEXTRINASE INHIBITOR PROTEIN

According to one aspect of the invention, the nucleic acid molecule expressed in the plant cell, plant or part of a plant comprises a nucleotide sequence encoding a plant limit dextrinase inhibitor protein, a fragment or variant thereof. The nucleic acid molecule expressed in the plant cell can comprise a nucleotide sequence encoding a full length plant limit dextrinase inhibitor protein. Examples of such sequences include SEQ ID NO: 1, or variants thereof and the corresponding the amino acid sequences of SEQ ID NO: 2 or variants thereof.

In an embodiment of the invention, the nucleic acid molecules of the invention are expressed in a plant cell and are transcribed only in the sense orientation. A plant that expresses a recombinant plant limit dextrinase inhibitor nucleic acid may be engineered by transforming a plant cell with a nucleic acid construct comprising a regulatory region operably associated with a nucleic acid molecule, the sequence of which encodes a plant limit dextrinase inhibitor protein or a fragment thereof. In plants derived from such cells, starch synthesis is altered in ways described in section 5.6. The term "operably associated" is used herein to mean that transcription controlled by the associated regulatory region would produce a functional mRNA, whose translation would produce the plant limit dextrinase inhibitor protein. Starch may be altered in particular parts of a plant, including but not limited to seeds, tubers, leaves, roots and stems or modifications thereof.

In an embodiment of the invention, a plant is engineered to constitutively express a plant limit dextrinase inhibitor protein in order to alter the maximum starch granule size of the plant. In a preferred embodiment, the maximum starch granule size is at least 2%, 5%, 10%, 20%,

30%, 40% or 50% greater than that of a non-engineered control plant(s). In another preferred embodiment, the maximum starch granule size is at least 2%, 5%, 10%, 20%, 30%, 40% or 50% less than that of a non-engineered control plant(s).

In an embodiment of the invention, a plant is engineered to constitutively express a plant limit dextrinase inhibitor protein in order to alter the starch granule size distribution of the plant. In a preferred embodiment, the average of the starch granule size distribution is at least 2%, 5%, 10%, 20%, 30%, 40% or 50% greater than that of a non-engineered control plant(s). In another preferred embodiment, the average starch granule size content is at least 2%, 5%, 10%, 20%, 30%, 40% or 50% less than that of a non-engineered control plant(s). The average starch granule size includes the mean, median or mode.

In an embodiment of the invention, a plant is engineered to constitutively express a plant limit dextrinase inhibitor protein in order to alter the starch content of the plant. In a preferred embodiment, the starch content is at least 2%, 5%, 10%, 20%, 30% or 40% greater than that of a non-engineered control plant(s). In another preferred embodiment, the starch content is at least 2%, 5%, 10%, 20%, 30% or 40% less than that of a non-engineered control plant(s).

In another aspect of the invention, where the nucleic acid molecules of the invention are expressed in a plant cell and are transcribed only in the sense orientation, the starch content of the plant cell and plants derived from such a cells exhibit altered starch composition. The altered starch composition comprises an increase in the ratio of amylose to amylopectin. In one embodiment of the invention, the ratio of amylose to amylopectin increases by at least 2%, 5%, 10%, 20%, 30%, 40%, or 50% in comparison to a non-engineered control plant(s).

In yet another embodiment of the present invention, it may be advantageous to transform a plant with a nucleic acid construct operably linking a modified or artificial promoter to a nucleic acid molecule having a sequence encoding a plant limit dextrinase inhibitor protein or a fragment thereof. Such promoters typically have unique expression patterns and/or expression levels not found in natural promoters because they are constructed by recombining structural elements from different promoters. *See, e.g.*, Salina et al., 1992, *Plant Cell* 4:1485-93, for examples of artificial promoters constructed from combining cis-regulatory elements with a promoter core.

In a preferred embodiment of the present invention, the associated promoter is a strong endosperm and/or embryo-specific plant promoter such that the plant limit dextrinase inhibitor protein is overexpressed in the transgenic plant.

In yet another preferred embodiment of the present invention, the overexpression of plant limit dextrinase inhibitor protein in starch producing organs and organelles may be

engineered by increasing the copy number of the plant limit dextrinase inhibitor gene. One approach to producing such transgenic plants is to transform with nucleic acid constructs that contain multiple copies of the complete plant limit dextrinase inhibitor gene with native or heterologous promoters. Another approach is to repeatedly transform successive generations of a plant line with one or more copies of the complete plant limit dextrinase inhibitor gene constructs. Yet another approach is to place a complete plant limit dextrinase inhibitor gene in a nucleic acid construct containing an amplification-selectable marker (ASM) gene such as the glutamine synthetase or dihydrofolate reductase gene. Cells transformed with such constructs are subjected to culturing regimes that select cell lines with increased copies of complete plant limit dextrinase inhibitor gene. *See, e.g.*, Donn et al., 1984, *J. Mol. Appl. Genet.* 2:549-62, for a selection protocol used to isolate a plant cell line containing amplified copies of the GS gene. Cell lines with amplified copies of the plant limit dextrinase inhibitor gene can then be regenerated into transgenic plants.

5.5 TRANSGENIC PLANTS THAT SUPPRESS ENDOGENOUS PLANT LIMIT DEXTRINASE INHIBITOR PROTEIN EXPRESSION

The nucleic acid molecules of the invention may also be used to alter activity of the plant limit dextrinase inhibitor protein of a plant cell, plant, or part of a plant by modifying transcription or translation of the plant limit dextrinase inhibitor gene. In an embodiment of the invention, an antagonist which is capable of altering the expression of a nucleic acid molecule of the invention is introduced into a plant in order to alter the plastid division process and hence the synthesis of starch. The antagonist may be protein, nucleic acid, chemical antagonist, or any other suitable moiety. In an embodiment of the invention, an antagonist which is capable of altering the expression of a nucleic acid molecule of the invention is provided to alter the plastid division process and hence the synthesis of starch. The antagonist may be protein, nucleic acid, chemical antagonist, or any other suitable moiety. Typically, the antagonist will function by inhibiting or enhancing transcription from the plant limit dextrinase inhibitor gene, either by affecting regulation of the promoter or the transcription process; inhibiting or enhancing translation of any RNA product of the plant limit dextrinase inhibitor gene; inhibiting or enhancing the activity of the plant limit dextrinase inhibitor protein itself or inhibiting or enhancing the protein-protein interaction of the plant limit dextrinase inhibitor protein and downstream enzymes of the starch biosynthesis pathway. For example, where the antagonist is a protein it may interfere with transcription factor binding to the plant limit dextrinase inhibitor gene promoter, mimic the activity of a transcription factor, compete with or mimic the plant limit dextrinase inhibitor

protein, or interfere with translation of the plant limit dextrinase inhibitor RNA, interfere with the interaction of the plant limit dextrinase inhibitor protein and downstream enzymes. Antagonists which are nucleic acids may encode proteins described above, or may be transposons which interfere with expression of the plant limit dextrinase inhibitor gene.

The suppression may be engineered by transforming a plant with a nucleic acid construct encoding an antisense RNA or ribozyme complementary to a segment or the whole of plant limit dextrinase inhibitor gene RNA transcript, including the mature target mRNA. In another embodiment, plant limit dextrinase inhibitor gene suppression may be engineered by transforming a plant cell with a nucleic acid construct encoding a ribozyme that cleaves the plant limit dextrinase inhibitor gene mRNA transcript.

In another embodiment, the plant limit dextrinase inhibitor mRNA transcript can be suppressed through the use of RNA interference, referred to herein as RNAi. RNAi allows for selective knock out of a target gene in a highly effective and specific manner. The RNAi technique involves introducing into a cell double-stranded RNA (dsRNA) which corresponds to exon portions of a target gene such as an endogenous plant limit dextrinase inhibitor gene. The dsRNA causes the rapid destruction of the target gene's messenger RNA, i.e. an endogenous plant limit dextrinase inhibitor gene mRNA, thus preventing the production of the plant limit dextrinase inhibitor protein encoded by that gene. The RNAi constructs of the invention confer expression of dsRNA which correspond to exon portions of an endogenous plant limit dextrinase inhibitor gene. The strands of RNA that form the dsRNA are complimentary strands encoded by a coding region, i.e., exons encoding sequence, on the 3' end of the plant limit dextrinase inhibitor gene.

The dsRNA has an effect on the stability of the mRNA. The mechanism of how dsRNA results in the loss of the targeted homologous mRNA is still not well understood (Cogoni and Macino, 2000, Genes Dev 10: 638-643; Guru, 2000, Nature 404, 804-808; Hammond et al., 2001, Nature Rev Gen 2: 110-119). Current theories suggest a catalytic or amplification process occurs that involves an initiation step and an effector step.

In the initiation step, input dsRNA is digested into 21-23 nucleotide "guide RNAs". These guide RNAs are also referred to as siRNAs, or short interfering RNAs. Evidence indicates that siRNAs are produced when a nuclease complex, which recognizes the 3' ends of dsRNA, cleaves dsRNA (introduced directly or via a transgene or virus) ~22 nucleotides from the 3' end. Successive cleavage events, either by one complex or several complexes, degrade the RNA to 19-20 bp duplexes (siRNAs), each with 2-nucleotide 3' overhangs. RNase III-type endonucleases cleave dsRNA to produce dsRNA fragments with 2-nucleotide 3' tails, thus an RNase III-like

activity appears to be involved in the RNAi mechanism. Because of the potency of RNAi in some organisms, it has been proposed that siRNAs are replicated by an RNA-dependent RNA polymerase (Hammond et al., 2001, Nature Rev Gen 2:110-119; Sharp, 2001, Genes Dev 15: 485-490).

In the effector step, the siRNA duplexes bind to a nuclease complex to form what is known as the RNA-induced silencing complex, or RISC. The nuclease complex responsible for digestion of mRNA may be identical to the nuclease activity that processes input dsRNA to siRNAs, although its identity is currently unclear. In either case, the RISC targets the homologous transcript by base pairing interactions between one of the siRNA strands and the endogenous mRNA. It then cleaves the mRNA ~12 nucleotides from the 3' terminus of the siRNA (Hammond et al., 2001, Nature Rev Gen 2:110-119; Sharp, 2001, Genes Dev 15: 485-490).

Methods and procedures for successful use of RNAi technology in post-transcriptional gene silencing in plant systems has been described by Waterhouse et al. (Waterhouse et al., 1998, Proc Natl Acad Sci U S A, 95(23):13959-64).

For all of the aforementioned suppression or antisense constructs, it is preferred that such nucleic acid constructs express specifically in organs where starch synthesis occurs (i.e. tubers, seeds, stems roots and leaves) and/or the plastids where starch synthesis occurs. Alternatively, it may be preferred to have the suppression or antisense constructs expressed constitutively. Thus, constitutive promoters, such as the nopaline, CaMV 35S promoter, may also be used to express the suppression constructs. A most preferred promoter for these suppression or antisense constructs is a maize ubiquitin promoter. Alternatively, a co-suppression construct promoter can be one that expresses with the same tissue and developmental specificity as the plant limit dextrinase inhibitor gene.

In accordance with the present invention, desired plants with suppressed target gene expression may also be engineered by transforming a plant cell with a co-suppression construct. A co-suppression construct comprises a functional promoter operatively associated with a complete or partial plant limit dextrinase inhibitor nucleic acid molecule. According to the present invention, it is preferred that the co-suppression construct encodes fully functional plant limit dextrinase inhibitor gene mRNA or enzyme, although a construct encoding an incomplete plant limit dextrinase inhibitor gene mRNA may also be useful in effecting co-suppression.

In accordance with the present invention, desired plants with suppressed target gene expression may also be engineered by transforming a plant cell with a construct that can effect site-directed mutagenesis of the plant limit dextrinase inhibitor gene. For discussions of nucleic

acid constructs for effecting site-directed mutagenesis of target genes in plants see, *e.g.*, Mengiste et al., 1999, Biol. Chem. 380:749-758; Offringa et al., 1990, *EMBO J.* 9:3077-84; and Kanevskii et al., 1990, *Dokl. Akad. Nauk. SSSR* 312:1505-7. It is preferred that such constructs effect suppression of plant limit dextrinase inhibitor genes by replacing the endogenous plant limit dextrinase inhibitor gene nucleic acid molecule through homologous recombination with either an inactive or deleted plant limit dextrinase inhibitor protein coding nucleic acid molecule.

In yet another embodiment, antisense technology can be used to inhibit plant limit dextrinase inhibitor gene mRNA expression. Alternatively, the plant can be engineered, *e.g.*, via targeted homologous recombination, to inactivate or "knock-out" expression of the plant's endogenous plant limit dextrinase inhibitor protein. The plant can be engineered to express an antagonist that hybridizes to one or more regulatory elements of the gene to interfere with control of the gene, such as binding of transcription factors, or disrupting protein-protein interaction. The plant can also be engineered to express a co-suppression construct. The suppression technology may also be useful in down-regulating the native plant limit dextrinase inhibitor gene of a plant where a foreign plant limit dextrinase inhibitor gene has been introduced. To be effective in altering the activity of a plant limit dextrinase inhibitor protein in a plant, it is preferred that the nucleic acid molecules are at least 50, preferably at least 100 and more preferably at least 150 nucleotides in length. In one aspect of the invention, the nucleic acid molecule expressed in the plant cell can comprise a nucleotide sequence of the invention which encodes a full length plant limit dextrinase inhibitor protein and wherein the nucleic acid molecule has been transcribed only in the antisense direction.

In another aspect of the invention, the nucleic acid molecules of the invention are expressed in a plant cell engineered expressing an antisense RNA homologous to the coding region of an endogeneous limit dextrinase inhibitor gene or using the dsRNA technology described herein and the starch of the plant cell and plants derived from such cells exhibit altered properties. The altered starch content comprises a decrease in the ratio of amylose to amylopectin. In one embodiment of the invention, the ratio of amylose to amylopectin decreases by at least 10%, 20%, 30%, 40%, or 50% in comparison to a non-engineered control plant(s).

In a particular embodiment, the nucleic acid molecules of the invention are expressing an antisense RNA homologous to a portion of the coding region of an endogeneous limit dextrinase inhibitor or using the dsRNA technology described herein, in conjunction with a developmental specific promoter directed towards later stages of seed development, in cereals crops. In this embodiment, the ratio of small starch granules to large starch granules decreases. A decreased

ratio of small to large starch granules results in greater accessibility of starch granules, which has certain industrial and commercial advantages related to extraction and processing of starch.

The progeny of the transgenic or genetically-engineered plants of the invention containing the nucleic acids of the invention are also encompassed by the invention.

5.6 MODIFIED STARCH

The invention encompasses methods of altering starch synthesis in a plant and the resulting modified starch produced using limit dextrinase inhibitor genes in accordance with the invention. In the context of the present invention, "altering starch synthesis" means altering any aspect of starch production in the plant, from initiation by the starch primer to downstream aspects of starch production such as elongation, branching and storage, such that it differs from starch synthesis in the native plant. In the invention, this is achieved by altering the activity of limit dextrinase by changing the expression of the limit dextrinase inhibitor, which includes, but is not limited to, its function in initiating starch synthesis, its temporal and spatial distribution and specificity, and its interaction with downstream factors in the synthesis pathway. The effects of altering the activity of the limit dextrinase inhibitor may include, for example, increasing or decreasing the starch yield of the plant; increasing or decreasing the rate of starch production; altering temporal or spatial aspects of starch production in the plant; altering the initiation sites of starch synthesis; changing the optimum conditions for starch production; and altering the type of starch produced, for example in terms of the ratio of its different components. For example, the endosperm of mature wheat and barley grains contain two major classes of starch granules: large, early formed "A" granules and small, later formed "B" granules. Type A starch granules in wheat are about 20 μm diameter and type B around 5 μm in diameter (Tester, 1997, in : Starch Structure and Functionality, Frazier et al., eds., Royal Society of Chemistry, Cambridge, UK). Rice starch granules are typically less than 5 μm in diameter, while potato starch granules can be greater than 80 μm in diameter. The quality of starch in wheat and barley is greatly influenced by the ratio of A-granules to B-granules. Altering the activity of the limit dextrinase inhibitor will influence the number of granule initiation sites, which will be an important factor in determining the number and size of formed starch granules. The degree to which the granule initiation activity of the plant is affected will depend at least upon the nature and of the nucleic acid molecule or antagonist introduced into the plant, and the amount present. By altering these variables, a person skilled in the art can regulate the degree to which starch synthesis is altered according to the desired end result.

The methods of the invention (i.e. engineering-a plant to express a construct comprising a plant limit dextrinase inhibitor nucleic acid) can, in addition to altering the total quantity of starch, alter the fine structure of starch in several ways including but not limited to, altering the ratio of amylose to amylopectin, altering the length of amylose chains, altering the length of chains of amylopectin fractions of low molecular weight or high molecular weight fractions, or altering the ratio of low molecular weight or high molecular weight chains of amylopectin. The methods of the invention can also be utilized to alter the granule structure of starch, i.e. the ratio of large to small starch granules from a plant or a portion of a plant. The alteration in the structure of starch can in turn affect the functional characteristics of starch such as viscosity, elasticity or rheological properties of the starch as measured using viscometric analysis. The modified starch can also be characterised by an alteration of more than one of the above-mentioned properties.

In an embodiment the length of amylose chains in starch extracted from a plant engineered to express a construct comprising a plant limit dextrinase inhibitor nucleic acid is decreased by at least 50, 100, 150, 200, 250, or 300 glucose units in length in comparison to amylose from non-modified starch from a plant of the same genetic background. In another embodiment, the length of amylose chains in starch is increased by at least 50, 100, 150, 200, 250, or 300 glucose units in length in comparison to amylose from non-modified starch from a plant of the same genetic background.

In an embodiment of the invention, the ratio of amylose to amylopectin decreases by at least 10%, 20%, 30%, 40%, or 50% in comparison to a non-engineered control plant(s).

In a preferred embodiment, the ratio of low molecular weight chains to high molecular weight chains of amylopectin is altered by at least 10%, 20%, 30%, 40%, or 50% in comparison to a non-engineered control plant(s).

In another preferred embodiment the average length of low molecular weight chains of amylopectin is altered by at least 5, 10, 15, 20, or 25 glucose units in length in comparison to a non-engineered control plant(s). In yet another preferred embodiment the average length of high molecular weight chains of amylopectin is altered by at least 10, 20, 30, 40, 50, 60, 70 or 80 glucose units in length in comparison to a non-engineered control plant(s).

According to one aspect of the invention, the ratio of small starch granules to large granules is altered by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more in comparison to a non-engineered control plant(s).

The embodiments described in each section above apply to the other aspects of the invention, mutatis mutandis.

6 EXAMPLES

Example 1. Identification of *limit dextrinase inhibitor* homologous gene sequences.

The sequence comparison and identification program tblastn was used with the *limit dextrinase inhibitor* gene amino acid sequence from *Hordeum vulgare* var Bomi ((MacGregor *et al.*, 2000) against the Genbank database. A number of possible cDNA sequences were obtained including those for *Triticum aestivum*, *Hordeum sponteneum*, *Oryza sativa*, *Triticum durum* and *Zea mays* In addition, a BLASTP search also identified two amino acid sequences for limit dextrinase inhibitor-like proteins from *Eleusine coracana* and *Secale cereale.* The amino acid sequences were aligned using ClustalW and the alignment is shown in Figure 1. All of the sequences except for those from *Eleusine coracana* and *Secale cereale* have an approximately 20 amino acid signal peptide at the N-terminal end, which is cleaved off to give the mature protein.

Example 2. Isolation of barley *limit dextrinase inhibitor* cDNA fragments.

Cloning of LDI gene by RT-PCR

Total RNA was extracted from barley (var. Golden Promise) grains 2 and 4 weeks post anthesis with a LiCl method as described by (Cathala *et al.*, 1983).

2 µg of RNA was treated with Rnase-free DNase I (Amersham Pharmacia Biotech) and used to synthesize 30 µl first strand cDNA using random hexamer primers (Roche) and M-MLV reverse transcriptase (Promega) using the reaction conditions recommended by the manufacturer.

It will be recognised by one skilled in the art that other mRNA extraction and cDNA synthesis methods exist which could be employed to produce cDNA from tissue of *Hordeum vulgare.*

A 3 µl aliquot of the cDNA product was used in a standard PCR reaction containing 2 mM $MgCl_2$, 8% (v/v) DMSO and primers Inhib-5 (5'-ACCAATAAACTAGTATCAACAATGGCATCCGACCA-3' SEQ ID No 17) and Inhib-6 (5'-CCAACCTTTTTTATTCATCAATCGGCCACA-3' SEQ ID No 18), which were designed against the *Hordeum vulgare* Limit dextrinase inhibitor sequence (SEQ ID No 3.), using Taq polymerase (Bioline) as recommended by the manufacturer. The PCR program used was 5 min at 94 °C, followed by 35 cycles of 94 °C for 30 sec, 63.5 °C for 30 sec and 72 °C for 1 min, finalized by 7 min at 72 °C. The product length was 623 bp. The amplified product was cloned into a pGEM-T Easy vector (Promega) and verified by sequencing.

It will be recognised by one skilled in the art that other methods exist which could be employed to produce cloned DNA fragments.

The sequence of this cDNA clone is shown in SEQ ID No 1. Compared to the published sequence (SEQ ID No. 3) there are five single base pair substitutions, which in turn lead to two amino acid substitutions as shown in a comparison of SEQ ID No. 2 and SEQ ID No. 4.

Example 3. Construction of vectors for barley transformation.

The antisense expression vector pYSUanti was constructed using the ubiquitin promoter (promoter, 5'-untranslated exon, and first intron of the maize ubiquitin (*Ubi-1*) gene) of the pAHC20 vector (Christensen & Quail, 1996), the cloned *LDI* gene and an OCS-terminator (octopine synthase). The OCS-terminator was PCR amplified using the BinAR vector (Höfgen & Willmitzer, 1990) as a template and primers OCSF (5'-TCGGATTCCATTGCCCAGCTATCTGTC-3' SEQ ID No 19) and OCS-R (5'-ATGGGCCCTAACAATCAGTAAATTGAACG-3' SEQ ID No 20) with an introduced *Apa* I site (underlined). The PCR was carried out using Pfu polymerase (Stratagene) as recommended by the manufacturer. The 544 bp PCR product was purified using a High Pure PCR product purification Kit (Roche), digested with the appropriate restriction enzymes (see Figure 1) and ligated into the pCR2.1-TOPO cloning vector (Invitrogen). The LDI gene was cut and cloned in an antisense direction with respect to the promoter. Restriction enzymes used are shown in Figure 1. All DNA cloning and manipulations were performed using standard protocols (Ausubel *et al.*, 1989).

Example 4. Transformation of barley

Stable barley transformation of half immature embryos (IEs) of the spring barley variety "Golden Promise" was performed with a biolistic device (Biolistic. PDS- 1000/He, BioRad) using DNA coated gold particles (1 μm), as described by (Wan & Lemaux, 1994). Gold particles were coated with 25 μg DNA using a protocol according to (Lemaux *et al.*, 1999) using a 1:1 molar ratio of pYSUanti and pAHC20 (Christensen & Quail, 1996). The latter carries the *bar* gene (encoding phosphinotricin acetyltransferase), giving resistance to the herbicides BASTA (PPT) and bialaphos (Murakami *et al.*, 1986; Thompson *et al.*, 1987) under the control of the maize ubiquitin (*Ubi-1*) promoter and first intron and terminated by the *nos* terminator (Bevan *et al.*, 1983). Preparation of IEs for bombardment was carried out as described in (Wan & Lemaux, 1994). Each plate with around 100 half IEs was bombarded once using a 1100 psi rupture disc and 28-29 mm Hg vacuum using bombardment conditions as published previously (Lemaux *et al.*, 1999). Selection against bialaphos and regeneration procedures were performed as described in (Cho *et al.*, 1998; Lemaux *et al.*, 1999).

Example 5. Analysis of transformed plants for presence of the limit dextrinase inhibitor construct.

Analysis of regenerated Barley transformants.

Total genomic DNA was isolated from either leaf material of primary transformants and/or isolated embryos of mature grains (T1 and T2 generations) using a modified method described by (Dellaporta *et al.*, 1983). The presence of transgenes was determined by PCR using the genomic DNA as a template. The oligonucleotide primers used were BAR-I (5'-CGGTACCGGCAGGCTGAAGTCCA-3' SEQ ID No 21) and BAR-II (5'-CCGGGGATCTACCATGAGCCCAGA-3' SEQ ID No 22) for the *bar* gene, Inhib-6 (5'-CCAACCTTTTTTATTCATCAATCGGCCACA-3' SEQ ID No 18) and OCS-II (5'-GAATGAACCGAAACCGGCGGTA-3' SEQ ID No 23) for the *LDI* gene in antisense direction (see Figure 2). As a control of the DNA quality primers TUB-F (5'-TACCACCTCCCTGAGGTTTG-3' SEQ ID No 24) and TUB-R (5'-CCATGCCTAGGGTCACACTT3' SEQ ID No 25) were used to amplify the *tubulin* gene. The PCR conditions used were the same as for cloning of the *LDI* gene, but DMSO was omitted from the PCR reactions for the *tubulin* gene. PCR products were 534 (*bar*), 817 (*LDI* antisense) and 217 bp (*tubulin*) respectively.

The presence of the *LDI* gene in the antisense direction and the *bar* gene was confirmed in regenerated transgenic barley plants using PCR analysis as shown in Figure 3. In total seven independent transgenic lines were produced, of which five carried both the *LDI* gene in antisense direction and the *bar* gene (U1-5), one carried only the *LDI* gene in antisense direction (U7) and one showed the *bar* gene only (U6). Two lines (U2, U5) were found to be sterile and therefore could not be analyzed further.

Primary transgenic plants (T_0 generation) were also tested by BASTA® painting. A 2 cm section of a barley leaf blade was treated with a 0.5 % (v/v) BASTA solution (Bayer, contains 150 g/l glufosinate ammonium) in 0.1 % Tween 20. Plants were examined 7-14 days after application.

All lines positive in the PCR reactions for the *bar* gene as expected also showed resistance to BASTA® painting.

Table 1 gives an overview of the analysis of T_0, T_1 and T_2 generation transgenic lines. The transgenic lines did not show any obvious morphological phenotypic changes. PCR analysis

of DNA derived from embryos of single mature grains resulted in the identification of two independent homozygous lines (U3, U4) in the T_2 generation.

Table 1. Analysis of T_0, T_1, and T_2 generation barley plants transformed with plasmid pYSUanti.

Transgenic barley lines	T_0			T_1		T_2	
	PCR		BASTA®	PCR		PCR	
	LDI (+/-)	bar (+/-)	(r/s)	LDI (+/-)	bar (+/-)	LDI (+/-)	bar (+/-)
U1	+	+	r	n.d.	n.d.		
U2*	+	+	r	-	–		
U3	+	+	r	24/8	24/8	12/0	12/0
U4	+	+	r	5/0	5/0	22/0	22/0
U5*	+	+	r	–	–		
U6	-	+	r	n.d.	n.d.		
U7	+	-	s	6/0	0/0		

LDI: PCR analysis with primers Inhib-6 and OCS-II for the *LDI* gene in antisense direction; *bar*: PCR analysis with primers BAR-I and BAR-II for the *bar* gene; (+/–): positive or negative amplification; (r/s): resistant or sensitive to BASTA® painting; *: sterile lines; n.d.: not determined; T_2 generation plants derived from positive T_1 generation plants.

Example 6. Detailed analysis of succeeding generations of transformed plants

Partial purification of limit dextrinase (LD)

LD was partially purified using 500 g of green 9 days old barley malt from variety Alexis with a protocol as described by (Kristensen *et al.*, 1998) up to the ion exchange chromatography step by using a fast protein liquid chromatography system (FPLC, Amersham Pharmacia Biotech). The activity of the partially purified LD was determined using the Limit Dextrizyme method (Megazyme) according to manufacturer's recommendations.

Assay of LDI activity

15 mg of ground endosperm was extracted with 0.5 ml 0.1 M NaOAc (pH 5.5) at 4 °C for 30 min. The extract was heated to 70°C for 40 min, centrifuged at 1000 .g for 5 min and the supernatant retained (LDI extract) and 1,10-phenanthroline to 10 mM added. The protein content of the LDI extracts was measured using a Bradford assay (BioRad) according to manufacturer's instructions. 10 µg protein of the LDI extract were mixed with 4.3 mU of partially purified LD and the volume made up to 0.5 ml in 0.1 M maleic acid, 0.02% (w/v) Na azide (pH 5.5). The

mixture was left to stand for 1 h at room temperature and then assayed with the Limit Dextrizyme method (Megazyme) according to manufacturers recommendations.

LD activity in developing endosperm

Five endosperms (2 wpa) were extracted with 1 ml/100 mg 0.1 M maleic acid, 0.02% (w/v) Na azide (pH 5.5). After 5 h at 40 °C, 250 µl of each extract were assayed for LD activity using the Limit Dextrizyme method as recommended by the manufacturer (Megazyme).

Analysis of protein expression by Western blotting.

Proteins were separated by SDS-PAGE on a resolving gel (6 . 9 . 0.1 cm) of 10% or 12% (w/v) polyacrylamide by the method of (Laemmli, 1970) and silver stained. The proteins were transferred from the gel to a nitrocellulose membrane (Amersham Pharmacia Biotech) with a transblotter (BioRad) and the membrane was incubated with rabbit antiserum that contained polyclonal antibodies raised against purified LDI from mature barley grains (kind gift from Canadian Grain Research Laboratory, Winnipeg, Canada). The immunoreactive protein bands were detected by incubation with alkaline phosphatase-conjugated antibodies against rabbit IgG (Sigma) and detected with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

Results.

T_1 generation grains of line U3 showed a Mendelian segregation ratio of 1:3 on 32 grains tested by PCR as shown in Table 1, suggestive of a single locus insertion. Six of these grains were also individually tested for LDI activity by LD assays and presence of LDI by immunoblotting of LDI extracts. The results are shown in Figure 4. Four out of the six grains analyzed (1, 2, 3 and 5) showed the *LDI* and *bar* genes in the PCR analysis (Figure 4C and D). These findings correlated with less LDI protein present as shown by Western analysis (Figure 4A and B) and less LDI activity as shown by enhanced % LD activity after incubation with grain extracts (Figure 4F). The two grains negative in the PCR reactions (4 and 6) showed wildtype levels of LDI protein presence in the immunoblot and LDI activity in the LD assay.

The homozygous T_2 generation lines U3 and U4 were also analyzed for LDI levels and activity. For this purpose starch from 5-10 grains of each line and wildtype grains was used to make LDI extracts that were then subjected to immunoblotting and LDI activity assays. The results of the latter experiments are shown in Figure 5 and 6 respectively. Both lines contained significantly less LDI protein present in comparison to wildtype levels (see Figure 5A and B).

These results correlate with the reduced LDI activity of these lines as shown in Figure 6. Lines U3 showed 34% and U4 29% less LDI activity in the developing grain compared to non transformed wildtype grain.

Free LD activity in developing endosperm two weeks post anthesis was measured in wildtype and T_2 generation transgenic lines U3 and U4 and found to be up to 50% higher than in wildtype endosperm (Table 2).

Table 2. Free Limit Dextrinase activity in developing endosperm 2 weeks post anthesis.

sample	mU LD/100 mg
wt	2.06 ± 0.14
U3	2.94 ± 0.15
U4	3.04 ± 0.29

Free LD activity in mU per 100 mg endosperm. wt: wildtype; U3/U4: T_2 generation grains of homozygous line U3 or U4. Each value represents the mean ± SE of three replicate experiments.

Example 7. Microscopic analysis of starch granule size and number.

Mature barley grains were fractured using a razor blade to initiate the fracture. The resulting cross-sections of wildtype and transgenic barley grains (outer endosperm regions) were viewed under an environmental scanning electron microscope (model Philips XL 30 Environmental Scanning Electron Microscope) at 0.4 Torr and 1600 x magnification.

Ratio of A to B starch granules

Starch from the endosperm of wildtype and transgenic barley grains was stained with a 0.1% I/ 1% KI solution and the A and B starch granules were counted under a light microscope (Axiophot Photomicroscope, Zeiss) using a gridded ocular.

Environmental scanning electron microscopy of the outer endosperm region of single transgenic and wildtype grains was carried out. A much-reduced level of the small B granules was present in the transgenic grains of homozygous T_2 generation line U4 and positive T_1 generation line U3 (see Figure 7B and D) in comparison to wildtype and transformation negative segregants (Figure 7A and C). The ratio of A to B granules was also counted in wildtype and transgenic grains, and it was found to be lower in the transgenic grains thus confirming the electron microscopy findings. Wildtype and negative segregants of T_1 generation line U3 showed a ratio of 1 : 17.08 ± 1.24 and 1 : 16.08 ± 1.92 respectively, whereas in transgenic grains the ratio dropped as low as 1 : 1.36 ± 0.56 in homozygous T_2 generation line U4 as shown in Table 3.

Table 3. Ratio of A to B starch granules in mature endosperms of wildtype and transgenic barley grains.

sample	A : B granules ratio	Total number of starch granules per grain
wt	1 : 17.08 ± 1.24	278 x10^6 ± 38.8 x 10^6
U3 T_1–	1 : 16.08 ± 1.92	Not determined
U3 T_1+	1 : 5.60 ± 0.19	Not determined
U3	1 : 2.13 ± 0.25	92.7 x10^6 ± 10.2 x 10^6
U4	1 : 1.36 ± 0.56	22.1 x10^6 ± 4 x 10^6

wt: wildtype; U3 T_1–/+: wildtype/transgenic T_1 generation grain of heterozygous transgenic line U3. U3/U4: homozygous transgenic T_2 generation grains of lines U3 or U4. Each value represents the mean ± SE of six replicate experiments.

Example 8. Analysis of starch structure.

Determination of distribution of lengths of α-1,4-glucan chains by HPAEC-PAD (Analysis of α-polyglucan structure II)

Barley starch samples were taken into boiling tubes with 5 mM sodium acetate pH 4.8 (1mg starch to 0.130 ml of buffer) and gelatinised by heating in a boiling water bath with periodic vortex mixing and then cooled. Isoamylase (15 µl) was added and digestion allowed to take place by incubating at 37 °C for 4 hours. The digest was stopped by boiling for 2 min and the debranched starch analysed by HPLC using a Dionex system equipped with a pulsed electrochemical detector. The solvents were 150 mM NaOH and 150mM NaOH, 1.0M Sodium Acetate. The HPLC conditions used were as follows:

HPLC Conditions

Column: Dionex PA 100
Detection: PED - Integrated Amperometry
Flow rate: 1.0 ml/min
Solvents:
1) 150mM NaOH
2) 150mM NaOH, 1.0M Sodium Acetate
3) Not used
4) Not used

Gradient Profile

TIME (MIN)	% 1	%2
0	100	0

1	100	0.
6	89	11
130	65	35
135	0	100
150	100	0
165	100	0

PED Parameters

WAVEFORM TABLE:

TIME (SEC)	POTENTIAL (V)
0.00	0.10
0.50	0.10
0.51	0.60
0.59	0.60
0.60	-0.60
0.65	-0.60

INTEGRATION:

BEGIN (SEC)	END (SEC)
0.30	0.50

Results of this analysis shows that the starch profiles of starch from the transgenic plants are significantly different to starch from control plants.

Example 9. Analysis of starch amylose content.

Molecular size separation of α-polyglucans by Sepharose CL2B chromatography

(Analysis of α-polyglucan structure I)

Sepharose CL2B analysis was in principle performed as described by (Denyer *et al.*, 1995), except that 0.39 ml fractions were collected at a rate of one fraction per min. 5 mg starch from 5-10 ground endosperms was dissolved in 0.5 ml 0.5 M NaOH and centrifuged at 13000 x g for 1 min. 50 µl of the supernatant was applied to a 12 ml Sepharose CL2B (Sigma) column and eluted with 0.1 M NaOH. The absorbance of fractions after the addition of iodine solution was measured at 595 nm. Additionally λmax values of the peak fractions were measured in the range of 450-700 nm.

Determination of amylose/amylopectin ratio and total starch content of barley

grains

Approximately 15 mg of ground endosperm of 5-10 mature grains was assayed using the amylose/amylopectin assay Kit (Megazyme) based on the glucose oxidase-peroxidase method as recommended by the manufacturer and total starch calculated in relation to a glucose standard as described by Megazyme.

Starch derived from homozygous lines U3 and U4 was subjected to Sepharose CL 2B chromatography, and fractions identified and quantified by staining with iodine. The resulting profiles are shown in Figure 8 and statistical analysis of this data in Table 4. The fractionation of wildtype α-glucan showed two distinct peaks of which the first one (fractions 14-24) is likely to be the amylopectin peak, as it has a λ_{max} of 558 ± 1.8 in fraction 17. The second peak was broader and considered to be predominantly amylose, because of a higher λ_{max} of 605.83 ± 4.65 (fraction 38).

Table 4. Percentage composition of the amylopectin fraction (I) and amylose fraction (II) separated by Sepharose CL 2B chromatography in mature endosperms of wildtype and transgenic barley grains of homozygous T_2 generation lines.

sample	I (%)	II (%)	II/I	λ_{max1}	λ_{max2}
wt	31.97 ±.2.09	53.73 ± 0.62	1.68	558.00 ± 1.80	605.83 ± 4.65
U3	44.06 ± 3.50	43.05 ± 0.51	0.98	585.83 ± 5.39	596.50 ± 1.80
U4	39.32 ± 4.84	46.39 ± 0.37	1.18	580.17 ± 4.54	590.33 ± 4.51

The amylopectin (I) and amylose (II) fraction correspond to fractions 14-24 and 25-45 respectively, in the Sepharose CL 2B chromatogram, as shown in Figure 7. The amount of carbohydrates in fractions I and II were expressed as percentages of total carbohydrates in the chromatogram. The data was that shown in Figure 3. λ_{max1} and λ_{max2} values were measured using fractions 17 and 38 in each chromatogram

In the two homozygous transgenic lines U3 and U4, starch subjected to Sepharose CL 2B fractionation showed a different profile; the amylose peak was reduced (see Figure 7). Also the λ_{max} values for fractions 17 in each chromatogram increased to 585.83 ± 5.39 (U3) and 580.17 ± 4.54 (U4). The λ_{max} values for fraction 38 decreased to 596.50 ± 1.8 (U3) and 590.33 ± 4.51 (U4). This suggests that less amylose is present in starch of the transgenic lines and a change in the structure of the starch, because the spectral properties of starch-bound iodine depend on the physical nature of the starch.

In view of this finding, the total starch content and the relative amounts of amylose and amylopectin in starch from the two homozygous transgenic lines was measured and compared to wildtype starch values (Table 5). The starch content (as a percentage of flour weight) of the

transgenic lines was reduced from 63.72 ± 4.20 % (wildtype) to 29.32 ± 2.07 % (U3) and 40.11 ± 5.25 % (U4). Also the amylose content decreased from 17.44 ± 0.67 % (wildtype) to 11.36 ± 0.58 % (U3) and 4.20 ± 1.31 % (U4).

Table 5. Total starch content and composition of amylose and amylopectin in starch from mature endosperms of wildtype and transgenic barley grains.

sample	total starch content (%)	amylose (%)	amylopectin (%)	SE
wt	63.72 ± 4.20	17.44	82.56	± 0.67
U3	29.32 ± 2.07	11.36	88.64	± 0.58
U4	40.11 ± 5.25	4.20	95.80	± 1.31

Total starch content in percent of weight of ground endosperms used. Amylose and amylopectin represent percentage of total starch (100%). wt: wildtype; U3/U4: T_2 generation grains of homozygous line U3 or U4. Each value represents the mean ± SE of three replicate experiments.

Example 10. Analysis of starch functionality.

Preparation of starch from barley grain

Starch was extracted from barley using 1% Sodium bisulphite solution. The starch was allowed to settle, the supernatant decanted off and the starch washed by resuspending in 200 ml of ice-cold water. The resulting starch pellet was left to air dry. Once dried the starch was stored at -20 °C.

Viscometric analysis of starch.

Starch samples were analysed for functionality by testing rheological properties using viscometric analysis.

Example 11. *Arabidopsis* transformation.

Arabidopsis thaliana c.v. Columbia plants were transformed according to the method of Clough and Brent 1998Plant J. 16(6):735-743 (1998) with slight modification.

Growing Plants

Plants were grown to a stage at which bolts were just emerging. Phytagar *0.1%* was added to the seeds and these were vernalized overnight at 4^0C. About 10-15 seeds were added per 3x5 inch pot. Seed was added onto the soil with a pipette, about 4-5 seeds per ml was dispersed. Seeds were germinated as usual (ie under humidity pots were covered until first leaves appeared and then over a two day period the lid was cracked and then removed). Plants were grown for about 4 weeks in the greenhouse (long day condition) until bolts emerged. The first bolts were cut to

encourage growth of multiple secondary bolts. Bolts containing many unopened flower buds were chosen for dipping.

Growing the Agrobacterium culture

Aliquots of the *Agrobacterium* strain carrying the constructs containing the limit dextrinase inhibitor gene were grown first as a 5ml culture in YEP containing Gentamycin (15ug/ml) and Kanamycin 20ug/ml. Next day, 2ml freshly grown culture was added to 400ml YEP media (10g Yeast Extract, 10g peptone 5g NaCl, pH 7.0) in a 2litre flask. and the flask was incubated at 28^0C incubator with shaking overnight. Next day OD 600 of the cells was measured and found to be 1.8. Cells were divided into 2X Oakridge bottles and harvested by centrifugation at 5000rpm for 10 min in a GSA rotor at room temperature The Pellet was resuspended in 3 volumes of infiltration media so that the final concentration of the culture was 0.6. Infiltration media was prepared by adding the following: ½ Murashige and Skoog Salts, 1x Gamborg's Vitamins and 0.44uM Benzylamino Purine (10ul per L of a 1mg/ml stock), pH was adjusted to 5.7 with NaOH. Then 0.02% Silwet (200ul per 1L) was added and mixed into the solution.

Arabidopsis transformation by Dipping

500 ml of resuspended *Agrobacterium* was poured into a tray and plants were inverted into Agrobacterium solution in batches of 10 for 15 minutes. After 15 minutes the plants were lifted and the excess solution drained. The plants were transferred on their sides to a fresh tray containing tissue paper to allow further soaking of the solution and then transferred to propagating trays. The plants were immediately covered with lids to maintain humidity. After two days the lid was removed and the plants allowed to grow normally. They were not watered for one week until the soil looked dry. After flowering was complete and the siliques on the plants were dry, all the seeds from one pot were harvested. The seeds were completely dried by keeping harvested seed in an envelope for one week

Transformed plants were analysed for effects on starch metabolism in the leaves.

Example 12. Transformation of potato.

Solanum tuberosum was transformed with constructs containing limit dextrinase inhibitor using the method of leaf disc cocultivation essentially as described by Horsch *et al.* (Science 227: 1229-1231, 1985). The youngest two fully-expanded leaves from a 5-6 week old soil grown potato plant were excised and surface sterilised by immersing the leaves in 8% 'Domestos'

for 10 minutes. The leaves were then rinsed four times in sterile distilled water. Discs were cut from along the lateral vein of the leaves using a No. 6 cork borer. The discs were placed in a suspension of *Agrobacterium*, containing one of the four plasmids listed above for approximately 2 minutes. The leaf discs were removed from the suspension, blotted dry and placed on petri dishes (10 leaf discs/plate) containing callusing medium (Murashige and Skoog agar containing 2.5µg/ml BAP, 1 µg/ml dimethylaminopurine, 3% (w/v) glucose). After 2 days the discs were transferred onto callusing medium containing 500µg/ml Claforan and 50µg/ml Kanamycin. After a further 7 days the discs were transferred (5 leaf discs/plate) to shoot regeneration medium consisting of Murashige and Skoog agar containing 2.5µg/ml BAP, 10 µg/ml GA3, 500µg/ml Claforan, 50µg/ml Kanamycin and 3% (w/v) glucose. The discs were transferred to fresh shoot regeneration media every 14 days until shoots appeared. The callus and shoots were excised and placed in liquid Murashige and Skoog medium containing 500µg/ml Claforan and 3% (w/v) glucose. Rooted plants were weaned into soil and grown up under greenhouse conditions to provide tuber material for analysis. Alternatively microtubers were produced by taking nodal pieces of tissue culture grown plants onto Murashige and Skoog agar containing 2.5µg/ml Kanamycin and 6% (w/v) sucrose. These were placed in the dark at 19° C for 4-6 weeks when microtubers were produced in the leaf axils.

Transformed plants were analysed for effects on starch metabolism in the tubers

Example 13. Transformation of wheat

Wheat was transformed with *Agrobacterium* including the *limit dextrinase inhibitor* containing plasmid using the seed inoculation method described in WO 00/63398 (RhoBio S.A.).

Transformed plants were analysed for effects on starch metabolism in the endosperm.

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Arg Cys Glu Ala Leu Arg Val Leu Met Asp Gly Val Arg Ala Glu Glu
85 90 95
Gly His Val Val Glu Gly Arg Leu Gly Asp Arg Arg Asp Cys Pro Arg
100 105 110
Glu Ala Gln Arg Glu Phe Ala Ala Thr Leu Val Thr Ala Ala Glu Cys
115 120 125
Asn Leu Pro Thr Val Ser Gly Val Gly Ser Thr Leu Gly Ala Thr Gly
130 135 140
Arg Trp Met Thr Ile Glu Leu Pro Lys
145 150

<210> 7
<211> 444
<212> DNA

<213> Hordeum spontaneum

<220>
<221> CDS
<222> (1)..(444)

<400> 7
atg gcg ttc aag tac cag ctc ctc ctc tcg gcc gcc gtc atg ctc gcc 48
Met Ala Phe Lys Tyr Gln Leu Leu Leu Ser Ala Ala Val Met Leu Ala
1 5 10 15

att ctc gcc gcc act gtc acc agt ttc ggg gat atg tgt gct cca ggg 96
Ile Leu Ala Ala Thr Val Thr Ser Phe Gly Asp Met Cys Ala Pro Gly
20 25 30

gat gcg ttg cca gcc aac cct ctc aga gcc tgc cgc acc tat gtg gtt 144
Asp Ala Leu Pro Ala Asn Pro Leu Arg Ala Cys Arg Thr Tyr Val Val
35 40 45

agt caa atc tgc cat gta ggc cct aga cta tcc acc tgg gac atg aag 192

Ser Gln Ile Cys His Val Gly Pro Arg Leu Ser Thr Trp Asp Met Lys
50 55 60

agg cgg tgc tgc gac gag ctg tcg gcc atc ccg gcg tac tgc aga tgc 240
Arg Arg Cys Cys Asp Glu Leu Ser Ala Ile Pro Ala Tyr Cys Arg Cys
65 70 75 80

gag gcg ctg cgt atc atc atg gat ggg aca gta act tgg cag ggt gtg 288
Glu Ala Leu Arg Ile Ile Met Asp Gly Thr Val Thr Trp Gln Gly Val
85 90 95

ttc ggt gcc tac ttc aag gac atg ccc aac tgc cct agg gtg atg caa 336
Phe Gly Ala Tyr Phe Lys Asp Met Pro Asn Cys Pro Arg Val Met Gln
100 105 110

acg agc tac gcc gcc aac ctc gtc aac ccg cag gag tgc aac cta tgg 384
Thr Ser Tyr Ala Ala Asn Leu Val Asn Pro Gln Glu Cys Asn Leu Trp
115 120 125

act atc cac ggc agc ccg tcc tgc ccc gaa ctg cag ccc gga tat gaa 432
Thr Ile His Gly Ser Pro Ser Cys Pro Glu Leu Gln Pro Gly Tyr Glu
130 135 140

gtg gtc ttg taa 444
Val Val Leu
145

<210> 8
<211> 147
<212> PRT
<213> Hordeum spontaneum

<400> 8
Met Ala Phe Lys Tyr Gln Leu Leu Leu Ser Ala Ala Val Met Leu Ala
1 5 10 15
Ile Leu Ala Ala Thr Val Thr Ser Phe Gly Asp Met Cys Ala Pro Gly
20 25 30
Asp Ala Leu Pro Ala Asn Pro Leu Arg Ala Cys Arg Thr Tyr Val Val
35 40 45
Ser Gln Ile Cys His Val Gly Pro Arg Leu Ser Thr Trp Asp Met Lys
50 55 60
Arg Arg Cys Cys Asp Glu Leu Ser Ala Ile Pro Ala Tyr Cys Arg Cys
65 70 75 80
Glu Ala Leu Arg Ile Ile Met Asp Gly Thr Val Thr Trp Gln Gly Val
85 90 95
Phe Gly Ala Tyr Phe Lys Asp Met Pro Asn Cys Pro Arg Val Met Gln
100 105 110
Thr Ser Tyr Ala Ala Asn Leu Val Asn Pro Gln Glu Cys Asn Leu Trp
115 120 125
Thr Ile His Gly Ser Pro Ser Cys Pro Glu Leu Gln Pro Gly Tyr Glu
130 135 140
Val Val Leu
145

<210> 9
<211> 483
<212> DNA
<213> Oryza sativa

<220>
<221> CDS
<222> (1)..(483)

<400> 9

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atg gct tcc aac aag gta gtg ttc tca gtg ttg ctt ctc gcc gtc gtc   48
Met Ala Ser Asn Lys Val Val Phe Ser Val Leu Leu Leu Ala Val Val
  1               5                  10                  15

tcc gtg ctc gcg gcg acg gcg acc atg gcg gag tac cac cac caa gac   96
Ser Val Leu Ala Ala Thr Ala Thr Met Ala Glu Tyr His His Gln Asp
            20                  25                  30

cag gtg gtc tac acc ccg ggc ccg ctc tgt cag cca gga atg ggc tac  144
Gln Val Val Tyr Thr Pro Gly Pro Leu Cys Gln Pro Gly Met Gly Tyr
        35                  40                  45

ccg atg tac ccg ctc ccg cgt tgc cgg gcg ttg gtg aag cgc cag tgc  192
Pro Met Tyr Pro Leu Pro Arg Cys Arg Ala Leu Val Lys Arg Gln Cys
    50                  55                  60

gtc ggc cgt ggc acg gcc gcc gcc gcc gag cag gtc cgg cga gac tgc  240
Val Gly Arg Gly Thr Ala Ala Ala Ala Glu Gln Val Arg Arg Asp Cys
65                  70                  75                  80

tgc cgg cag ctc gcc gcc gtc gac gac agc tgg tgc agg tgc gag gcg  288
Cys Arg Gln Leu Ala Ala Val Asp Asp Ser Trp Cys Arg Cys Glu Ala
                85                  90                  95

atc agc cac atg ctg gga ggc atc tac agg gag ctc ggc gcc ccc gat  336
Ile Ser His Met Leu Gly Gly Ile Tyr Arg Glu Leu Gly Ala Pro Asp
            100                 105                 110

gtc ggg cac ccc atg tcc gag gtg ttc cgc ggc tgc cgg aga ggg gac  384
Val Gly His Pro Met Ser Glu Val Phe Arg Gly Cys Arg Arg Gly Asp
        115                 120                 125

ttg gag cgc gcg gcg gcg agc ctc ccg gcg ttc tgc aac gtg gac atc  432
Leu Glu Arg Ala Ala Ala Ser Leu Pro Ala Phe Cys Asn Val Asp Ile
    130                 135                 140

ccc aac ggc gga ggt ggt gtc tgc tac tgg ctg gcg aga tct ggc tac  480
Pro Asn Gly Gly Gly Gly Val Cys Tyr Trp Leu Ala Arg Ser Gly Tyr
145                 150                 155                 160

tag                                                             483
```

<210> 10
<211> 160
<212> PRT
<213> Oryza sativa

<400> 10

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Met Ala Ser Asn Lys Val Val Phe Ser Val Leu Leu Leu Ala Val Val
  1               5                  10                  15
Ser Val Leu Ala Ala Thr Ala Thr Met Ala Glu Tyr His His Gln Asp
            20                  25                  30
Gln Val Val Tyr Thr Pro Gly Pro Leu Cys Gln Pro Gly Met Gly Tyr
        35                  40                  45
Pro Met Tyr Pro Leu Pro Arg Cys Arg Ala Leu Val Lys Arg Gln Cys
    50                  55                  60
Val Gly Arg Gly Thr Ala Ala Ala Ala Glu Gln Val Arg Arg Asp Cys
65                  70                  75                  80
Cys Arg Gln Leu Ala Ala Val Asp Asp Ser Trp Cys Arg Cys Glu Ala
```

```
                85                  90                  95
Ile Ser His Met Leu Gly Gly Ile Tyr Arg Glu Leu Gly Ala Pro Asp
            100                 105                 110
Val Gly His Pro Met Ser Glu Val Phe Arg Gly Cys Arg Arg Gly Asp
        115                 120                 125
Leu Glu Arg Ala Ala Ala Ser Leu Pro Ala Phe Cys Asn Val Asp Ile
    130                 135                 140
Pro Asn Gly Gly Gly Gly Val Cys Tyr Trp Leu Ala Arg Ser Gly Tyr
145                 150                 155                 160
```

<210> 11
<211> 707
<212> DNA
<213> Triticum durum

<220>
<221> CDS
<222> (27)..(533)

<400> 11

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agcgaaccag acttggctag aatacc atg gcg tgc aag tcc agc tgc agc ctc  53
                              Met Ala Cys Lys Ser Ser Cys Ser Leu
                                1               5

ctc ctc ttg gcc gcc gtc ctg ctc tcc gtc ttg gcc gct gct tcc gcc  101
Leu Leu Leu Ala Ala Val Leu Leu Ser Val Leu Ala Ala Ala Ser Ala
 10                  15                  20                  25

tcc ggc agc tgc gtc cca ggg gtg gct ttt cgg acc aat ctt ctg cca  149
Ser Gly Ser Cys Val Pro Gly Val Ala Phe Arg Thr Asn Leu Leu Pro
                30                  35                  40

cac tgc cgc gac tat gtg tta caa caa act tgt ggc acc ttc acc cct  197
His Cys Arg Asp Tyr Val Leu Gln Gln Thr Cys Gly Thr Phe Thr Pro
            45                  50                  55

ggg tca aag tta ccc gaa tgg atg aca tct gcg tcg ata tac tcc cct  245
Gly Ser Lys Leu Pro Glu Trp Met Thr Ser Ala Ser Ile Tyr Ser Pro
        60                  65                  70

ggg aaa ccg tac ctc gcc aag ttg tat tgc tgc cag gag ctc gca gaa  293
Gly Lys Pro Tyr Leu Ala Lys Leu Tyr Cys Cys Gln Glu Leu Ala Glu
    75                  80                  85

att tct cag cag tgc cgg tgc gag gcg ctg cgc tac ttc ata gcg ttg  341
Ile Ser Gln Gln Cys Arg Cys Glu Ala Leu Arg Tyr Phe Ile Ala Leu
 90                  95                  100                 105

ccg gta ccg tct cag cct gtg gac ccg agg tcc ggc aat gtt ggt gag  389
Pro Val Pro Ser Gln Pro Val Asp Pro Arg Ser Gly Asn Val Gly Glu
                110                 115                 120

agc ggc ctc atc gat ctg ccc gga tgc ccc agg gag atg caa tgg gac  437
Ser Gly Leu Ile Asp Leu Pro Gly Cys Pro Arg Glu Met Gln Trp Asp
            125                 130                 135

ttc gtc aga tta ctc gtc gcc ccg ggg cag tgc aac ttg gcg acc att  485
Phe Val Arg Leu Leu Val Ala Pro Gly Gln Cys Asn Leu Ala Thr Ile
        140                 145                 150
```

cac aat gtt cga tac tgc ccc gcc gtg gaa cag cct ctg tgg atc tag 533
His Asn Val Arg Tyr Cys Pro Ala Val Glu Gln Pro Leu Trp Ile
155 160 165

agataaaatc agtcgctcgt gaataagcat gcatgttgca tccataggcg tgtggtgtgc 593

atgtatacat atgtgagctc cgcgcgctca acatgtgtgg gctatctgct atgaatgaga 653

ataaagagaa tcattctgtg gttctttaat ttcaactaaa aaaaaaaaaa aaaa 707

<210> 12
<211> 168
<212> PRT
<213> Triticum durum

<400> 12
Met Ala Cys Lys Ser Ser Cys Ser Leu Leu Leu Leu Ala Ala Val Leu
1 5 10 15
Leu Ser Val Leu Ala Ala Ala Ser Ala Ser Gly Ser Cys Val Pro Gly
20 25 30
Val Ala Phe Arg Thr Asn Leu Leu Pro His Cys Arg Asp Tyr Val Leu
35 40 45
Gln Gln Thr Cys Gly Thr Phe Thr Pro Gly Ser Lys Leu Pro Glu Trp
50 55 60
Met Thr Ser Ala Ser Ile Tyr Ser Pro Gly Lys Pro Tyr Leu Ala Lys
65 70 75 80
Leu Tyr Cys Cys Gln Glu Leu Ala Glu Ile Ser Gln Gln Cys Arg Cys
85 90 95
Glu Ala Leu Arg Tyr Phe Ile Ala Leu Pro Val Pro Ser Gln Pro Val
100 105 110
Asp Pro Arg Ser Gly Asn Val Gly Glu Ser Gly Leu Ile Asp Leu Pro
115 120 125
Gly Cys Pro Arg Glu Met Gln Trp Asp Phe Val Arg Leu Leu Val Ala
130 135 140
Pro Gly Gln Cys Asn Leu Ala Thr Ile His Asn Val Arg Tyr Cys Pro
145 150 155 160
Ala Val Glu Gln Pro Leu Trp Ile
165

<210> 13
<211> 712
<212> DNA
<213> Zea mays

<220>
<221> CDS
<222> (33)..(500)

<400> 13
catccatcga gaggccgtcg acaggggaat ta atg gcg tcg tcg tct agc agc 53
Met Ala Ser Ser Ser Ser Ser
1 5

agc cac cgc cgc ctc atc ctc gca gcc gcc gtc ctg ctc tcc gtg ctc 101
Ser His Arg Arg Leu Ile Leu Ala Ala Ala Val Leu Leu Ser Val Leu
10 15 20

```
gcg gct gcc agc gcc agc gcc ggg acc tcc tgc gtg ccg ggg tgg gcc   149
Ala Ala Ala Ser Ala Ser Ala Gly Thr Ser Cys Val Pro Gly Trp Ala
     25                  30                  35

atc ccg cac aac ccg ctc ccg agc tgc cgc tgg tac gtg acc agc cgg   197
Ile Pro His Asn Pro Leu Pro Ser Cys Arg Trp Tyr Val Thr Ser Arg
 40                  45                  50                  55

acc tgc ggc atc ggg ccg cgc ctc ccg tgg ccg gag ctg aag agg aga   245
Thr Cys Gly Ile Gly Pro Arg Leu Pro Trp Pro Glu Leu Lys Arg Arg
                 60                  65                  70

tgc tgc cgg gag ctg gcg gac atc ccg gcg tac tgc cgg tgc acg gcg   293
Cys Cys Arg Glu Leu Ala Asp Ile Pro Ala Tyr Cys Arg Cys Thr Ala
             75                  80                  85

ctg agc atc ctc atg gac ggc gcg atc ccg cct ggc ccg gac gcg cag   341
Leu Ser Ile Leu Met Asp Gly Ala Ile Pro Pro Gly Pro Asp Ala Gln
         90                  95                 100

ctg gag ggc cgc cta gag gac ctg ccg ggc tgc ccg cgg gag gtg cag   389
Leu Glu Gly Arg Leu Glu Asp Leu Pro Gly Cys Pro Arg Glu Val Gln
    105                 110                 115

agg gga ttc gcc gcc acc ctc gtc acg gag gcc gag tgc aac ctg gcc   437
Arg Gly Phe Ala Ala Thr Leu Val Thr Glu Ala Glu Cys Asn Leu Ala
120                 125                 130                 135

acc atc agc ggc gtc gcc gaa tgc ccc tgg att ctc ggc ggc gga acg   485
Thr Ile Ser Gly Val Ala Glu Cys Pro Trp Ile Leu Gly Gly Gly Thr
                140                 145                 150

atg ccc tcc aag taa ctgcgaagag catagtgcat gaggaatgag cttgtagcta   540
Met Pro Ser Lys
            155

gctcatatgt ctgaataata agcacagcaa gaagatgaat gcatttctcg gatcgttcat 600

ccggaacaat aattaaaggg gatccggatt tgttcttgtg atataattaa cgattcctgt 660

tatacttgga agtagctagg ctcgtcccca tccaatgcaa gcaaaaaaaa aa         712


<210> 14
<211> 155
<212> PRT
<213> Zea mays

<400> 14
Met Ala Ser Ser Ser Ser Ser Ser His Arg Arg Leu Ile Leu Ala Ala
  1               5                  10                  15
Ala Val Leu Leu Ser Val Leu Ala Ala Ala Ser Ala Ser Ala Gly Thr
             20                  25                  30
Ser Cys Val Pro Gly Trp Ala Ile Pro His Asn Pro Leu Pro Ser Cys
         35                  40                  45
Arg Trp Tyr Val Thr Ser Arg Thr Cys Gly Ile Gly Pro Arg Leu Pro
     50                  55                  60
Trp Pro Glu Leu Lys Arg Arg Cys Cys Arg Glu Leu Ala Asp Ile Pro
 65                  70                  75                  80
```

```
Ala Tyr Cys Arg Cys Thr Ala Leu Ser Ile Leu Met Asp Gly Ala Ile
                    85                  90                  95
Pro Pro Gly Pro Asp Ala Gln Leu Glu Gly Arg Leu Glu Asp Leu Pro
            100                 105                 110
Gly Cys Pro Arg Glu Val Gln Arg Gly Phe Ala Ala Thr Leu Val Thr
        115                 120                 125
Glu Ala Glu Cys Asn Leu Ala Thr Ile Ser Gly Val Ala Glu Cys Pro
    130                 135                 140
Trp Ile Leu Gly Gly Gly Thr Met Pro Ser Lys
145                 150                 155
```

<210> 15
<211> 122
<212> PRT
<213> Eleusine coracana

<400> 15

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Ser Val Gly Thr Ser Cys Ile Pro Gly Met Ala Ile Pro His Asn Pro
  1               5                  10                  15

Leu Asp Ser Cys Arg Trp Tyr Val Ala Lys Arg Ala Cys Gly Val Gly
             20                  25                  30

Pro Arg Leu Ala Thr Gln Glu Met Lys Ala Arg Cys Cys Arg Gln Leu
         35                  40                  45

Glu Ala Ile Pro Ala Tyr Cys Arg Cys Glu Ala Val Arg Ile Leu Met
     50                  55                  60

Asp Gly Val Val Thr Pro Ser Gly Gln His Glu Gly Arg Leu Leu Gln
 65                  70                  75                  80

Asp Leu Pro Gly Cys Pro Arg Gln Val Gln Arg Ala Phe Ala Pro Lys
                 85                  90                  95

Leu Val Thr Glu Val Glu Cys Asn Leu Ala Thr Ile His Gly Gly Pro
            100                 105                 110

Phe Cys Leu Ser Leu Leu Gly Ala Gly Glu
        115                 120
```

<210> 16
<211> 121
<212> PRT
<213> Secale cereale

<400> 16

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Ser Val Gly Gly Gln Cys Val Pro Gly Leu Ala Met Pro His Asn Pro
  1               5                  10                  15

Leu Gly Ala Cys Arg Thr Tyr Val Val Ser Gln Ile Cys His Val Gly
             20                  25                  30

Pro Arg Leu Phe Thr Trp Asp Met Lys Arg Arg Cys Cys Asp Glu Leu
         35                  40                  45

Leu Ala Ile Pro Ala Tyr Cys Arg Cys Glu Ala Leu Arg Ile Leu Met
     50                  55                  60
```

Asp Gly Val Val Thr Gln Gln Gly Val Phe Glu Gly Gly Tyr Leu Lys
65 70 75 80

Asp Met Pro Asn Cys Pro Arg Val Thr Gln Arg Ser Tyr Ala Ala Thr
85 90 95

Leu Val Ala Pro Gln Glu Cys Asn Leu Pro Thr Ile His Gly Ser Pro
100 105 110

Tyr Cys Pro Thr Leu Gln Ala Gly Tyr
115 120

<210> 17
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 17
accaataaac tagtatcaac aatggcatcc gacca 35

<210> 18
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 18
ccaacctttt ttattcatca atcggccaca 30

<210> 19
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 19
tcggattcca ttgcccagct atctgtc 27

<210> 20
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 20
atgggcccta acaatcagta aattgaacg 29

<210> 21
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 21
cggtaccggc aggctgaagt cca 23

<210> 22
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 22
ccggggatct accatgagcc caga 24

<210> 23
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 23
gaatgaaccg aaaccggcgg ta 22

<210> 24
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 24
taccacctcc ctgaggtttg 20

<210> 25
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 25
ccatgcctag ggtcacactt 20

Claims

1. A method of producing a plant having starch granule modification comprising introducing into a plant a nucleotide sequence comprising a limit dextrinase inhibitor gene as shown in SEQ ID No.1, a fragment thereof, or a sequence having at least 40% identity thereto.

2. A method of producing a plant according to Claim 1, wherein said starch granule modification is any one or more of an altered number of starch granules, an altered size of starch granules, an altered composition of starch granules, or an increased size uniformity of starch granules in comparison to a non-engineered plant.

3. A method of producing a plant having an altered ability to degrade starch in comparison with a non-engineered plant, the method comprising introducing into a plant a nucleotide sequence comprising a limit dextrinase inhibitor gene as shown in SEQ ID No.1, or a fragment thereof, or a sequence having at least 40% identity thereto.

4. A method according to Claim 1, 2 or 3, wherein said limit dextrinase inhibitor gene is from a plant.

5. A method according to any one of Claims 1- 4, wherein said limit dextrinase inhibitor gene has the sequence shown in any one of SEQ ID Nos. 3, 5, 7, 9, 11 or 13.

6. A method according to any one of Claims 1-4, wherein said nucleotide sequence encodes a protein comprising an amino acid sequence shown in any one of SEQ ID No.2, 4, 6, 8, 10, 12 or 14, or a fragment or variant thereof

7. A method according to any one of Claims 4-6, wherein said limit dextrinase inhibitor gene is from barley, maize, wheat or rice..

8. A method according to any one of the preceding claims, wherein said limit dextrinase inhibitor gene comprises a regulatory element involved in the control of expression of said gene.

9. A method according to Claim 8, wherein said regulatory element is a promoter which causes expression of said limit dextrinase inhibitor gene in a starch storage tissue.

10. A method according to any one of the preceding claims, wherein said limit dextrinase inhibitor gene is native to the plant into which it is introduced.

11. A method according to any one of Claims 1-9, wherein said limit dextrinase inhibitor gene is foreign to the plant into which it is introduced.

12. A method according to any one of the preceding claims, wherein expression of a gene involved in starch degradation is up-regulated.

13. A method according to any one of Claims 1-11, wherein expression of a gene involved in starch degradation is down-regulated.

14. A method according to Claim 13, wherein said expression of a gene involved in starch degradation is down-regulated by antisense down-regulation, co-suppression or double stranded RNA technology.

15. A method according to any one of Claims 11-14, wherein said gene involved in starch degradation is a starch debranching enzyme.

16. A method according to any one of Claims 11-15, wherein said gene involved in starch degradation is limit dextrinase.

17. A method according to any one of the preceding claims, wherein said plant is grown and starch is harvested from said plant.

18. A method according to Claim 17, wherein said plant is grown until fruit, seed or tubers are yielded and starch is harvested from the fruit, seed or tubers.

19. A nucleotide sequence comprising the sequence shown in SEQ ID No. 1, a fragment or complement thereof, or a sequence having at least 99% identity thereto.

20. A nucleotide sequence encoding a polypeptide comprising the amino acid sequence shown in SEQ ID No. 2, a fragment or complement thereof, or a sequence having at least 99% identity thereto.

21. A nucleotide sequence according to Claim 19 or 20, wherein said sequence is from barley.

22. A protein comprising an amino acid sequence as shown in SEQ ID No. 2, a fragment thereof, or an amino acid sequence having at least 99% identity thereto.

23. A vector comprising the nucleotide sequence of any one of Claims 19-21.

24. A vector according to Claim 23, wherein a regulatory element is operably associated with said nucleotide sequence, the regulatory element being capable of directing expression of said nucleotide sequence.

25. A vector according to Claim 23 or 24, wherein said vector is a plasmid, cosmid, virus or phage.

26. A vector according to any one of Claims 23-25, wherein said vector comprises a selectable marker gene.

27. A vector according to Claim 26, wherein said vector comprises a promoter to direct expression of said selectable marker gene in cells other than the seed.

28. A vector according to any one of Claims 23-27, wherein said vector encodes for a fusion protein comprising a limit dextrinase inhibitor protein fused to a further protein product.

29. A vector according to Claim 28, wherein said further protein product is a transit peptide or a reporter gene product.

30. A plant produced according to the method of any one of Claims 1-18.

31. A genetically-engineered plant comprising the nucleotide sequence of any one of Claims 19-21.

32. A plant comprising the vector of any one of Claims 23-29.

33. A plant according to any one of Claims 30-32, wherein said plant is maize, wheat, rice, sorghum, barley, banana, apple, tomato, pear, cassava, potato, yam, turnip, rape seed, canola, sunflower, oil palm, coconut, linseed, groundnut, soya bean or pea.

34. A plant according to any one of Claims 30-33, wherein the maximum size of starch granule is up to 50% greater than that of a non-engineered control plant.

35. A plant according to any one of Claims 30-33, wherein the maximum size of starch granule is up to 50% less than that of a non-engineered plant.

36. A plant according to any one of Claims 30-33, wherein the average size distribution of starch granules is up to 50% greater than that of a non-engineered plant.

37. A plant according to any one of Claims 30-33, wherein the average size distribution of starch granules is up to 50% less than that of a non-engineered plant.

38. A plant according to any one of Claims 30-33, wherein starch content is up to 40% greater than that of a non-engineered plant.

39. A plant according to any one of Claims 30-33, wherein starch content is up to 40% less than that of a non-engineered plant.

40. A plant according to any one of Claims 30-33, wherein the ratio of amylose to amylopectin is increased compared to that of a non-engineered plant.

41. A plant according to any one of Claim 40, wherein said ratio of amylose to amylopectin is increased by up to 50% compared to that of a non-engineered plant.

42. Propagating material of a plant produced according to the method of any one of Claims 1-18.

43. Propagating material of a plant according to Claim 42, wherein said propagating material is a seed or tuber.

44. A plant cell from a plant produced according to the method of any one of Claims 1-18.

45. A genetically-engineered plant cell comprising the nucleotide sequence of any one of Claims 19-21.

46. A plant cell comprising the vector of any one of Claims 23-29.

47. Use of a nucleotide sequence comprising a limit dextrinase inhibitor gene as shown in SEQ ID No. 1, a fragment or variant thereof, or a sequence having at least 40% identity thereto, for altering the number, size or composition of starch granules in a plant.

48. Starch obtained from the plant of any one of Claims 30-41, wherein the length of amylose chains is decreased by at least 50 glucose units in comparison to amylose from non-modified starch from a non-engineered plant.

49. Starch obtained from the plant of any one of Claims 30-41, wherein the length of amylose chains is increased by at least 50 glucose units in comparison to amylose from non-modified starch from a non-engineered plant.

50. Starch obtained from the plant of any one of Claims 30-41, wherein the ratio of amylose to amylopectin is decreased by at least 10% in comparison to starch from a non-engineered plant.

51. Starch obtained from the plant of any one of Claims 30-41, wherein the ratio of low molecular weight chains to high molecular weight chains is altered by at least 10% in comparison to starch from a non-engineered plant.

52. Starch obtained from the plant of any one of Claims 30-41, wherein the average length of low molecular weight chains of amylopectin is altered by at least 5 glucose units in comparison to starch from a non-engineered plant.

53. Starch obtained from the plant of any one of Claims 30-41, wherein the average length of high molecular weight chains of amylopectin is altered by at least 10 glucose units in comparison to starch from a non-engineered plant.

54. Starch obtained from the plant of any one of Claims 30-41, wherein the ratio of small starch granules to large granules is altered by at least 10% in comparison to starch from a non-engineered plant.

Figure 1

```
                                  ....|....|....|....|....|....|....|....|....|....|
                                            10        20        30        40        50
Hordeum vulgare                   ---MASD-HR RFVLSGAVLL SVLAVAAATL E---------- SVKDECQLGV
Oryza sativa                      ---MASN-KV VFSVLLLAVV SVLAATATMA EYHHQDQVVY TPGPLCQPGM
Hordeum spontaneum                ---MAFK--Y QLLLSAAVML AILAATVT-- ---------- SFGDMCAPGD
Eleusine coracana                 ---------- ---------- ---------- ---------- SVGTSCIPGM
Secale cereale                    ---------- ---------- ---------- ---------- SVGGQCVPGL
Triticum durum                    ---MACKSSC SLLLLAAVLL SVLAA--A-- ---------- SASGSCVPGV
Zea mays                          MASSSSSHR RLILAAAVLL SVLAAASA-- ---------- SAGTSCVPGW
Tritcum aestivum                  ---MASN-HR RFLLSGAVLL SVLAAVAA-L E---------- SVEDECQPGV

                                  ....|....|....|....|....|....|....|....|....|....|
                                            60        70        80        90       100
Hordeum vulgare                   DFPHNPLATC HTYVIKRVCG ---RGPSRPM LV-------- ------KERC
Oryza sativa                      GYPMYPLPRC RALVKRQCVG ---RGTAAAA EQ-------- -----VRRDC
Hordeum spontaneum                ALPANPLRAC RTYVVSQIC- --HVGPRLST WD-------- -----MKRRC
Eleusine coracana                 AIPHNPLDSC RWYVAKRACG ---VGPRLAT QE-------- -----MKARC
Secale cereale                    AMPHNPLGAC RTYVVSQIC- --HVGPRLFT WD-------- -----MKRRC
Triticum durum                    AFRTNLLPHC RDYVLQQTCG TFTPGSKLPE WMTSASIYSP GKPYLAKLYC
Zea mays                          AIPHNPLPSC RWYVTSRTCG ---IGPRLPW PE-------- -----LKRRC
Tritcum aestivum                  AFPHNALATC HTYVIKRVCG ---RGPSRPM LV-------- ------KERC

                                  ....|....|....|....|....|....|....|....|....|....|
                                           110       120       130       140       150
Hordeum vulgare                   CRELAAVP-D HCRCEALRIL MDGVRTPE-- ----GRVVEG RLGDRRDCPR
Oryza sativa                      CRQLAAVDDS WCRCEAISHM LGGIYRELG- ---APDVGHP MSEVFRGCRR
Hordeum spontaneum                CDELSAIP-A YCRCEALRII MDGTVTWQ-- ----GVFG-A YFKDMPNCPR
Eleusine coracana                 CRQLEAIP-A YCRCEAVRIL MDGVVTP--- ---SGQHEGR LLQDLPGCPR
Secale cereale                    CDELLAIP-A YCRCEALRIL MDGVVTQQ-- ----GVFEGG YLKDMPNCPR
Triticum durum                    CQELAEIS-Q QCRCEALRYF IALPVPSQPV DPRSGNVGES GLIDLPGCPR
Zea mays                          CRELADIP-A YCRCTALSIL MDGAIPPGP- ---DAQLEGR -LEDLPGCPR
Tritcum aestivum                  CRELAVVP-D YCRCEALRVL MDGVRAEE-- ----GHVVEG RLGDRRDCPR

                                  ....|....|....|....|....|....|....|....|....|....|...
                                           160       170       180       190
Hordeum vulgare                   EEQRAFAATL VTAAECNLSS VQAPGVRLVL LADG------ ---
Oryza sativa                      GDLERAAASL PAFCNVDIPN GGG-GVCYWL ARSGY----- ---
Hordeum spontaneum                VMQTSYAANL VNPQECNLWT IHGSPSCPEL QPGYEVVL-- ---
Eleusine coracana                 QVQRAFAPKL VTEVECNLAT IHGGPFCLSL LGAGE----- ---
Secale cereale                    VTQRSYAATL VAPQECNLPT IHGSPYCPTL QAGY------ ---
Triticum durum                    EMQWDFVRLL VAPGQCNLAT IHNVRYCPAV EQPLWI---- ---
Zea mays                          EVQRGFAATL VTEAECNLAT ISGVAECPWI LGGGTMPSK- ---
Tritcum aestivum                  EAQREFAATL VTAAECNLPT VS--GVGSTL GATGRWMTIE LPK
```

Figure 2

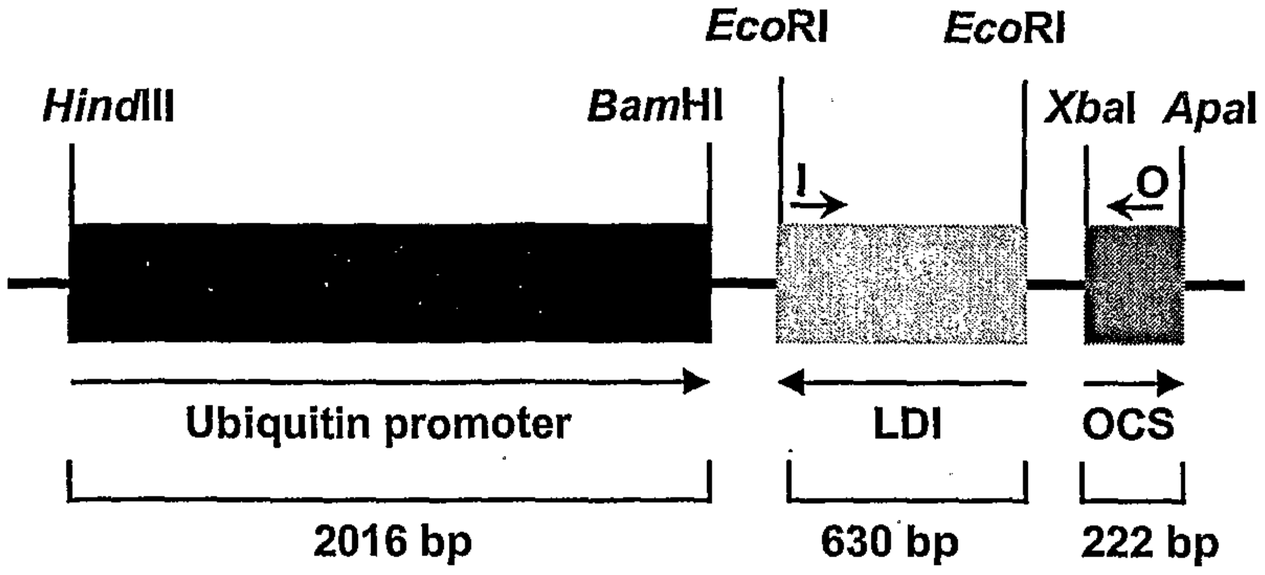


Figure 3

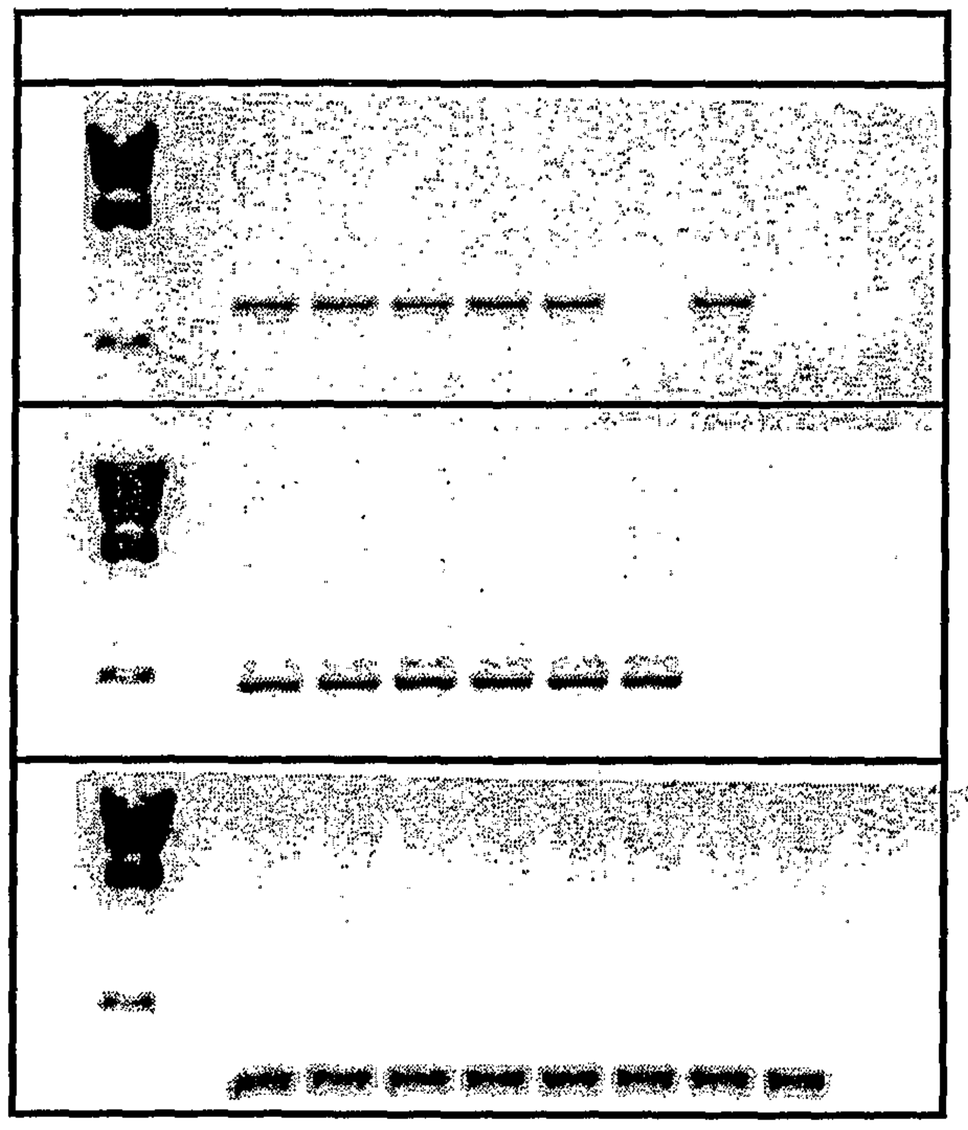
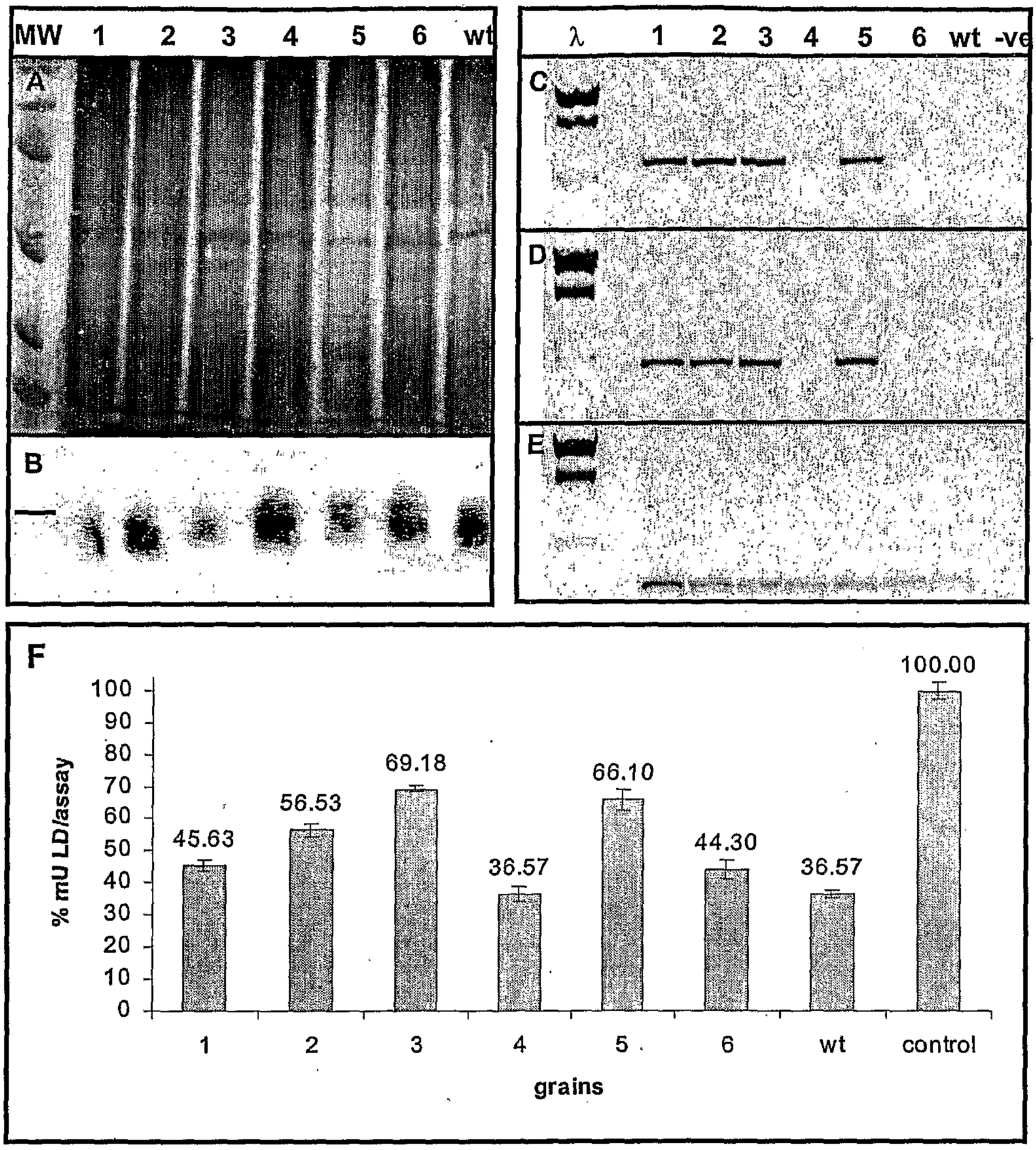


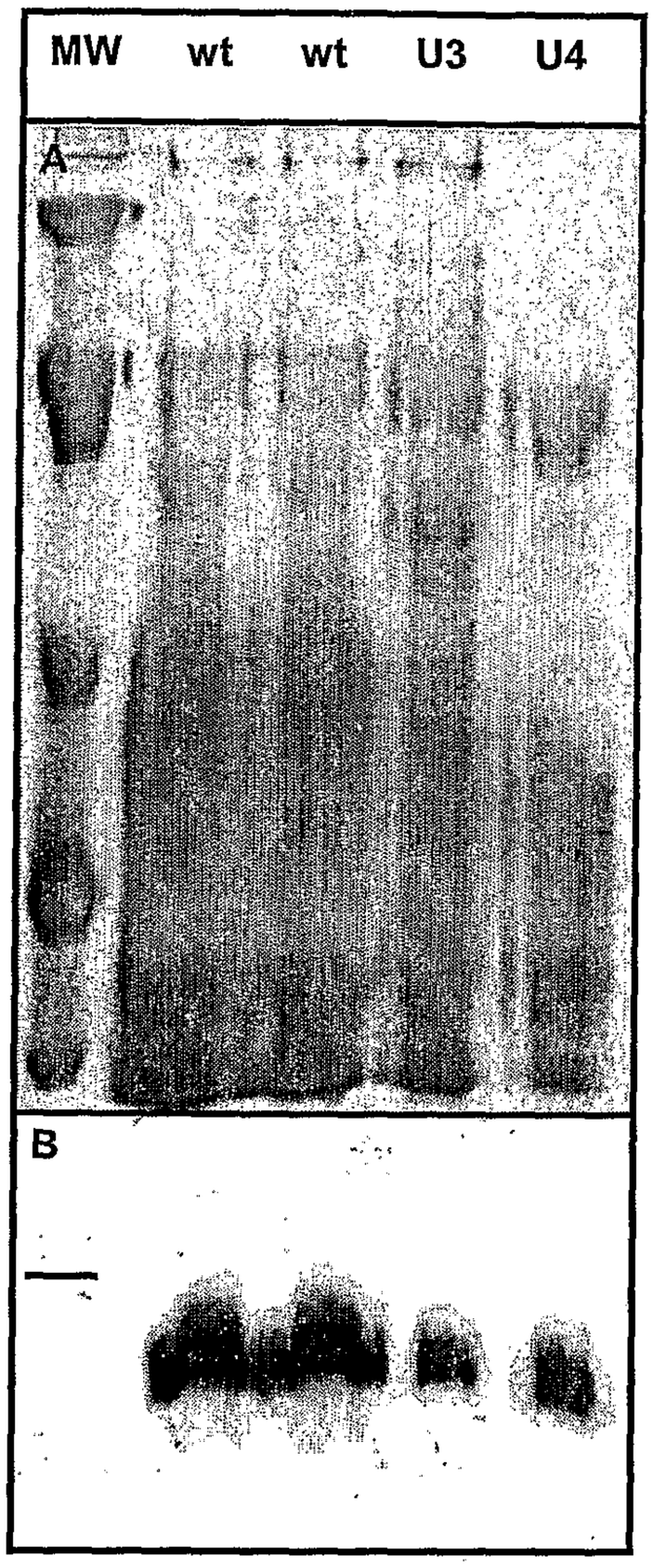
Figure 4



(A) 10% SDS-PAGE of LDI extracts corresponding to 5 μg protein.
(B) Immunoblot developed with antiserum against LDI; bar represents 21.5 kD.
(C) PCR of genomic DNA with primers Inhib-6 + OCS-II for the *LDI* gene in antisense direction (817 bp).
(D) PCR of genomic DNA primers BAR-I + BAR-II for the *bar* gene (534 bp).
(E) PCR of genomic DNA primers TUB-F+ TUB-R for the *tubulin* gene (217 bp).
(F) LDI activity assay. The control represents the amount of LD used for each assay. LDI extracts corresponding to 10 μg protein were mixed with LD and assayed for remaining LD activity. Each value represents the mean ± SE of three replicate experiments.

1-6: six individual grains; **wt**: wildtype; **MW:** molecular weight marker sizes in kD are 97.4, 66.2, 45, 31, 21.5 and 14.4; λ: λ/*Hind* III molecular weight marker; **-ve**: negative control of PCR.

Figure 5



(A) 12% SDS-PAGE of LDI extracts corresponding to 5µg protein.
(B) Immunoblot developed with antiserum against LDI; bar represents 21.5 kD.

U3: homozygous T_2 generation transgenic line U3; **U4**: homozygous T_2 generation transgenic line U4; **wt**: wildtype; **MW**: molecular weight marker sizes in kD are 97.4, 66.2, 45, 31, 21.5 and 14.4.

Figure 6

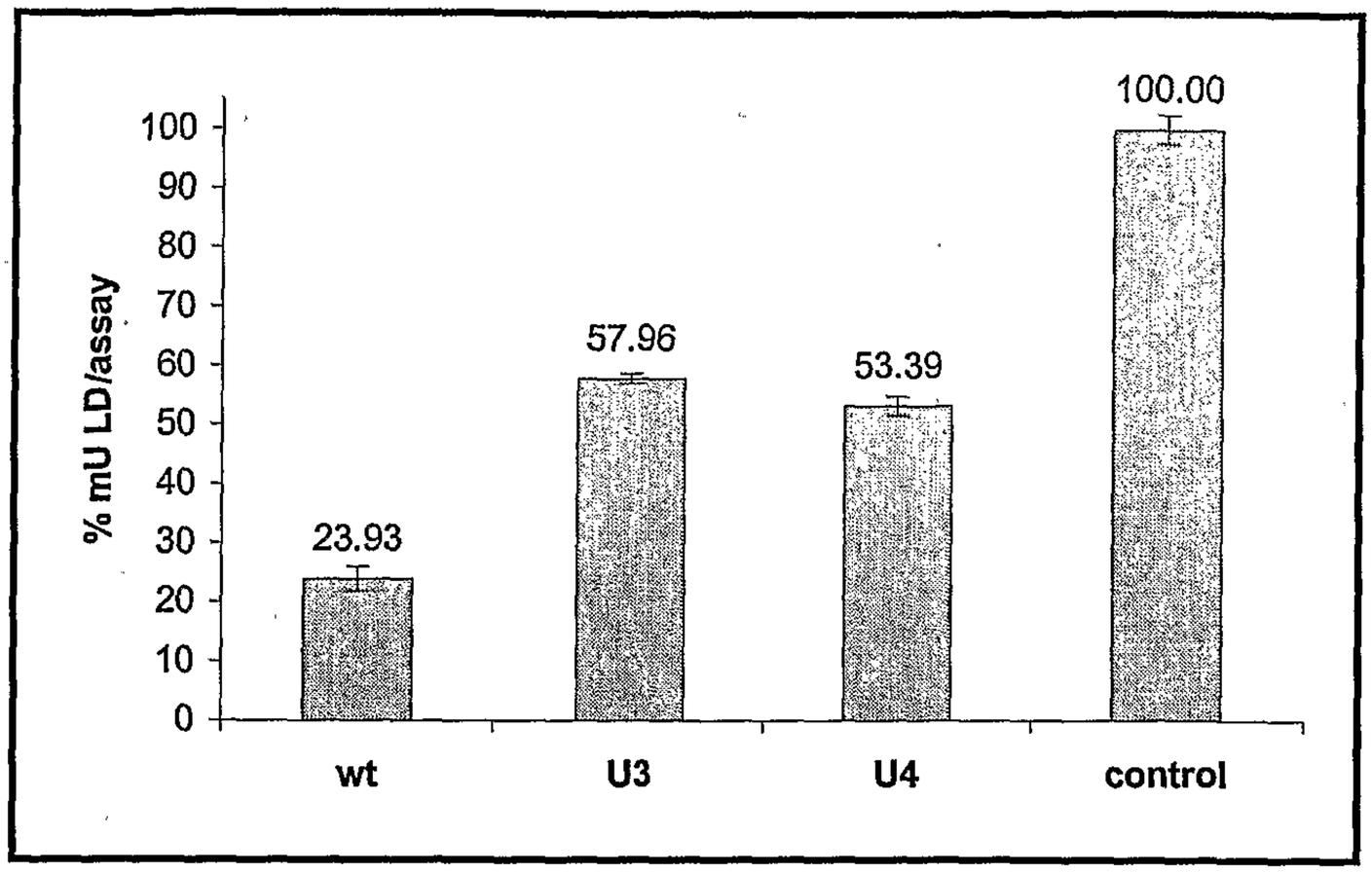
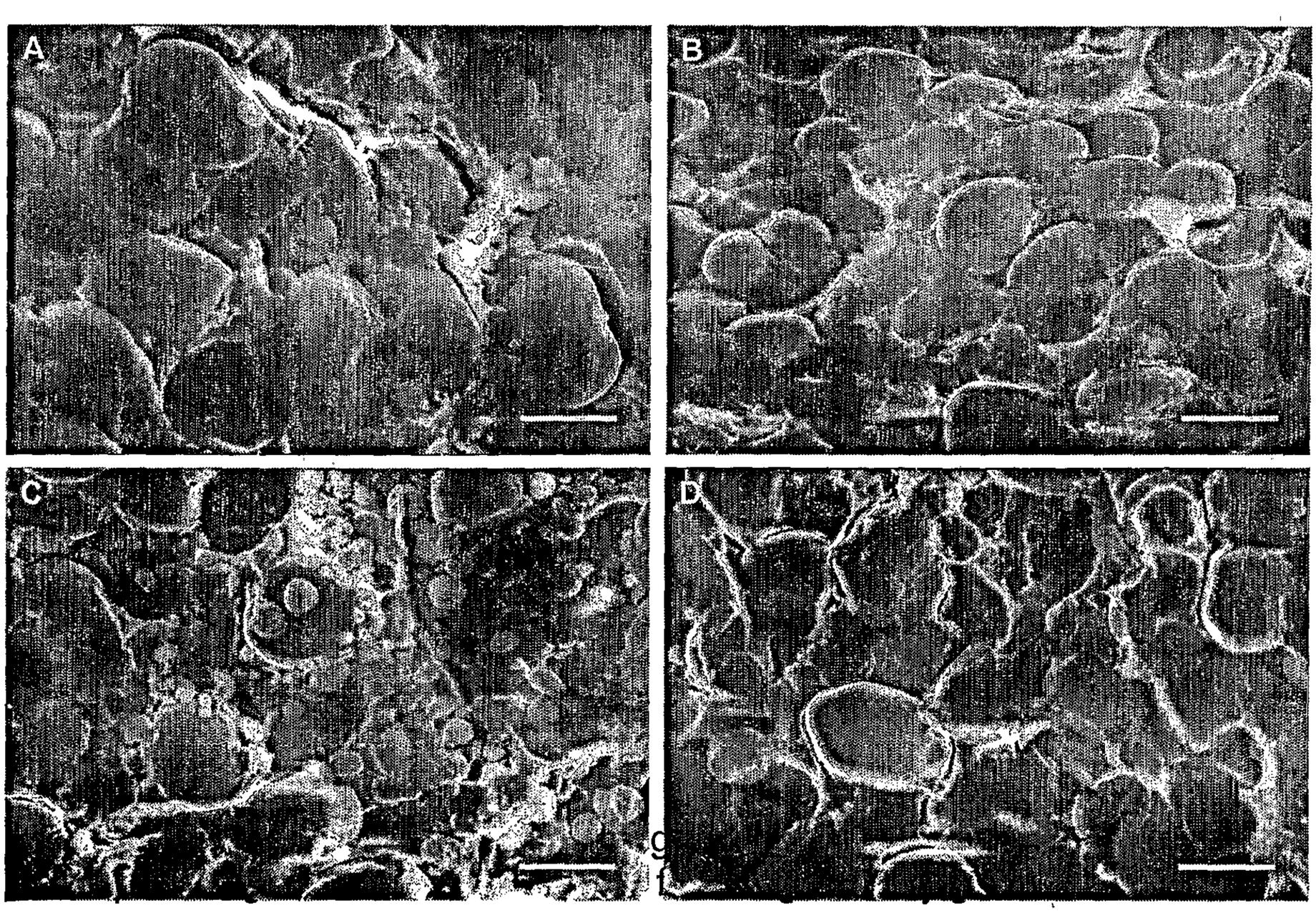


Figure 7



(A) wildtype barley grain.
(B) transgenic T_2 generation grain of homozygous line U4.
(C) wildtype T_1 grain of heterozygous transgenic line U3.
(D) transgenic T_1 grain of heterozygous line U3.
Bar is 10 μm long.

Figure 8

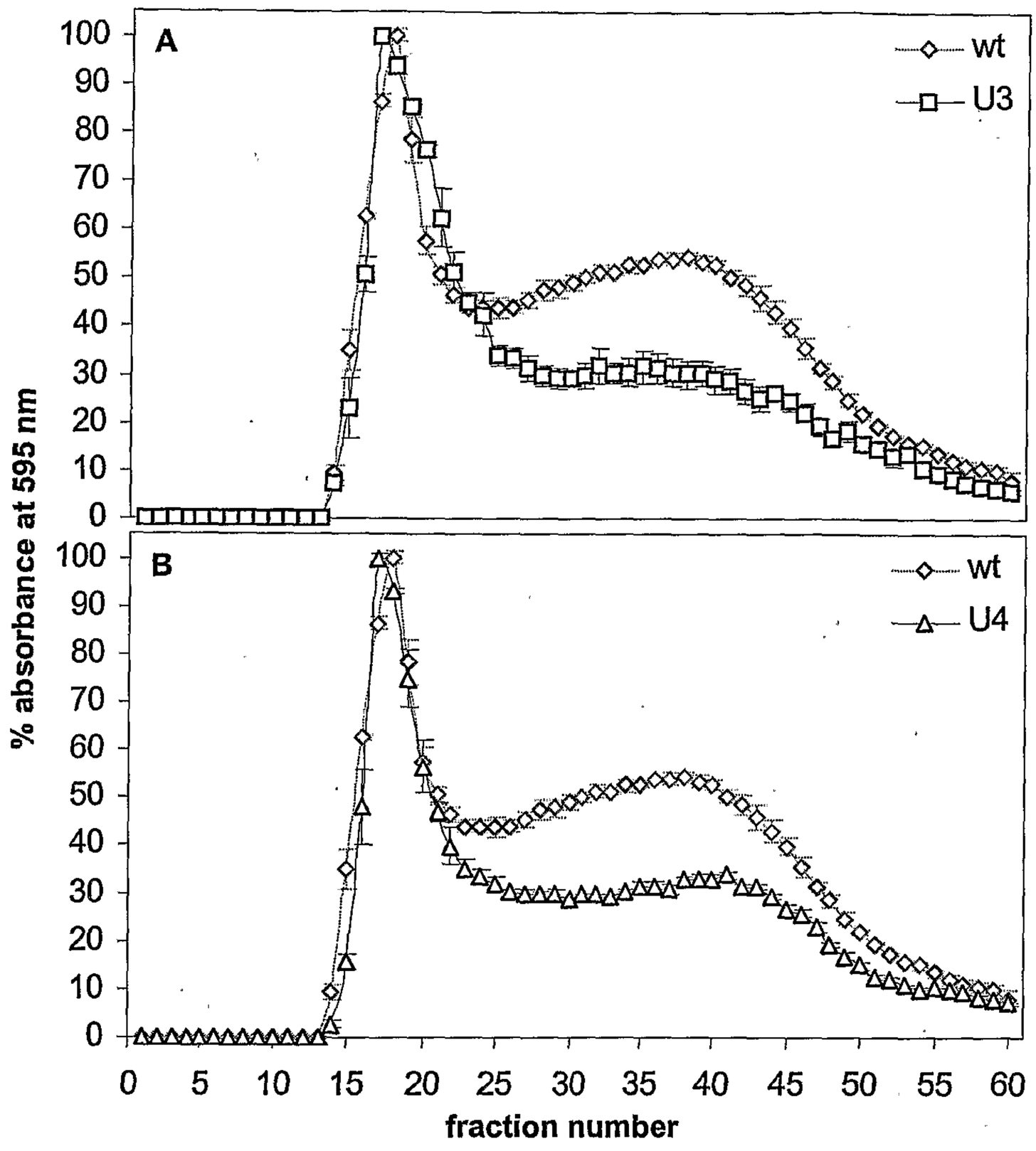

A
wt
U3
B
wt
U4
% absorbance at 595 nm
fraction number
0
10
20
30
40
50
60
70
80
90
100
0
5
10
15
20
25
30
35
40
45
50
55
60

Figure 9

DNA % Identity						
	Hordeum vulgare SEQ. ID. No. 3	*Hordeum vulgare SEQ. ID. No. 1*	*Triticum aestivum*	*Zea mays*	*Oryza sativa*	*Hordeum sponteneum*
Hordeum vulgare SEQ. ID. No. 3	100	76.34				
Hordeum vulgare SEQ. ID. No. 1	98.84	100	77.27	63.45	42.35	43.09
Triticum aestivum			100	65.38	45.57	46.05
Zea mays				100	41.37	46.81
Oryza sativa					100	49.28
Hordeum sponteneum						100

% amino acid identities								
	Hordeum vulgare SEQ. ID. No. 4	*Hordeum vulgare SEQ. ID. No. 2*	*Hordeum sponteneum*	*Oryza sativa*	*Triticum aestivum*	*Zea mays*	*Secale cereale*	*Eleusine coracana*
Hordeum vulgare SEQ. ID. No. 4	100							
Hordeum vulgare SEQ. ID. No. 2	98.64	100	42.86	34.01	80.95	53.06	40.14	42.18
Hordeum sponteneum		43.54	100	29.92	43.54	48.98	66.67	46.26
Oryza sativa		33.75	25.63	100	31.88	33.13	23.75	25
Triticum aestivum		78.43	41.83	33.33	100	54.25	39.22	39.22
Zea mays		50.97	46.45	35.48	53.55	100	43.87	52.9
Secale cereale		48.36	79.51	29.51	50.82	55.74	100	62.3
Eleusine coracana		51.22	54.47	29.27	50.41	66.67	61.79	100

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                                 ....|....| ....|....| ....|....| ....|....| ....|....|
                                          10         20         30         40         50
Hordeum vulgare                  ---MASD-HR RFVLSGAVLL SVLAVAAATL E--------- SVKDECQLGV
Oryza sativa                     ---MASN-KV VFSVLLLAVV SVLAATATMA EYHHQDQVVY TPGPLCQPGM
Hordeum spontaneum               ---MAFK--Y QLLLSAAVML AILAATVT-- ---------- SFGDMCAPGD
Eleusine coracana                ---------- ---------- ---------- ---------- SVGTSCIPGM
Secale cereale                   ---------- ---------- ---------- ---------- SVGGQCVPGL
Triticum durum                   ---MACKSSC SLLLLAAVLL SVLAA--A-- ---------- SASGSCVPGV
Zea mays                         MASSSSSSHR RLILAAAVLL SVLAAASA-- ---------- SAGTSCVPGW
Tritcum aestivum                 ---MASN-HR RFLLSGAVLL SVLAAVAA-L E--------- SVEDECQPGV

                                 ....|....| ....|....| ....|....| ....|....| ....|....|
                                          60         70         80         90        100
Hordeum vulgare                  DFPHNPLATC HTYVIKRVCG ---RGPSRPM LV-------- ------KERC
Oryza sativa                     GYPMYPLPRC RALVKRQCVG ---RGTAAAA EQ-------- -----VRRDC
Hordeum spontaneum               ALPANPLRAC RTYVVSQIC- --HVGPRLST WD-------- -----MKRRC
Eleusine coracana                AIPHNPLDSC RWYVAKRACG ---VGPRLAT QE-------- -----MKARC
Secale cereale                   AMPHNPLGAC RTYVVSQIC- --HVGPRLFT WD-------- -----MKRRC
Triticum durum                   AFRTNLLPHC RDYVLQQTCG TFTPGSKLPE WMTSASIYSP GKPYLAKLYC
Zea mays                         AIPHNPLPSC RWYVTSRTCG ---IGPRLPW PE-------- -----LKRRC
Tritcum aestivum                 AFPHNALATC HTYVIKRVCG ---RGPSRPM LV-------- ------KERC

                                 ....|....| ....|....| ....|....| ....|....| ....|....|
                                         110        120        130        140        150
Hordeum vulgare                  CRELAAVP-D HCRCEALRIL MDGVRTPE-- ----GRVVEG RLGDRRDCPR
Oryza sativa                     CRQLAAVDDS WCRCEAISHM LGGIYRELG- ---APDVGHP MSEVFRGCRR
Hordeum spontaneum               CDELSAIP-A YCRCEALRII MDGTVTWQ-- ----GVFG-A YFKDMPNCPR
Eleusine coracana                CRQLEAIP-A YCRCEAVRIL MDGVVTP--- ---SGQHEGR LLQDLPGCPR
Secale cereale                   CDELLAIP-A YCRCEALRIL MDGVVTQQ-- ----GVFEGG YLKDMPNCPR
Triticum durum                   CQELAEIS-Q QCRCEALRYF IALPVPSQPV DPRSGNVGES GLIDLPGCPR
Zea mays                         CRELADIP-A YCRCTALSIL MDGAIPPGP- ---DAQLEGR -LEDLPGCPR
Tritcum aestivum                 CRELAVVP-D YCRCEALRVL MDGVRAEE-- ----GHVVEG RLGDRRDCPR

                                 ....|....| ....|....| ....|....| ....|....| ....|....|
                                         160        170        180        190
Hordeum vulgare                  EEQRAFAATL VTAAECNLSS VQAPGVRLVL LADG------ ---
Oryza sativa                     GDLERAAASL PAFCNVDIPN GGG-GVCYWL ARSGY----- ---
Hordeum spontaneum               VMQTSYAANL VNPQECNLWT IHGSPSCPEL QPGYEVVL-- ---
Eleusine coracana                QVQRAFAPKL VTEVECNLAT IHGGPFCLSL LGAGE----- ---
Secale cereale                   VTQRSYAATL VAPQECNLPT IHGSPYCPTL QAGY------ ---
Triticum durum                   EMQWDFVRLL VAPGQCNLAT IHNVRYCPAV EQPLWI---- ---
Zea mays                         EVQRGFAATL VTEAECNLAT ISGVAECPWI LGGGTMPSK- ---
Tritcum aestivum                 EAQREFAATL VTAAECNLPT VS--GVGSTL GATGRWMTIE LPK
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