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(54) Title: PLANT CELLS AND PLANTS WHICH SYNTHESIZE A STARCH WITH AN INCREASED FINAL VISCOSITY

(57) Abstract: The present invention relates to a plant cell which is genetically modified, the genetic modification leading to the reduction of the activity of one or more SSIII proteins which occur endogenously in the plant cell and to the reduction of the activity of one or more BEII proteins which occur endogenously in the plant cell and to the reduction of the activity of one or more BEII proteins which occur endogenously in the plant cell in comparison with corresponding plant cells, of wild-type plants, which have not been genetically modified. Further aspects of the invention relate to plants containing such plant cells, to a method for generating the plant cells and plants, and to the starch obtainable from them.

Plant cells and plants which synthesize a starch with an increased final viscosity

5 Description

The present invention relates to plant cells and plants which are genetically modified, the genetic modification leading to the reduction of the activity of SSIII and BEI and BEII proteins in comparison with 10 corresponding plant cells, of wild-type plants, which have not been genetically modified. Furthermore, the present invention relates to means and methods for the generation of such plant cells and plants. Such plant cells and plants synthesize a modified starch which is characterized in that it has an amylose content of at least 30% and a phosphate content which 15 is increased in comparison with starch from corresponding wild-type plants which have not been genetically modified and which have a final viscosity in the RVA analysis which is increased over the prior art and/or a modified side-chain distribution and/or an increased gel strength in the Texture Analyser and/or a modified granule morphology and/or a modified mean 20 granule size. The present invention thus also relates to the starch synthesized by the plant cells and plants according to the invention, and to methods for producing this starch.

In view of the increasing importance which is currently attached to plant 25 constituents as renewable raw materials, one of the tasks of biotechnology research is to attempt an adaptation of these vegetable raw materials to the requirements of the processing industry. In order to make possible the use of renewable raw materials in as many fields of application as possible, it is additionally necessary to arrive at very diverse substances.

The polysaccharide starch is a polymer of chemically uniform units, the glucose molecules. However, it takes the form of a highly complex mixture of different forms of molecules which differ with regard to their degree of polymerization and the occurrence of branches of the glucose chains.

5 Starch is therefore no uniform raw material. One distinguishes between two chemically different components of starch, amylose and amylopectin. In typical plants used for starch production such as, for example, maize, wheat or potato, amylose starch accounts for approximately 20%-30% and amylopectin starch for approximately 70%-80% of the starch synthesized.

10 Amylose has long been regarded as a linear polymer consisting of α -1,4-glycosidically linked α -D-glucose monomers. However, more recent studies have demonstrated the presence of α -1,6-glycosidic branch points (approx. 0.1%) (Hizukuri and Takagi, Carbohydr. Res. 134, (1984), 1-10; Takeda et al., Carbohydr. Res. 132, (1984), 83-92).

15

Various methods are available for determining the amylose content. Some of these methods are based on the iodine-binding capacity of amylose, which can determine potentiometrically (Banks & Greenwood, in W. Banks & C.T. Greenwood, Starch and its components (pp. 51-66), Edinburgh, 20 Edinburgh University Press), amperometrically (Larson et al., Analytical Chemistry 25(5), (1953), 802-804) or spectrophotometrically (Morrison & Laignelet, J. Cereal Sc. 1, (1983), 9-20). The amylose content may also be determined calorimetrically by means of DSC (differential scanning calorimetry) measurements (Kugimiya & Donovan, Journal of Food Science 46, (1981), 765-770; Sievert & Holm, Starch/Stärke 45 (4), (1993), 136-139). It is furthermore possible to determine the amylose content via the use of SEC (size exclusion chromatography) chromatography of native or debranched starch. This method has been recommended in particular for determining the amylose content of genetically modified starches 30 (Gérard et al., Carbohydrate Polymers 44, (2001), 19-27).

In contrast to amylose, amylopectin shows a higher degree of branching and has approximately 4% of branch points brought about by the occurrence of additional α -1,6-glycosidic linkages. Amylopectin constitutes a complex mixture of glucose chains with different branching patterns.

- 5 Another important difference between amylose and amylopectin is their molecular weight. While amylose, depending on the origin of the starch, has a molecular weight of 5×10^5 – 10^6 Da, the molecular weight of amylopectin is between 10^7 and 10^8 Da. The two macromolecules can be distinguished on the basis of their molecular weight and their different 10 physico-chemical properties, and the simplest way of visualizing this is through their different iodine-binding properties.

In addition to the amylose/amylopectin ratio and the phosphate content, the functional properties of starch are affected greatly by the molecular 15 weight, the side-chain distribution pattern, the ionic content, the lipid and protein content, the mean granule size and the granule morphology and the like. Important functional properties which may be mentioned in this context are solubility, the retrogradation behaviour, the water-binding capacity, the film-forming properties, viscosity, the gelatinization 20 properties, freeze-thaw-stability, acid stability, gel strength and the like. Granule size may also be of importance for various applications.

The skilled workers frequently resort to different methods to determine the gelatinization properties, one of which is the final viscosity. Depending on 25 the method used, absolute values in particular, but also relative values, may differ between one and the same starch sample. A rapid and effective method for analysing the gelatinization properties is the RVA analysis. Depending on the choice of the parameters and the temperature profile in the RVA analysis, different RVA profiles are obtained for one and the 30 same sample. It should be mentioned that in some cases different profiles

were used in the prior art mentioned hereinbelow when determining the gelatinization properties.

An overview over different plant species with a reduction of the enzymes 5 participating in starch biosynthesis can be found in Kossmann and Lloyd (2000, Critical Reviews in Plant Sciences 19(3), 171-126).

To date, plants have been described in which the activity of an SSIII 10 protein (Abel et al., 1996, The Plant Journal 10(6), 9891-991; Lloyd et al., 1999, Biochemical Journal 338, 515-521) or the activity of a BEI protein 15 (Kossmann et al. 1991, Mol Gen Genet 230, 39-44); Safford et al., 1998, Carbohydrate Polymers 35, 155-168, or the activity of a BEII protein (Jobling et al., 1999, The Plant Journal 18, or the activity of a BEI and a BEII protein (Schwall et al., 2000, Nature Biotechnology 18, 551- 554; WO 96/34968), or the activity of a BEI and an SSIII (WO 00/08184) protein are reduced .

In plants in which the activity of an SSIII protein is reduced, a relative shift 20 of the amylopectin side chains from longer chains towards shorter chains (Lloyd et al., 1999, Biochemical Journal 338, 515-521), a 70% higher phosphate content, no changes in the amylose content (Abel et al., 1996, The Plant Journal 10(6), 9891-991) and a reduced final viscosity in the RVA analysis (Abel, 1995, PhD Thesis at the Freie Universität Berlin) are 25 observed in comparison with corresponding wild-type plants. In such plants, which are also described in WO 00/08184, a 197% increase in the phosphate content, 123% increase in the amylose content and a final viscosity in the RVA analysis which drops to 76% of that of the wild type can be observed in the isolated starch in comparison with untransformed wild-type plants. Moreover, the gel strength of the starch in question drops to 84% of the wild type.

30 The spectrophotometric analysis by the method of Morrison & Laignelet (1983, J. Cereal Sc. 1, 9-20) reveals an amylose content of up to a

maximum of 89.14% (corresponding to 344% of the wild type) and a starch phosphate content which corresponds to up to a maximum of 522% of the phosphate content of starch isolated from corresponding wild-type plants in 5 plants with a reduced activity of both a BEI and a BEII protein. The RVA analysis reveals a final viscosity value in these starches which is increased up to a maximum of 237%. Moreover, the modified granule morphology in starch grains isolated from such plants is distinguished by the fact that the granules have large grooves in the centre of the granule in question when viewed under 10 the microscope under polarized light.

As a result, the skilled worker is familiar with plant cells and plants and starches synthesized by them which have an increased amylose and phosphate content but whose final viscosity in the RVA analysis is increased by not more than up 15 to a maximum of 256% in comparison with wild-type plants which have not been genetically modified. Higher final viscosities in the RVA analysis have not been achieved to date. However, this would be desirable since less starch solids have to be employed, for example when using such a starch as thickener, gelling agent or binder, in order to achieve the desired effect. This 20 allows for example the reduction of the amount of additives in human and animal foods, in healthcare products and in cosmetics. It would also be possible to employ smaller amounts of starch when using such a starch in glues, leading to reduced costs for example in making, for example, paper, cardboard and insulating board.

25 The present invention relates to providing plant cells, plants and starch from suitable plant cells or plants with an increased amylose content and an increased phosphate content and, in the RVA analysis, a final viscosity which is increased by at least 270% and/or an increased gel strength of the gelatinized starch and/or a modified granule morphology.

30

This is achieved by providing the embodiments specified in the patent claims.

In the claims which follow and in the preceding description of the invention,
5 except where the context requires otherwise due to express language or
necessary implication, the word "comprise" or variations such as "comprises" or
"comprising" is used in an inclusive sense, i.e. to specify the presence of the
stated features but not to preclude the presence or addition of further features
in various embodiments of the invention.

10

It is to be understood that, if any prior art publication is referred to herein, such
reference does not constitute an admission that the publication forms a part of
the common general knowledge in the art, in Australia or any other country.

15 A first aspect of the present invention thus relates to a plant cell which is
genetically modified, the genetic modification leading to the reduction of the
activity of one or more SSIII proteins occurring endogenously in the plant cell
and to the reduction of the activity of one or more BEI proteins which occur
endogenously in the plant cell and to the reduction of the activity of one or more
20 BEII proteins which occur endogenously in the plant cell in comparison to
corresponding plant cells, of wild-type plants, which have not been genetically
modified.

In this context, the genetic modification can be any genetic modification which
25 leads to a reduction of the activity of one or more SSIII proteins which occur
endogenously in the plant cell and to the reduction of the activity of one or more
BEI proteins which occur endogenously in the plant cell and to the reduction of
the activity of one or more BEII proteins which occur endogenously in the plant
cell in comparison to corresponding plant cells, of wild-type plants, which have
30 not been genetically modified.

2003293991 18 Dec 2008

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For the purposes of the invention, the genetic modification may encompass for example the generation of plant cells according to the invention by subjecting one or more genes to mutagenesis. The type of mutation is irrelevant as long 5 as it leads to a reduction of the activity of an SSIII protein and/or a BEI protein and/or a BEII protein. In connection with the present invention, the term "mutagenesis" is understood as meaning any type of mutation, such as, for example, deletions, point mutations (nucleotide substitutions), insertions, inversions, gene conversions or chromosome translocation.

10

In this context, the mutation can be generated by using chemical agents or high-energy radiation (for example x-rays, neutron, gamma, UV radiation). Agents which can be employed for generating chemically induced mutations, and the mutations generated thereby by the action of the 5 mutagens in question, are described, for example, by Ehrenberg and Husain, 1981, (Mutation Research 86, 1-113), Müller, 1972 (Biologisches Zentralblatt 91 (1), 31-48). The generation of rice mutants using gamma rays, ethyl methanesulfonate (EMS), N-methyl-N-nitrosurea or sodium azide (NaN₃) is described, for example, in Jauhar and Siddiq (1999, Indian 10 Journal of Genetics, 59 (1), 23-28), in Rao (1977, Cytologica 42, 443-450), Gupta and Sharma (1990, Oryza 27, 217-219) and Satoh and Omura (1981, Japanese Journal of Breeding 31 (3), 316-326). The generation of wheat mutants using NaN₃ or maleic hydrazide is described in Arora et al. (1992, Annals of Biology 8 (1), 65-69). An overview over the generation of 15 wheat mutants using different types of high-energy radiation and chemical agents is given in Scarascia-Mugnozza et al. (1993, Mutation Breeding Review 10, 1-28). Svec et al. (1998, Cereal Research Communications 26 (4), 391-396) describes the use of N-ethyl-N-nitrosurea for generating mutants in triticale. The use of MMS and gamma radiation for generating 20 millet mutants is described in Shashidhara et al. (1990, Journal of Maharashtra Agricultural Universities 15 (1), 20-23). The generation of mutants in plant species whose propagation is predominantly vegetatively was described for example for potatoes which produce a modified starch (Hovenkamp-Hermelink et al. (1987, Theoretical 25 and Applied Genetics 75, 217-221) and for mint with an increased oil yield/modified oil quality (Dwivedi et al., 2000, Journal of Medicinal and Aromatic Plant Sciences 22, 460-463). All of these methods are suitable in principle for generating the plant cells according to the invention and the starch produced by them.

Mutations in the relevant genes, in particular in genes encoding a BEI protein and/or a BEII protein and/or an SSIII protein, can be identified with the aid of methods known to the skilled worker. Analyses based on hybridizations with probes (Southern blot), the amplification by means of 5 polymerase chain reaction (PCR), the sequencing of genomic sequences in question, and the search for individual nucleotide substitutions, may be employed in particular. One method of identifying mutations with the aid of hybridization patterns is, for example, the search for restriction fragment length polymorphisms (RFLP) (Nam et al., 1989, *The Plant Cell* 1, 699-10 705; Leister and Dean, 1993, *The Plant Journal* 4 (4), 745-750). An example of a method based on PCR is the analysis of amplified fragment length polymorphisms (AFLP) (Castiglioni et al., 1998, *Genetics* 149, 2039-2056; Meksem et al., 2001, *Molecular Genetics and Genomics* 265, 207-214; Meyer et al., 1998, *Molecular and General Genetics* 259, 150-15 160). The use of amplified fragments cleaved with the aid of restriction endonucleases (cleaved amplified polymorphic sequences, CAPS) may also be used for identifying mutations (Konieczny and Ausubel, 1993, *The Plant Journal* 4, 403-410; Jarvis et al., 1994, *Plant Molecular Biology* 24, 685-687; Bachem et al., 1996, *The Plant Journal* 9 (5), 745-753). Methods 20 for determining SNPs have been described, inter alia, by Qi et al. (2001, *Nucleic Acids Research* 29 (22), e116) Drenkard et al. (2000, *Plant Physiology* 124, 1483-1492) and Cho et al. (1999, *Nature Genetics* 23, 203-207). Methods which are particularly suitable are those which permit a 25 large number of plants to be analysed within a short time for mutations in specific genes. Such a method, known as TILLING (targetting induced local lesions in genomes), has been described by McCallum et al. (2000, *Plant Physiology* 123, 439-442).

The use of all of these methods is suitable in principle for the purposes of 30 the present invention.

Hoogkamp et al. (2000, Potato Research 43, 179-189) have isolated stable potato mutants which contain an amylose-free starch. These plants no longer synthesize active enzyme for a granule-bound starch synthase (GBSS I). After subjecting these plants to another mutagenesis, those 5 which additionally have mutations in genes which are involved in starch biosynthesis may be selected. Plants which synthesize starch with improved characteristics might thus be generated. Using the suitable method, it is also possible to identify and isolate the plant cells according to the invention which produce a starch according to the invention.

10

Moreover, the plant cells according to the invention may also be generated with the aid of homologous transposons, that is to say transposons which are naturally present in the plant cells in question. A detailed description of this method is given hereinbelow.

15

All of the abovementioned methods are suitable in principle for generating plant cells according to the invention and the modified starch synthesized by them. The present invention therefore also relates to methods for generating genetically modified plant cells which synthesize a modified 20 starch, this starch being characterized in that it has an amylose content of at least 30%, in that it has an increased phosphate content in comparison with starch from corresponding wild-type plant cells which have not been genetically modified and in that it has an increased final viscosity in the RVA analysis in comparison with starch from corresponding wild-type plant 25 cells which have not been genetically modified.

A further aspect of the present invention relates to methods for generating a plant cell which synthesizes a modified starch, comprising the genetic modification of the plant cell, the genetic modification leading to the 30 reduction of the activity of one or more SSIII proteins which occur endogenously in the plant cell and to the reduction of the activity of one or

more BEI proteins which occur endogenously in the plant cell and to the reduction of the activity of one or more BEII proteins which occur endogenously in the plant cell, in comparison with corresponding plant cells, of wild-type plants, which have not been genetically modified.

5

Yet a further aspect of the present invention relates to methods for generating a genetically modified plant which synthesizes a modified starch, in which

- a) a plant cell is generated as described above;
- 10 b) a plant is regenerated from, or using, the plant cell generated in accordance with a); and,
- c) if appropriate, further plants are generated from the plant generated in accordance with step b).

15 In connection with the present invention, the term "genetically modified" means that the genetic information of the plant cell is altered.

In this context, a reduction of the activity of one or more SSIII proteins which occur endogenously in the plant cell and a reduction of the activity 20 of one or more BEI proteins which occur endogenously in the plant cell and a reduction of the activity of one or more BEII proteins which occur endogenously in the plant cell is observed in the plant cells according to the invention in comparison with corresponding plant cells, of wild-type plants, which have not been genetically modified.

25

The genetic modifications for generating the plant cells according to the invention can be performed simultaneously or in consecutive steps. In this context, each genetic modification can lead to the reduction of the activity of one or more SSIII proteins and/or one or more BEI proteins and/or one 30 or more BEII proteins. The starting material may be either wild-type plants or wild-type plant cells in which no previous genetic modification in order to

reduce the activity of one or more SSIII proteins and/or one or more BEI proteins and/or one or more BEII proteins has been performed, or else genetically modified plant cells or plants in which the activity of one or more SSIII proteins and/or one or more BEI proteins and/or one or more BEII proteins has already been carried out by genetic modification. If such genetically modified plants (plant cells) constitute the starting material, the genetic modifications which are subsequently carried out preferably only relate to the activity of in each case one or more proteins whose activity has not been reduced yet (SSIII, BEI or BEII).

10

For example, a reduction of the expression of one or more SSIII genes which occur endogenously in the plant cell and a reduction of the expression of one or more BEI genes which occur endogenously in the plant cell and a reduction of the expression of one or more BEII genes which occur endogenously in the plant cell and/or a reduction of the activity of in each case one or more of the abovementioned proteins which occur in the plant cell is observed in genetically modified plant cells according to the invention in comparison with plant cells, of wild-type plants, which have not been genetically modified.

15

For the purposes of the present invention, the term "reduction of the activity" refers to a reduction of the expression of endogenous genes which encode SSIII, BEI and/or BEII proteins, and/or a reduction of the amount of SSIII, BEI and/or BEII protein in the cells and/or a reduction of the enzymatic activity of the SSIII, BEI and/or BEII proteins in the cells.

20

The reduction of the expression can be determined for example by measuring the amount of SSIII, BEI or BEII protein-encoding transcripts, for example by Northern blot analysis or RT-PCR. A reduction preferably means, in this context, a reduction of the amount of transcripts by at least 50%, in particular by at least 70%, preferably by at least 85% and

especially preferably by at least 95% in comparison to corresponding cells which have not been genetically modified.

The reduction of the amount of SSIII, BEI and/or BEII proteins which results in a reduced activity of these proteins in the plant cells in question
5 can be determined for example by immunological methods such as Western blot analysis, ELISA (enzyme-linked immunosorbent assay) or RIA (radio-immune assay). In this context, a reduction preferably means a reduction of the amount of SSIII, BEI and/or BEII protein by at least 50%, in particular by at least 70%, preferably by at least 85% and especially
10 preferably by at least 95% in comparison to corresponding cells which have not been genetically modified.

In connection with the present invention, SSIII protein is understood as meaning a class of soluble starch synthases (ADP-glucose-1,4-alpha-D-
15 glucan-4-alpha-D-glucosyltransferase; EC 2.4.1.21). Soluble starch synthases catalyze a glycosylation reaction, in which glucose residues of the substrate ADP-glucose are transferred to alpha-1,4-linked glucan chains, with formation of an alpha-1,4 linkage (ADP glucose + {(1,4)-alpha-D-glucosyl}(N) <=> ADP + {(1,4)-alpha-D-glucosyl}(N+1)).

20 SSIII proteins are described, for example, by Marshall et al. (The Plant Cell 8; (1996); 1121-1135), Li et al. (2000, Plant Physiology 123, 613-624), Abel et al. (The Plant Journal 10(6); (1996); 981-991) and in WO 0066745. The structure of SSIII proteins frequently shows a sequence of domains.
25 At the N terminus, SSIII proteins have a signal peptide for the transport into plastids. Towards the C terminus, this is followed by an N-terminal region, an SSIII-specific region and a catalytic domain (Li et al., 2000, Plant Physiology 123, 613-624). Further analyses which are based on primary sequence alignments (<http://hits.isb-sib.ch/cgi-bin/PFSCAN>),
30 revealed that the potato SSIII protein has what is known as a carbohydrate binding domain (CBM). This domain (Pfam motiv cbm 25) comprises the

amino acids 377 to 437 of the sequence of the potato SSIII protein shown in Seq ID No. 2. In connection with the present invention, an SSIII protein is therefore to be understood as meaning starch synthases which have at least 50%, preferably at least 60%, especially preferably at least 70%,
5 more preferably at least 80% and in particular at least 90% identity with the sequence shown in Seq ID No. 3.

The term homology, or identity, is understood as meaning the number of agreeing amino acids (identity) with other proteins, expressed in percent.

10 The identity is preferably determined by comparing the Seq. ID No. 3 with other proteins with the aid of computer programmes. If sequences which are compared with each other are different in length, the identity is to be determined in such a way that the number of amino acids which the short sequence shares with the longer sequence determines the percentage
15 identity. The identity can be determined routinely by means of known computer programmes which are publicly available such as, for example, ClustalW (Thompson et al., Nucleic Acids Research 22 (1994), 4673-4680). ClustalW is made publicly available by Julie Thompson (Thompson@EMBL-Heidelberg.DE) and Toby Gibson (Gibson@EMBL-
20 Heidelberg.DE), European Molecular Biology Laboratory, Meyerhofstrasse 1, D 69117 Heidelberg, Germany. ClustalW can likewise be downloaded from various internet pages, inter alia the IGBMC (Institut de Génétique et de Biologie Moléculaire et Cellulaire, B.P.163, 67404 Illkirch Cedex, France; <ftp://ftp-igbmc.u-strasbg.fr/pub/>) and the EBI
25 (<ftp://ftp.ebi.ac.uk/pub/software/>) and all mirrored EBI internet pages (European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SD, UK).

If the ClustalW computer programme Version 1.8 is used to determine the identity between, for example, the reference protein of the present
30 application and other proteins, the following parameters are to be set: KTUPLE=1, TOPDIAG=5, WINDOW=5, PAIRGAP=3, GAPOEN=10,

GAPEXTEND=0.05, GAPDIST=8, MAXDIV=40, MATRIX=GONNET, ENDGAPS(OFF), NOPGAP, NOHGAP.

One possibility of finding similar sequences is to carry out sequence database researches. Here, one or more sequences are entered as what 5 is known as a query. This query sequence is then compared with sequences present in the selected databases using statistical computer programmes. Such database queries (blast searches) are known to the skilled worker and can be carried out at different suppliers. If, for example, such a database query is carried out at the NCBI (National Center for 10 Biotechnology Information, <http://www.ncbi.nlm.nih.gov/>), the standard settings for the respective comparison query should be used. For protein sequence comparisons (blastp), these settings are: Limit entrez = not activated; Filter = low complexity activated; Expect value = 10; word size = 3; Matrix = BLOSUM62; Gap costs: Existence = 11, Extension = 1. The 15 result of such a query is, among other parameters, the degree of identity between the query sequence and the similar sequences found in the databases.

Thus, an SSIII protein is to be understood as meaning, in connection with the present invention, starch synthases which, when using at least one of 20 the above-described methods for determining the identity with the sequence shown in Seq ID No. 3, have at least 50%, preferably at least 60%, especially preferably at least 70%, more preferably at least 80% and in particular at least 90% identity.

25 For the purposes of the present invention, the term SSIII gene is understood as meaning a nucleic acid molecule (DNA, cDNA, RNA) which encodes an SSIII protein, preferably from potato. Nucleic acid molecules encoding an SSIII protein have been described for a variety of plant species such as, for example, potato (Abel et al., The Plant Journal 10(6); 30 (1996); 981-991), wheat (WO 00/66745, Li et al., 2000, Plant Physiology 123, 613-624; Genbank Acc. No AF258608; Genbank Acc. No

AF258609), maize (Gao et al., 1998, Plant Cell 10 (3), 399-412; Genbank Acc. No AF023159), *Vignia* (Genbank Acc. No AJ225088), rice (Genbank Acc. No AY100469; Genbank Acc. No AF43291) and *Arabidopsis* (Genbank Acc. No AC007296).

5

For the purposes of the present invention, the term "branching enzyme" or "BE protein" (α -1,4-glucan: α -1,4-glucan-6-glycosyltransferase, E.C. 2.4.1.18) is understood as meaning a protein which catalyzes a transglycosylation reaction in which α -1,4-linkages of an α -1,4-glucan donor are hydrolyzed and the α -1,4-glucan chains liberated in this process are transferred to an α -1,4-glucan acceptor chain, where they are converted into α -1,6 linkages.

15 The term "BEI protein" is to be understood as meaning, for the purposes of the present invention, an isoform I branching enzyme (branching enzyme = BE). The BEI protein is preferably derived from potato plants.

In this context, isoform terminology relies on the nomenclature proposed by Smith-White and Preiss (Smith-White and Preiss, Plant Mol Biol. Rep. 12, (1994), 67-71, Larsson et al., Plant Mol Biol. 37, (1998), 505-511). This 20 nomenclature assumes that all enzymes which have a higher degree of homology (identity) at the amino acid level with the maize BEI protein (GenBank Acc. No. D11081; Baba et al., Biochem. Biophys. Res. Commun. 181 (1), (1991), 87-94; Kim et al. Gene 216, (1998), 233-243) than to the maize BEII protein (Genbank Acc. No AF072725, U65948) are 25 referred to as isoform I branching enzymes, abbreviated to BEI proteins.

The term "BEII protein" is to be understood as meaning, for the purposes of the present invention, an isoform II branching enzyme (branching enzyme = BE). This enzyme preferably originates from potato plants. In 30 connection with the present invention, all enzymes which, at the amino acid level, have a higher degree of homology (identity) with the maize BEII

protein (Genbank Acc. No AF072725, U65948) than with the maize BEI protein (Genbank Acc. No. D 11081, AF 072724) shall be referred to as BEII protein.

5 The term "BEI gene" is understood as meaning, for the purposes of the present invention, a nucleic acid molecule (cDNA, DNA) which encodes a "BEI protein", preferably a BEI protein from potato plants. Such nucleic acid molecules have been described for a large number of plants, for example for maize (Genbank Acc. No. D 11081, AF 072724), rice 10 (Genbank Acc. No. D11082), pea (Genbank Acc. No. X80010) and potato. Various forms of the BEI gene, or the BEI protein, from potato have been described, for example, by Khoshnoodi et al., Eur. J. Biochem. 242 (1), 148-155 (1996), Genbank Acc. No. Y 08786 and by Kossmann et al., Mol. Gen. Genet. 230, (1991), 39-44). In potato plants, the BEI gene is 15 expressed predominantly in the tubers and to a very minor degree in the leaves (Larsson et al., Plant Mol. Biol. 37, (1998), 505-511).

The term "BEII gene" is to be understood as meaning, for the purposes of the present invention, a nucleic acid molecule (for example cDNA, DNA) 20 which encodes a "BEII protein", preferably a BEII protein from potato plants. Such nucleic acid molecules have been described for a large number of plants, for example for potato (GenBank Acc. No. AJ000004, AJ011888, AJ011889, AJ011885, AJ011890, EMBL GenBank A58164), maize (AF 072725, U65948), barley (AF064561), rice (D16201) and wheat 25 (AF 286319). In potato plants, the BEII gene is expressed predominantly in the tubers and to a very minor degree in the leaves (Larsson et al., Plant Mol. Biol. 37, (1998), 505-511).

The term "transgenic" is to be understood as meaning, in the present 30 context, that the genetic information of the plant cells according to the invention deviates from corresponding plant cells which have not been

genetically modified owing to the introduction of a foreign nucleic acid molecule or several foreign nucleic acid molecules into the cell.

In a further embodiment of the present invention, the genetic modification 5 of the transgenic plant cell according to the invention consists in the introduction of one or more foreign nucleic acid molecules whose presence and/or expression leads to the reduction of the activity of SSIII and BEI and BEII proteins in comparison to corresponding plant cells, of wild-type plants, which have not been genetically modified. Specifically, 10 the term "genetic manipulation" is understood as meaning the introduction of homologous and/or heterologous nucleic acid molecules and/or foreign nucleic acid molecules which have been subjected to mutagenesis into a plant cell, where said introduction of these molecules leads to the reduction of the activity of an SSIII protein and/or a BEI protein and/or BEII 15 protein.

The term "foreign nucleic acid molecule" or "of foreign nucleic acid molecules" is understood as meaning, for the purposes of the present invention, such a molecule which either does not occur naturally in the 20 plant cells in question, or which does not naturally occur in the plant cells in the specific spatial arrangement, or which is localized at a site in the genome of the plant cell where it does not occur naturally. The foreign nucleic acid molecule is preferably a recombinant molecule which consists of various elements whose combination, or specific spatial arrangement, 25 does not naturally occur in plant cells.

The foreign nucleic acid molecule(s) which is, or are, used for the genetic modification may take the form of a hybrid nucleic acid construct or of several separate nucleic acid constructs, in particular of what are known as single, dual and triple constructs. Thus, the foreign nucleic acid 30 molecule may be, for example, what is known as a "triple construct", which is understood as meaning one single vector for plant transformation which

contains not only the genetic information for inhibiting the expression of one or more endogenous SSIII genes, but also the genetic information for inhibiting the expression of one or more BEI genes and of one or more BEII genes, or whose presence, or expression, leads to the reduction of 5 the activity of one or more SSIII, BEI and BEII proteins.

In a further embodiment, the foreign nucleic acid molecule may be what is known as a "dual construct", which is understood as meaning a vector for plant transformation which contains the genetic information for inhibiting 10 the expression of two out of the three target genes (SSIII, BEI, BEII gene) or whose presence, or expression, leads to the reduction of the activity of two out of the three target proteins (SSIII, BEI, BEII proteins). The inhibition of the expression of the third target gene and/or the reduction of the activity of the third target protein is effected, in this embodiment of the 15 invention, with the aid of a separate, foreign nucleic acid molecule which contains the relevant genetic information for inhibiting this third target gene.

In a further embodiment of the invention, it is not a triple construct which is 20 introduced into the genome of the plant cell, but several different foreign nucleic acid molecules are introduced, one of these foreign nucleic acid molecules being, for example, a DNA molecule which constitutes, for example, a cosuppression construct which brings about a reduction of the expression of one or more endogenous SSIII genes, and a further foreign 25 nucleic acid molecule being a DNA molecule which encodes, for example, an antisense RNA which brings about a reduction of the expression of one or more endogenous BEI and/or BEII genes. When constructing the foreign nucleic acid molecules, however, the use of any combination of antisense, cosuppression, ribozyme and double-stranded RNA constructs 30 or in-vivo mutagenesis which leads to a simultaneous reduction of the gene expression of endogenous genes encoding one or more SSIII, BEI

and BEII proteins, or which leads to a simultaneous reduction of the activity of one or more SSIII, BEI and BEII proteins, is also suitable in principle.

The foreign nucleic acid molecules can be introduced into the genome of 5 the plant cell either simultaneously ("cotransformation") or else one after the other, i.e. in succession at different times ("supertransformation").

The foreign nucleic acid molecules can also be introduced into different 10 individual plants of one species. This may give rise to plants in which the activity of one target protein, or two target proteins, (BEI, BEII, SSIII) is reduced. Subsequent hybridizing may then give rise to plants in which the activity of all three of the target proteins is reduced.

Instead of a wild-type plant cell or plant, a mutant which is distinguished by 15 already showing a reduced activity of one or more target proteins (BEI, BEII, SSIII) may further be used for introducing a foreign nucleic acid molecule or for generating the plant cells or plants according to the invention. The mutants may take the form of spontaneously occurring mutants or else of mutants which have been generated by the specific 20 application of mutagens. Possibilities of generating such mutants have been described further above.

The plant cells according to the invention and their starch can be generated, or produced, by using what is known as insertion mutagenesis 25 (review article: Thorneycroft et al., 2001, Journal of experimental Botany 52 (361), 1593-1601). Insertion mutagenesis is to be understood as meaning, in particular, the insertion of transposons or what is known as transfer DNA (T-DNA) into a gene encoding a BEI protein and/or BEII protein and/or an SSIII protein, thus reducing the activity of said proteins in 30 the cell in question.

The transposons may take the form of transposons which occur naturally in the cell (endogenous transposons) or else those which do not occur naturally in said cell but have been introduced into the cell by means of recombinant methods, such as, for example, by transforming the cell 5 (heterologous transposons). Modifying the expression of genes by means of transposons is known to the skilled worker. A review of the utilization of endogenous and heterologous transposons as tools in plant biotechnology can be found in Ramachandran and Sundaresan (2001, *Plant Physiology and Biochemistry* 39, 234-252). The possibility of identifying mutants in 10 which specific genes have been inactivated by transposon insertion mutagenesis can be found in a review by Maes et al. (1999, *Trends in Plant Science* 4 (3), 90-96). The generation of rice mutants with the aid of endogenous transposons is described by Hirochika (2001, *Current Opinion in Plant Biology* 4, 118-122). The identification of maize genes with the aid 15 of endogenous retrotransposons is shown, for example, in Hanley et al. (2000, *The Plant Journal* 22 (4), 557-566). The possibility of generating mutants with the aid of retrotransposons and methods for identifying mutants are described by Kumar and Hirochika (2001, *Trends in Plant Science* 6 (3), 127-134). The activity of heterologous transposons in 20 different species has been described both for dicotyledonous and for monocotyledonous plants, for example for rice (Greco et al., 2001, *Plant Physiology* 125, 1175-1177; Liu et al., 1999, *Molecular and General Genetics* 262, 413-420; Hiroyuki et al., 1999, *The Plant Journal* 19 (5), 605-613; Jeon and Gynheung, 2001, *Plant Science* 161, 211-219), barley 25 (2000, Koprek et al., *The Plant Journal* 24 (2), 253-263), *Arabidopsis thaliana* (Aarts et al., 1993, *Nature* 363, 715-717, Schmidt and Willmitzer, 1989, *Molecular and General Genetics* 220, 17-24; Altmann et al., 1992, *Theoretical and Applied Genetics* 84, 371-383; Tissier et al., 1999, *The Plant Cell* 11, 1841-1852), tomato (Belzile and Yoder, 1992, *The Plant Journal* 2 (2), 173-179) and potato (Frey et al., 1989, *Molecular and*

General Genetics 217, 172-177; Knapp et al., 1988, Molecular and General Genetics 213, 285-290).

In principle, the plant cells and plants according to the invention, and the starch produced by them, can be generated, or produced, with the aid of both homologous and heterologous transposons, the use of homologous transposons also including those transposons which are already naturally present in the plant genome.

10 T-DNA insertion mutagenesis is based on the fact that certain segments (T-DNA) of Ti plasmids from *Agrobacterium* are capable of integrating into the genome of plant cells. The site of integration into the plant chromosome is not fixed but may take place at any position. If the T-DNA integrates in a segment of the chromosome which constitutes a gene function, this may lead to a modification of the gene expression and thus also to an altered activity of a protein encoded by the gene in question. In particular, the integration of a T-DNA into the coding region of a protein frequently means that the protein in question can no longer be synthesized in active form, or not at all, by the cell in question. The use of T-DNA insertions for the generation of mutants is described, for example, for *Arabidopsis thaliana* (Krysan et al., 1999, The Plant Cell 11, 2283-2290; Atipiroz-Leehan and Feldmann, 1997, Trends in genetics 13 (4), 152-156; Parinov and Sundaresan, 2000, Current Opinion in Biotechnology 11, 157-161) and rice (Jeon and An, 2001, Plant Science 161, 211-219; Jeon et al., 2000, The Plant Journal 22 (6), 561-570). Methods for identifying mutants which have been generated with the aid of T-DNA insertion mutagenesis are described, inter alia, by Young et al.,(2001, Plant Physiology 125, 513-518); Parinov et al. (1999, The Plant cell 11, 2263-2270), Thorneycroft et al. (2001, Journal of Experimental Botany 52, 1593-1601), and McKinney et al. (1995, The Plant Journal 8 (4),613-622).

In principle, T-DNA mutagenesis is suitable for generating the plant cells according to the invention and for producing the starch produced by them.

In a further embodiment of the present invention, the presence and/or the 5 expression of one or more foreign nucleic acid molecules leads to the inhibition of the expression of endogenous genes which encode SSIII proteins, BEI proteins and BEII proteins.

The plant cells according to the invention can be generated by various 10 methods with which the skilled worker is familiar, for example by those which lead to an inhibition of the expression of endogenous genes encoding an SSIII, BEI or BEII protein. They include, for example, the expression of a corresponding antisense RNA or a double-stranded RNA construct, the provision of molecules or vectors which confer a 15 cosuppression effect, the expression of a suitably constructed ribozyme which specifically cleaves transcripts encoding an SSIII, BEI or BEII protein, or what is known as "in-vivo mutagenesis". Moreover, the reduction of the SSIII and/or BEI and/or BEII activity in the plant cells may also be brought about by the simultaneous expression of sense and 20 antisense RNA molecules of the specific target gene to be repressed, preferably the SSIII and/or BEI and/or BEII gene. The skilled worker is familiar with these methods.

Moreover, it is known that the generation *in planta* of double-stranded RNA molecules of promoter sequences *in trans* can lead to methylation 25 and transcriptional inactivation of homologous copies of this promoter (Mette et al., EMBO J. 19, (2000), 5194-5201).

Other methods for reducing the activity of proteins are described hereinbelow.

All of these methods are based on the introduction of one or more foreign 30 nucleic acid molecules into the genome of plant cells.

To inhibit gene expression by means of antisense or cosuppression technology it is possible to use, for example, a DNA molecule which encompasses all of the sequence encoding an SSIII and/or BEI and/or BEII protein including any flanking sequences which may be present, or 5 else DNA molecules which only encompass parts of the coding sequence, which must be long enough in order to bring about an antisense effect, or cosuppression effect, in the cells. Sequences which are suitable generally have a minimum length of not less than 15 bp, preferably a length of 100-500 bp, and for effective antisense or cosuppression inhibition in particular 10 sequences which have a length of over 500 bp.

Another possibility which is suitable for antisense or cosuppression approaches is the use of DNA sequences with a high degree of homology with the endogenous sequences which encode SSIII, BEI or BEII proteins 15 and which occur endogenously in the plant cell. The minimum degree of homology should exceed approximately 65%. The use of sequences with homology levels of at least 90%, in particular between 95 and 100%, is to be preferred.

20 The use of introns, i.e. noncoding regions of genes, which encode SSIII, BEI and/or BEII proteins is also feasible for achieving an antisense or cosuppression effect.

The use of intron sequences for inhibiting the gene expression of genes which encode starch biosynthesis proteins has been described in the 25 international patent applications WO97/04112, WO97/04113, WO98/37213, WO98/37214.

The skilled worker is familiar with methods for achieving an antisense and cosuppression effect. The cosuppression inhibition method has been described, for example, in Jorgensen (Trends Biotechnol. 8 (1990), 340-344), Niebel et al., (Curr. Top. Microbiol. Immunol. 197 (1995), 91-103), 30 Flavell et al. (Curr. Top. Microbiol. Immunol. 197 (1995), 43-46), Palaqui

and Vaucheret (Plant. Mol. Biol. 29 (1995), 149-159), Vaucheret et al., (Mol. Gen. Genet. 248 (1995), 311-317), de Borne et al. (Mol. Gen. Genet. 243 (1994), 613-621).

5 The expression of ribozymes for reducing the activity of specific enzymes in cells is also known to the skilled worker and described, for example, in EP-B1 0321201. The expression of ribozymes in plant cells has been described, for example, in Feyter et al. (Mol. Gen. Genet. 250, (1996), 329-338).

10

Moreover, the reduction of the SSIII and/or BEI and/or BEII activity in the plant cells may also be achieved by what is known as "in-vivo mutagenesis", where an RNA-DNA oligonucleotide hybrid ("chimeroplast") is introduced into cells by means of transforming cells (Kipp, P.B. et al.,

15 Poster Session at the " 5th International Congress of Plant Molecular Biology, 21st-27th September 1997, Singapore; R. A. Dixon and C.J. Arntzen, Meeting report on "Metabolic Engineering in Transgenic Plants", Keystone Symposia, Copper Mountain, CO, USA, TIBTECH 15, (1997), 441-447; International Patent Application WO 9515972; Kren et al.,

20 Hepatology 25, (1997), 1462-1468; Cole-Strauss et al., Science 273, (1996), 1386-1389; Beetham et al., 1999, PNAS 96, 8774-8778).

Part of the DNA component of the RNA-DNA oligonucleotide is homologous with a nucleic acid sequence of an endogenous SSIII, BEI and/or BEII gene, but contains a mutation in comparison with the nucleic acid sequence of an endogenous SSIII, BEI and/or BEII gene or contains 25 a heterologous region which is surrounded by the homologous regions.

Owing to base pairing of the homologous regions of the RNA-DNA oligonucleotide and of the endogenous nucleic acid molecule, followed by homologous recombination, the mutation or heterologous region contained 30 in the DNA component of the RNA-DNA oligonucleotide can be transferred

into the genome of a plant cell. This leads to a reduction of the activity of one or more SSIII, BEI and/or BEII proteins.

Moreover, the reduction of the SSIII and/or BEI and/or BEII activity in the plant cells may also be caused by the simultaneous expression of sense 5 and antisense RNA molecules of the specific target gene to be repressed, preferably the SSIII and/or BEI and/or BEII gene.

This may be achieved for example by the use of chimeric constructs which contain "inverted repeats" of the respective target gene or parts of the target gene. The chimeric constructs encode sense and antisense RNA 10 molecules of the target gene in question. Sense and antisense RNA are synthesized simultaneously *in planta* as one RNA molecule, it being possible for sense and antisense RNA to be separated from each other by a spacer and to form a double-stranded RNA molecule.

It has been demonstrated that the introduction of inverted-repeat DNA 15 constructs in the genome of plants is a highly effective method for repressing the genes corresponding to the inverted-repeat DNA constructs (Waterhouse et al., Proc. Natl. Acad. Sci. USA 95, (1998), 13959-13964; Wang and Waterhouse, Plant Mol. Biol. 43, (2000), 67-82; Singh et al., Biochemical Society Transactions Vol. 28 part 6 (2000), 925- 927; Liu et 20 al., Biochemical Society Transactions Vol. 28 part 6 (2000), 927-929; Smith et al., (Nature 407, (2000), 319-320; International Patent Application WO99/53050 A1). Sense and antisense sequences of the target gene(s) may also be expressed separately from one another by means of identical or different promoters (Nap, J-P et al, 6th International Congress of Plant 25 Molecular Biology, Quebec, 18th-24th June, 2000; Poster S7-27, Session S7).

The reduction of the SSIII and/or the BEI and/or the BEII activity in the plant cells can thus also be achieved by generating double-stranded RNA 30 molecules of SSIII and/or BEI and/or BEII genes. To this end, it is preferred to introduce, into the genome of plants, inverted repeats of DNA

molecules of SSIII and/or BEI and/or BEII genes or cDNAs, the DNA molecules to be transcribed (SSIII, BEI or BEII gene or cDNA, or fragments of these genes or cDNAs) being under the control of a promoter which governs the expression of said DNA molecules.

5

Moreover, it is known that the formation of double-stranded RNA molecules of promoter DNA molecules in plants *in trans* can lead to methylation and transcriptional inactivation of homologous copies of these promoters, hereinbelow referred to as target promoters (Mette et al., 10 EMBO J. 19, (2000), 5194-5201).

Thus, it is possible, via the inactivation of the target promoter, to reduce the gene expression of a specific target gene (for example SSIII, BEI or BEII gene) which is naturally under the control of this target promoter.

This means that the DNA molecules which encompass the target 15 promoters of the genes to be repressed (target genes) are in this case – in contrast to the original function of promoters in plants – not used as control elements for the expression of genes or cDNAs, but as transcribable DNA molecules themselves.

To generate the double-stranded target promoter RNA molecules *in* 20 *planta*, where they may be present in the form of RNA hairpin molecules, it is preferred to use constructs which contain inverted repeats of the target promoter DNA molecules, the target promoter DNA molecules being under the control of a promoter which governs the gene expression of said target promoter DNA molecules. These constructs are subsequently introduced 25 into the genome of plants. Expression of the inverted repeats of said target promoter DNA molecules leads to the formation of double-stranded target promoter RNA molecules *in planta* (Mette et al., EMBO J. 19, (2000), 5194-5201). The target promoter can thus be inactivated.

The reduction of the SSIII and/or BEI and/or BEII activity in the plant cells 30 can thus also be achieved by generating double-stranded RNA molecules of promoter sequences of SSIII and/or BEI and/or BEII genes. To this end,

it is preferred to introduce, into the genome of plants, inverted repeats of promoter DNA molecules of SSIII and/or BEI and/or BEII promoters, the target promoter DNA molecules to be transcribed (SSIII, BEI and/or BEII promoter) being under the control of a promoter which governs the 5 expression of said target promoter DNA molecules.

The skilled worker furthermore knows to achieve the activity of one or more SSIII, BEI and/or BEII proteins by expressing nonfunctional derivatives, in particular trans-dominant mutants, of such proteins and/or 10 by expressing antagonists/inhibitors of such proteins.

Antagonists/inhibitors of such proteins encompass for example antibodies, antibody fragments or molecules with similar binding characteristics. For example, a cytoplasmic scFv antibody was employed for modulating the activity of the phytochrome A protein in genetically modified tobacco plants 15 (Owen, Bio/Technology 10 (1992), 790-4; Review: Franken, E, Teuschel, U. and Hain, R., Current Opinion in Biotechnology 8, (1997), 411-416; Whitelam, Trends Plant Sci. 1 (1996), 268-272).

Useful promoters for the expression of nucleic acids which reduce the 20 activity of a target gene are, for example, the promoter of the cauliflower mosaic virus 35S RNA and the maize ubiquitin promoter for constitutive expression, the patatin gene promoter B33 (Rocha-Sosa et al., EMBO J. 8 (1989), 23-29), the MCPI promoter of the potato metallocarbopeptidase 25 inhibitor gene (Hungarian Patent Application HU9801674) or the potato GBSSI promoter (International Patent Application WO 92/11376) for tuber-specific expression in potatoes, or a promoter which ensures expression uniquely in photosynthetically active tissues, for example the ST-LS1 30 promoter (Stockhaus et al., Proc. Natl. Acad. Sci. USA 84 (1987), 7943-7947; Stockhaus et al., EMBO J. 8 (1989), 2445-2451), the Ca/b promoter (see, for example, US 5656496, US 5639952, Bansal et al., Proc. Natl. Acad. Sci. USA 89, (1992), 3654-3658) and the Rubisco SSU promoter

(see, for example, US 5034322, US 4962028), or, for endosperm-specific expression, the glutelin promoter (Leisy et al., Plant Mol. Biol. 14, (1990), 41-50; Zheng et al., Plant J. 4, (1993), 357-366; Yoshihara et al., FEBS Lett. 383, (1996), 213-218), the Shrunken-1 promoter (Werr et al., EMBO J. 4, (1985), 1373-1380), the wheat HMG promoter, the USP promoter, the phaseolin promoter or promoters of zein genes from maize (Pedersen et al., Cell 29, (1982), 1015-1026; Quattrocco et al., Plant Mol. Biol. 15 (1990), 81-93).

The expression of the foreign nucleic acid molecule(s) is particularly 10 advantageous in those plant organs which store starch. Examples of such organs are the tuber of the potato plant or the kernels, or endosperm, of maize, wheat or rice plants. This is why it is preferred to use promoters which confer expression in these organs.

However, it is also possible to use promoters which are activated only at a 15 point in time determined by external factors (see, for example, WO 93/07279). Promoters which may be of particular interest in this context are promoters of heat-shock proteins, which permit simple induction. Others which can be [lacuna] are seed-specific promoters such as, for 20 example, the *Vicia faba* USP promoter, which ensures seed-specific expression in *Vicia faba* and other plants (Fiedler et al., Plant Mol. Biol. 22, (1993), 669-679; Bäumlein et al., Mol. Gen. Genet. 225, (1991), 459-467). Fruit-specific promoters such as, for example, those described in WO91/01373 may furthermore also be employed.

Another element which may be present is a termination sequence, which 25 serves for the correct termination of transcription and for the addition of a poly-A tail to the transcript, which is believed to have a function in stabilizing the transcripts. Such elements are described in the literature (cf., for example, Gielen et al., EMBO J. 8, (1989), 23-29) and can be substituted as desired.

The transgenic plant cells according to the invention synthesize a modified starch whose physico-chemical properties, in particular the amylose content and the amylose/amyopectin ratio, the phosphorus content, the viscosity behaviour, the gel strength, the granule size and/or the granule morphology is modified in comparison with starch synthesized in wild-type plants so that it is better suited to specific uses.

The present invention therefore also relates to a genetically modified plant cell according to the invention, in particular to a transgenic plant cell which synthesizes a modified starch.

Surprisingly, it has been found that the starch composition in the plant cells according to the invention is modified in such a way that the amylose content amounts to at least 30% and the phosphate content is increased and the end viscosity in the RVA analysis is increased in comparison with starch from plant cells from corresponding wild-type plants, so that this starch is better suited to specific uses.

In particular the starches according to the invention have the advantage that they gelatinize completely under standard conditions despite the increased amylose content. This markedly improves the processability of the starch in comparison with other starches with an increased amylose content. An increased temperature or increased pressure is therefore not necessary for gelatinizing the starch according to the invention. This is why the use of specific apparatuses such as, for example, jet cookers, extruders or autoclaves can be dispensed with when breaking down these starches. Another advantage of the starches according to the invention is that, when subjected to processing with hot rollers, they may be applied to the latter in the form of a suspension. Other starches with an increased amylose content would gelatinize, when subjected to this type of processing, to a limited extent only, if at all, and would not be capable of being applied to the rollers in question in the form of a paste or film.

The starches according to the invention are particularly suitable for all applications where the thickening ability, the gelling characteristics or the binding characteristics of added substances are of importance. The starch according to the invention is therefore particularly suitable for the 5 production of foodstuffs such as, for example, baked goods, instant food, blancmange, soups, confectionery, chocolate, icecream, batter for fish or meat, frozen desserts or extruded snacks. Moreover, the starch according to the invention is suitable for the production of glues, for applications in textile processing, as additive to building materials, for applications in the 10 field of animal nutrition, as additive for cosmetics, and in papermaking.

The starch which has been isolated from plant cells according to the invention is particularly suitable for the production of pregelatinized starch. Pregelatinized starches are physically modified starches which are produced predominantly by wet-heat treatment. As opposed to native 15 starch, they form dispersions/pastes or gels with cold water, depending on the concentration of the pregelatinized starch used and as a function of the starch type used for producing the pregelatinized starch. Owing to these characteristics, a series of possible applications exist for pregelatinized starches in the food industry and in addition in many fields 20 of industry. The use of pregelatinized starch, also referred to as cold-swelling starch, instead of native starch frequently has the advantage that production processes can be simplified and shortened.

The production of, for example, instant desserts and instant blancmange requires pregelatinized starches which, after being stirred into cold fluid 25 such as, for example, water or milk, form firm gels in a short time as is the case with, for example, a blancmange which requires boiling. These demands are not met by the commercial pregelatinized starches made with wheat starch, potato starch or corn starch. To obtain the abovementioned characteristics, additives to the pregelatinized starch 30 such as gelatin, alginate, carrageenan and/or inorganic salts are required in the case of the pregelatinized starches which are currently commercially

available. This addition of what are known as adjuvants is not required for example after the production of pregelatinized starches using starches according to the invention which are isolated from plant cells according to the invention.

5

The present invention also relates to a plant cell according to the invention with a modified starch with modified granule morphology.

For the purposes of the present invention, the term granule morphology is intended to refer to size and surface structure of native starch granules.

10 Starch is stored in the storage organs such as, for example, tubers, roots, embryos or endosperm of plants, as a crystalline structure in granular form. Starch granules in which these granular structures are retained after the starch has been isolated from plant cells are referred to as native starch. The mean granule size (determined by the method described 15 hereinbelow) of the native starch according to the invention is markedly lower than that of native starch isolated from wild-type plants. In the scanning electron micrograph (see Figs. 4 and 5) it can be seen clearly that, surprisingly, native starch granules according to the invention have a rough surface with many pores. The surface structure of native starch 20 granules isolated from wild-type plants, in contrast, is predominantly smooth in structure and no pores are discernible.

Both the presence of smaller granules and the rough surface with its pores lead to the fact that the surface area of starch granules according to the invention is considerably larger – at the same volume – than the surface 25 area of starch granules isolated from wild-type plants. The starch according to the invention is therefore particularly suited to the use as carrier for, for example, flavourings, pharmacologically active substances, prebiotics, probiotic microorganisms, enzymes or colorants. These starches are also particularly suitable for coagulating substances and in 30 papermaking.

A further possible application for the starches according to the invention is in the field of drilling for raw materials. Thus, when drilling for crude oil, adjuvants and/or lubricants must be employed which avoid overheating of the drill or drill column. Owing to its particular gelatinization properties, the 5 starch according to the invention is therefore also particularly suited to the use in this field.

The present invention also relates to a plant cell according to the invention which contains a modified starch with an amylose content of at least 30% 10 and which has an increased phosphate content and an increased final viscosity in the RVA analysis in comparison with starch from corresponding plant cells, from wild-type plants, which have not been genetically modified.

15 In connection with the present invention, the amylose content is determined by the method of Hovenkamp-Hermelink et al. (Potato Research 31, (1988), 241-246) described further below for potato starch. This method may also be applied to isolated starches from other plant species. Methods for isolating starches are known to the skilled worker.

20 For the purposes of the present invention, "phosphate content" of the starch refers to the content of phosphate covalently bonded in the form of starch phosphate monoesters.

In connection with the present invention, the term "increased phosphate 25 content" means that the total phosphate content of covalently bonded phosphate and/or the phosphate content in C-6 position of the starch synthesized in the plant cells according to the invention is increased, by preference by at least 270%, more preferably by at least 300%, especially preferably by at least 350% in comparison with starch from plant cells of 30 corresponding wild-type plants.

For the purposes of the present invention, the term "phosphate content in C6 position" is understood as meaning the content of phosphate groups which are bonded at the carbon atom position "6" of the glucose monomers of the starch. In principle, the positions C2, C3 and C6 of the 5 glucose units can be phosphorylated in starch *in vivo*. In connection with the present invention, the determination of the phosphate content in C6 position (= C6-P content) can be carried out via the determination of glucose-6-phosphate by means of a visual-enzymatic test (Nielsen et al., Plant Physiol. 105, (1994), 111-117) (see below).

10

In connection with the present invention, the term "total phosphate content" of the starch refers to the content of phosphate bound covalently in C2, C3 and C6 position of the glucose units in the form of starch phosphate monoesters. The content of phosphorylated non-glucans such 15 as, for example, phospholipids, does not come under the term "total phosphate content" in accordance with the invention. Phosphorylated non-glucans must therefore be removed quantitatively before determining the total phosphate content. Methods for separating the phosphorylated non-glucans (for example phospholipids) and the starch are known to the 20 skilled worker. Methods for determining the total phosphate content are known to the skilled worker and described hereinbelow.

In a further embodiment of the invention, the plant cells according to the invention synthesize a starch which have a phosphate content of 40-25 120 nmol, in particular 60-110 nmol, preferably 80-100 C6-P per mg starch in C6 position of the glucose monomers of the starch.

30 A protocol for carrying out the RVA analysis is described further below. Mention must be made in particular that the RVA analysis of potato starches frequently operates with an 8% starch suspension (w/w). The documentation included with the apparatus "RVAsuper3" (Instructions,

Newport Scientific Pty Ltd., Investment Support Group, Warried NSW 2102, Australia) recommends a suspension containing approximately 10% of starch for the analysis of potato starch.

Surprisingly, it has been found in the case of the starch from potato plants 5 in relation to the present invention, that it was not possible to use an 8% starch suspension (2 g of starch in 25 ml of water) for the analysis since the final viscosity achieved values beyond the range of the apparatus. This is why only 6% starch suspensions (1.5 g of starch in 25 ml of water) were employed for the RVA analysis instead of 8% starch suspensions. In 10 connection with the present invention, "increased end viscosity in the RVA analysis" is therefore understood as meaning an increase by at least 150%, especially by at least 200%, in particular by at least 250%, in comparison with wild-type plants which have not been genetically modified. The increase of the end viscosities relates to 6% starch 15 suspensions in this context.

In connection with the present invention, a potato starch is furthermore understood as meaning one with an at least 300 RVU, especially 400 RVU, in particular 500 RVU final viscosity in the RVA analysis with a 6% starch content. The determination of the RVU values will be discussed 20 in detail hereinbelow.

In a further preferred embodiment, the present invention relates to plant cells according to the invention which synthesize a modified starch which, after gelatinization in water, forms a gel with an increased gel strength in comparison with a gel made with starch of corresponding wild-type plant 25 cells which have not been genetically modified.

For the purposes of the present invention, the term "increased gel strength" is understood as meaning an increase of the gel strength by preference by at least 300%, in particular by at least 500%, more 30 preferably by at least 700% and especially preferably by at least 800%, up to a maximum of not more than 2000% or by not more than 1500% in

comparison with the gel strength of starch from corresponding wild-type plant cells which have not been genetically modified.

In connection with the present invention, the gel strength shall be determined with the aid of a Texture Analyser under the conditions 5 described hereinbelow.

To prepare starch gels, the crystalline structure of native starch must first be destroyed by heating in aqueous suspension with constant stirring. This was carried out with the aid of a Rapid Visco Analyser (Newport Scientific Pty Ltd., Investmet Support Group, Warriewood NSW 2102, Australia). As 10 already mentioned further above, the 8% starch suspension was replaced by an only 6% starch suspension in the case of starch from potato plants since the final viscosities of the 8% suspensions were outside the operating range of the apparatus. To determine gel strength, the starch suspensions gelatinized in the Rapid Visco Analyser were stored over a 15 certain period and then subjected to analysis using a Texture Analyser. Accordingly, 8% gelatinized starch suspensions were also replaced by 6% gelatinized starch suspensions for determining gel strength.

In a further embodiment of the present invention, the modified starch 20 synthesized in the plant cells according to the invention is distinguished not only by an increased amylose content in comparison with starch from corresponding wild-type plants and an increased phosphate content and an increased final viscosity in the RVA analysis, but also by a modified side chain distribution.

25 In a further embodiment, the present invention thus relates to plant cells according to the invention which synthesize a modified starch, the modified starch being characterized by a modified side chain distribution.

In one embodiment of the present invention, the term "modified side chain 30 distribution" is understood as meaning a reduction of the amount of short side chains with a DP (= degree of polymerization) of 6 to 11 by at least

10%, preferably by at least 15%, in particular by at least 30% and especially preferably by at least 50% in comparison with the amount of short side chains with a DP of 6 to 11 of amylopectin from wild-type plants and/or an increase in the content of short side chains with a DP of 6 to 22

5 by at least 5%, preferably by at least 10%, in particular by at least 15% and especially preferably by at least 30% in comparison with the amount of short side chains with a DP of 16 to 22 of amylopectin from wild-type plants.

10 The amount of short side chains is determined via determining the percentage of a specific side chain in the total of all side chains. The total of all side chains is determined via determining the total area under the peaks which represent the degrees of polymerization of DP 6 to 26 in the HPLC chromatogram. The percentage of a particular side chain in the total

15 of all side chains is determined via determining the ratio of the area under the peak which represents this side chain in the HPLC chromatogram to the total area. A programme which may be used for determining the peak areas is, for example, Chromelion 6.20 from Dionex, USA.

20 In a further embodiment of the present invention, the modified starch synthesized in the plant cells according to the invention is distinguished not only by an increased amylose content in comparison with starch from corresponding wild-type plants and an increased phosphate content and an increased final viscosity in the RVA analysis, but also by a modified

25 "side chain profile DP 12 to 18" and/or by a modified "side chain profile DP 19 to 24" and/or by a modified "side chain profile DP 25 to 30" and/or by a modified "side chain profile DP 37 to 42" and/or by a modified "side chain profile DP 62 to 123".

30 In connection with the present invention, the term modified "side chain profile DP 12 to 18" is understood as meaning a reduction of the amount

of amylopectin side chains with a DP of 12 to 18 by at least 25%, preferably by at least 35%, especially preferably by at least 45% and very especially preferably by at least 55% in comparison with the amount of amylopectin side chains with a DP of 12 to 18 from wild-type plants.

5

In connection with the present invention, the term modified "side chain profile DP 19 to 24" is understood as meaning a reduction of the amount of amylopectin side chains with a DP of 19 to 24 by at least 10%, preferably by at least 20% and especially preferably by at least 30% in 10 comparison with the amount of amylopectin side chains with a DP of 19 to 24 from wild-type plants.

In connection with the present invention, the term modified "side chain profile DP 25 to 30" is understood as meaning a reduction of the amount 15 of amylopectin side chains with a DP of 25 to 30 by at least 5% in comparison with the amount of amylopectin side chains with a DP of 25 to 30 from wild-type plants.

In connection with the present invention, the term modified "side chain 20 profile DP 37 to 42" is understood as meaning an increase of the amount of amylopectin side chains with a DP of 37 to 42 by at least 5%, preferably by at least 10% and especially preferably by at least 15% in comparison with the amount of amylopectin side chains with a DP of 37 to 42 from wild-type plants.

25

In connection with the present invention, the term modified "side chain profile DP 62 to 123" is understood as meaning an increase of the amount 20 of amylopectin side chains with a DP of 62 to 123 by at least 20%, preferably by at least 35%, especially preferably by at least 50% in 30 comparison with the amount of amylopectin side chains with a DP of 62 to 123 from wild-type plants.

The side chain profile is determined via determining the percentage of a specific group of side chains in the total of all side chains in the GPC chromatogram. To this end, the total area under the line of the GPC chromatogram is divided into individual segments, each of which

5 represents groups of side chains of different lengths. The chosen segments contain side chains with the following degree of polymerization (DP = number of glucose monomers within one side chain): DP≤11, DP12-18, DP19-24, DP25-30, DP31-36, DP37-42, DP43-48, DP49-55, DP56-61 and DP62-123. To correlate the elution volume with the molecular mass,

10 the GPC column used is calibrated with dextran standards (Fluka, Product# 31430). The dextrans used, their associated molecular mass and the elution volumes are shown in Fig. 9. Using the resulting calibration graph, the elution diagram is shown as a molecular weight distribution. To determine the molecular weight of the individual side chains, a molecular

15 weight of 162 was set for glucose. The total area under the line in the GPC chromatogram is set as 100% and the percentage of the areas of the individual segments is calculated based on the percentage of the total area.

20 In a further especially preferred embodiment, the amylopectin of starch according to the invention, from plant cells according to the invention or plants according to the invention, shows an increased amount of the amylopectin side chains with a DP of greater than 123 in comparison with the amount of side chains with a DP of greater than 123 from amylopectin

25 of wild-type plants.

The plant cells according to the invention may be used for the regeneration of intact plants.

The plants obtainable by regeneration of the transgenic plant cells according to the invention are likewise subject-matter of the present invention.

5 The plant cells according to the invention may belong to any plant species, i.e. to both monocotyledonous and dicotyledonous plants. They are preferably plant cells of agriculturally useful plants, i.e. plants which are grown by man for the purposes of nutrition or for technical, in particular industrial, purposes. The invention preferably relates to fibre-forming 10 plants (for example flax, hemp, cotton), oil-storing plants (for example oilseed rape, sunflower, soybean), sugar-storing plants (for example sugar beet, sugar cane, sugar millet) and protein-storing plants (for example legumes).

In a further preferred embodiment, the invention relates to fodder plants, in 15 particular to fodder grasses and forage grasses (alfalfa, clover and the like) and vegetable plants (for example tomato, lettuce, chicory).

In a further preferred embodiment, the invention relates to plant cells from starch-storing plants (for example wheat, barley, oats, rye, potato, maize, rice, pea, cassava), with plant cells from potato being especially preferred.

20 A multiplicity of techniques are available for introducing DNA into a plant host cell. These techniques encompass the transformation of plant cells with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, the fusion of protoplasts, injection, the 25 electroporation of DNA, the introduction of the DNA by means of the biolistic approach, and other possibilities.

The use of the agrobacteria-mediated transformation of plant cells has been studied intensively and described sufficiently in EP 120516; Hoekema, IN: The Binary Plant Vector System Offsetdrukkerij Kanters 30 B.V., Alblasserdam (1985), Chapter V; Fraley et al., Crit. Rev. Plant Sci. 4, 1-46 and in An et al. EMBO J. 4, (1985), 277-287. As regards the

transformation of potato, see, for example, Rocha-Sosa et al., EMBO J. 8, (1989), 29-33.).

The transformation of monocotyledonous plants by means of vectors based on transformation with agrobacterium has also been described (Chan et al., Plant Mol. Biol. 22, (1993), 491-506; Hiei et al., Plant J. 6, (1994) 271-282; Deng et al., Science in China 33, (1990), 28-34; Wilmink et al., Plant Cell Reports 11, (1992), 76-80; May et al., Bio/Technology 13, (1995), 486-492; Conner and Domisse, Int. J. Plant Sci. 153 (1992), 550-555; Ritchie et al., Transgenic Res. 2, (1993), 252-265). An alternative system for the transformation of monocotyledonous plants is the transformation by means of the biolistic approach (Wan and Lemaux, Plant Physiol. 104, (1994), 37-48; Vasil et al., Bio/Technology 11 (1993), 1553-1558; Ritala et al., Plant Mol. Biol. 24, (1994), 317-325; Spencer et al., Theor. Appl. Genet. 79, (1990), 625-631), protoplast transformation, the electroporation of partially permeabilized cells, the introduction of DNA by means of glass fibres. In particular the transformation of maize has been described repeatedly in the literature (cf., for example, WO95/06128, EP0513849, EP0465875, EP0292435; Fromm et al., Biotechnology 8, (1990), 833-844; Gordon-Kamm et al., Plant Cell 2, (1990), 603-618; Koziel et al., Biotechnology 11 (1993), 194-200; Moroc et al., Theor. Appl. Genet. 80, (1990), 721-726).

The successful transformation of other cereal species has also been described, for example for barley (Wan and Lemaux, see above; Ritala et al., see above; Krens et al., Nature 296, (1982), 72-74) and wheat (Nehra et al., Plant J. 5, (1994), 285-297). All of the abovementioned methods are suitable for the purposes of the present invention.

Any promoter which is active in plant cells is generally suitable for the expression of the foreign nucleic acid molecule(s). The promoter may be chosen in such a way that expression in the plants according to the invention takes place constitutively or only in a specific tissue, at a

particular point in time of plant development or at a point in time determined by external factors. As regards the plant, the promoter may be homologous or heterologous.

5 In a further embodiment of the invention, at least one antisense RNA is expressed in plant cells in order to reduce the activity of one or more SSIII proteins and/or BEI proteins and/or BEII proteins.

The present invention therefore also relates to a plant cell according to the
10 invention, wherein said foreign nucleic acid molecules are selected from the group consisting of

15 a) DNA molecules encoding at least one antisense RNA which brings about a reduction of the expression of at least one endogenous gene encoding SSIII proteins and/or BEI proteins and/or BEII proteins;

20 b) DNA molecules which, via a cosuppression effect, lead to a reduction of the expression of at least one endogenous gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s);

25 c) DNA molecules encoding at least one ribozyme which specifically cleaves transcripts of at least one endogenous gene encoding SSIII proteins and/or BEI proteins and/or BEII proteins; and

30 d) Nucleic acid molecules introduced by means of in-vivo mutagenesis which lead to a mutation or insertion of a heterologous sequence in at least one endogenous gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s), the mutation or insertion bringing about a reduction of the expression of at least one gene encoding SSIII protein(s) and/or BEI

protein(s) and/or BEII protein(s), or the synthesis of inactive SSIII and/or BEI and/or BEII proteins;

5 e) DNA molecules which simultaneously encode at least one antisense RNA and at least one sense RNA, where said antisense RNA and said sense RNA form a double-stranded RNA molecule which brings about a reduction of the expression of at least one endogenous gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s);

10 f) DNA molecules containing transposons, the integration of the transposon sequences leading to a mutation or an insertion in at least one endogenous gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s) which brings about a reduction of the expression of at least one gene encoding an SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s), or which results in the 15 synthesis of inactive SSIII and/or BEI and/or BEII proteins; and

15 g) T-DNA molecules which, owing to the insertion into at least one endogenous gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s), brings about a reduction of the expression of at least one endogenous gene encoding SSIII protein(s) and/or 20 BEI protein(s) and/or BEII protein(s), or which result in the synthesis of inactive SSIII and/or BEI and/or BEII proteins.

In a further aspect, the present invention relates to any kind of propagation material of plants according to the invention.

25

A further aspect of the present invention relates to the use of the nucleic acid molecules described herein for the generation of the plant cells and plants according to the invention.

30

A further aspect of the present invention relates to a composition comprising at least one of the above nucleic acid molecules, where the at

least one nucleic acid molecule, after introduction into a plant cell, leads to the reduction of at least one SSIII protein which occurs endogenously in the plant cell and at least one BEII protein which occurs endogenously in the plant cell and preferably furthermore to the reduction of at least one 5 BEI protein which occurs endogenously in the plant cell. The composition may comprise one or more nucleic acid constructs (cf. above).

A further aspect of the present invention relates to the use of the compositions according to the invention for the generation of the plant 10 cells and plants according to the invention, and to a host cell, in particular a plant cell, containing the composition according to the invention.

Yet a further aspect of the present invention relates to a transformation system in plant cells, comprising at least one nucleic acid molecule and at 15 least one plant cell, where the at least one nucleic acid molecule leads to the reduction of the activity of in each case at least one of the SSIII, BEI and BEII proteins which occur endogenously in the plant cell unless the activity of these proteins has been reduced already by an existing genetic modification of said plant cell. For the purposes of the present invention, 20 "transformation system" thus relates to a combination of at least one plant cell to be transformed and at least one nucleic acid molecule as described above which is used for the transformation. Further components with which the skilled worker in the field of the transformation of plant cells is familiar and which are required in the transformation process, including 25 buffers and the like, may be present in the transformation system according to the invention.

Description of the figures

Fig 1:

A graphic representation of the viscosity characteristics of starch from potato plants. The analysis was carried out using a Rapid Visco Analyser (Newport Scientific Pty Ltd., Investmet Support Group, Warriewood NSW 2102, Australia). The conditions under which the analysis was carried out are described under RVA analytical method 1 in the chapter "General Methods". The test starch was isolated from tubers of wild-type plants (WT), plants with a reduced activity of an SSIII protein and of a BEI protein (038VL008 and 038VL107) or from plants with a reduced activity of an SSIII protein and a BEI protein and a BEII protein (110CF003 and 108CF041). The starch was isolated by the method described under "Examples", "Starch extraction process for potatoes".

15

Fig 2:

A graphic representation of the viscosity characteristics of starch from potato plants. The analysis was carried out using a Rapid Visco Analyser (Newport Scientific Pty Ltd., Investmet Support Group, Warriewood NSW 2102, Australia). The conditions under which the analysis was carried out are described under RVA analytical method 2 in the chapter "General Methods". The test starch was isolated from tubers of wild-type plants (WT), plants with a reduced activity of an SSIII protein and of a BEI protein (038VL008 and 038VL107) or from plants with a reduced activity of an SSIII protein and a BEI protein and a BEII protein (110CF003 and 108CF041). The starch was isolated by the method described under "Examples", "Starch extraction process for potatoes".

30

Fig 3:

A graphic representation of the viscosity characteristics of starch from potato plants. The analysis was carried out using a Rapid Visco Analyser

(Newport Scientific Pty Ltd., Investmet Support Group, Warriewood NSW 2102, Australia). The conditions under which the analysis was carried out are described under RVA analytical method 3 in the chapter "General Methods". The test starch was isolated from tubers of wild-type plants 5 (WT), plants with a reduced activity of an SSIII protein and of a BEI protein (038VL008 and 038VL107) or from plants with a reduced activity of an SSIII protein and a BEI protein and a BEII protein (110CF003 and 108CF041). The starch was isolated by the method described under "Examples", "Starch extraction process for potatoes".

10

Fig 4:

Scanning-electron micrograph of a potato starch granule isolated from wild-type plants.

15 Fig 5:

Scanning-electron micrograph of a potato starch granule isolated from plants with a reduced activity of an SSIII protein and of a BEI protein and of a BEII protein (110CF003).

20 Fig 6:

Schematic representation of the vector pGSV71- α -BEII-basta, which was used for the retransformation of plants in which a reduced activity of an SSIII protein and of a BEI protein is already observed.
(RB, left T-DNA border, LB, right T-DNA border; CaMV35, cauliflower 25 mosaic virus 35S promoter; NOS, polyadenylation sequence of the *Agrobacterium tumefaciens* nopaline synthase gene; OCS, polyadenylation sequence of the *Agrobacterium tumefaciens* octopine synthase gene; B33, promoter of the potato patatin gene; BEII, coding sequences of the potato BEII gene; bar, sequence encoding a 30 *Streptomyces hygroscopicus* phosphinothricin acetyltransferase).

Fig 7:

Schematic representation of the vector pB33- α -BE- α -SSIII-Kan, which was used for the generation of transgenic plants with a reduced activity of an SSIII protein and a BEI protein (RB, left T-DNA border, LB, right T-DNA border; nos5', promoter of the *Agrobacterium tumefaciens* nopaline synthase gene; nptII, gene encoding the activity of a neomycin phosphotransferase; nos3, polyadenylation sequence of the *Agrobacterium tumefaciens* nopaline synthase gene; OCS, polyadenylation sequence of the *Agrobacterium tumefaciens* octopine synthase gene; B33, promoter of the potato patatin gene; BE, coding sequences of the potato BEI gene; SSIII, coding sequences of the potato SSIII gene).

Fig 8

15 The figure shows the entire elution diagram of the amylopectin from starches of lines 038VL008, 108CF041 and wild type. As shown in the figure, the amount of bigger side chains in line 108CF041 is markedly higher in contrast to the background 038VL008 and/or the corresponding wild type.

20

Fig 9

Calibration curve and table with corresponding dextran standards

Fig 10

25 The figure shows the entire elution diagram of the amylopectin from starches of lines 038VL008, 108CF041 and wild type. In contrast to Fig 8, the x axis does not show the elution volume, but the molecular weight. The elution diagram of Fig 8 as a function of the molecular weight distribution is shown with the aid of the calibration graph of Fig 9.

30

Fig 11

This represents the side chain profile distribution of the amylopectin from plants of line 038VL008 in comparison with the side chain profile of amylopectin from wild-type plants.

5 Fig 12

This represents the side chain profile distribution of the amylopectin from plants of line 108CF041 in comparison with the side chain profile of amylopectin from wild-type plants.

10 **Description of the sequences**

Seq ID 1:

Nucleic acid sequence of the potato (*Solanum tuberosum*) starch synthase SSIII with indication of the sequences which encode the corresponding 15 SSIII protein.

Seq ID 2:

Amino acid sequence of a potato SSIII protein.

Seq ID 3:

Amino acid sequence of the Pfam cbm25 binding domain of the potato 20 SSIII protein (*Solanum tuberosum*).

Seq ID 4:

Coding nucleic acid sequence of the potato (*Solanum tuberosum*) branching enzyme BEI.

Seq ID 5:

25 Amino acid sequence of the potato (*Solanum tuberosum*) branching enzyme BEI.

Seq ID 6:

Coding nucleic acid sequence of the potato (*Solanum tuberosum*) branching enzyme BEII.

30 Seq ID 7:

Amino acid sequence of the potato (*Solanum tuberosum*) branching enzyme BEII.

Seq ID 8:

PCR-amplified nucleic acid sequence of the potato (*Solanum tuberosum*)
5 branching enzyme BEII.

General Methods

The following methods were used in the examples:

5

Starch analysis**a) Determination of the amylose content and of the amylose/amyllopectin ratio**

Starch was isolated from potato plants by standard methods, and the 10 amylose content and the amylose:amyllopectin ratio was determined by the method described by Hovenkamp-Hermelink et al. (Potato Research 31, (1988), 241-246).

b) Determination of the phosphate content

15 In starch, the positions C2, C3 and C6 of the glucose units can be phosphorylated. To determine the C6-P content of starch, 50 mg of starch are hydrolysed for 4 h at 95°C in 500 µl of 0.7 M HCl. The samples are then centrifuged for 10 minutes at 15 500 g and the supernatants are removed. 7 µl of the supernatants are mixed with 193 µl of imidazole 20 buffer (100 mM imidazole, pH 7.4; 5 mM MgCl₂, 1 mM EDTA and 0.4 mM NAD). The measurement was carried out in a photometer at 340 nm. After the base absorption had been established, the enzyme reaction was started by addition of 2 units glucose-6-phosphate dehydrogenase (from Leuconostoc mesenteroides, Boehringer Mannheim). The change in 25 absorption is directly proportional to the concentration of the G-6-P content of the starch.

The total phosphate content was determined by the method of Ames (Methods in Enzymology VIII, (1966), 115-118).
30 Approximately 50 mg of starch are treated with 30 µl of ethanolic magnesium nitrate solution and ashed for 3 hours at 500°C in a muffle

oven. The residue is treated with 300 µl of 0.5 M hydrochloric acid and incubated for 30 minutes at 60°C. One aliquot is subsequently made up to 300 µl 0.5 M hydrochloric acid and this is added to a mixture of 100 µl of 10% ascorbic acid and 600 µl of 0.42% ammonium molybdate in 2 M sulphuric acid and incubated for 20 minutes at 45°C.

5 This is followed by a photometric determination at 820 nm with a phosphate calibration series as standard.

c) Determination of the gel strength (Texture Analyser)

10 1.5 g of starch (DM) are gelatinized in the RVA apparatus in 25 ml of an aqueous suspension (temperature programme: see item d) "Determination of the viscosity characteristics by means of a Rapld Visco Analyser (RVA)" and subsequently stored for 24 hours at room temperature in a sealed container. The samples are fixed under the probe (round piston with planar surface) of a Texture Analyser TA-XT2

15 from Stable Micro Systems (Surrey, UK) and the gel strength was determined using the following parameters:

-	Test speed	0.5 mm/s	
-	Depth of penetration	7 mm	
-	Contact surface	113 mm ²	
20	-	Pressure	2 g

d) Determination of the viscosity characteristics by means of a Rapld Visco Analyser (RVA)

25

Standard method

2 g of starch (DM) are taken up in 25 ml of H₂O (VE-type water, conductivity of at least 15 mega ohm) and used for the analysis in a Rapld Visco Analyser (Newport Scientific Pty Ltd., Investmet Support Group, 30 Warriewod NSW 2102, Australia). The apparatus is operated following the manufacturer's instructions. The viscosity values are indicated in RVUs in

accordance with the manufacturer's operating manual, which is incorporated into the description herewith by reference. To determine the viscosity of the aqueous starch solution, the starch suspension is first heated for one minute at 50°C (step 1), and then heated from 50°C to 5 95°C at a rate of 12°C per minute (step 2). The temperature is then held for 2.5 minutes at 95°C (step 3). Then, the solution is cooled from 95°C to 50°C at a rate of 12°C per minute (step 4). The viscosity is determined during the entire duration.

10 In particular in those cases where the limits of the measuring range of the RVA were insufficient when 2.0 g (DM) of starch in 25 ml of H₂O (VE-type water, conductivity of at least 15 mega ohm) were weighed in, only 1.5 g of starch (DM) were taken up in 25 ml of H₂O (VE-type water, conductivity of at least 15 mega ohm).

15

For reasons of comparison with the prior art, a modified temperature profile was additionally used in some cases.

The following temperature profiles were used:

20 RVA analytical method 1:

To determine the viscosity of a 6% aqueous starch solution, the starch suspension is first stirred for 10 seconds at 960 rpm and subsequently heated at 50°C at a stirring speed of 160 rpm, initially for a minute (step 1). The temperature was then raised from 50°C to 95°C at a heating rate of 25 12°C per minute (step 2). The temperature is held for 2.5 minutes at 95°C (step 3) and then cooled from 95°C to 50°C at 12°C per minute (step 4). In the last step (step 5), the temperature of 50°C is held for 2 minutes.

After the programme has ended, the stirrer is removed and the beaker covered. The gelatinized starch is now available for the texture analysis

30 after 24 hours.

RVA analytical method 2:

To determine the viscosity of a 6% aqueous starch solution, the starch suspension is first stirred for 10 seconds at 960 rpm and subsequently heated at 50°C at a stirring speed of 160 rpm, initially for two minutes

5 (step 1). The temperature was then raised from 50°C to 95°C at a heating rate of 1.5°C per minute (step 2). The temperature is held for 15 minutes at 95°C (step 3) and then cooled from 95°C to 50°C at 1.5°C per minute (step 4). In the last step (step 5), the temperature of 50°C is held for 15 minutes.

10 After the programme has ended, the stirrer is removed and the beaker covered. The gelatinized starch is now available for the texture analysis after 24 hours.

RVA analytical method 3:

15 To determine the viscosity of a 10% aqueous starch solution, the starch suspension is first stirred for 10 seconds at 960 rpm and subsequently heated at 50°C at a stirring speed of 160 rpm, initially for two minutes (step 1). The temperature was then raised from 50°C to 95°C at a heating rate of 1.5°C per minute (step 2). The temperature is held for 15 minutes

20 at 95°C (step 3) and then cooled from 95°C to 50°C at 1.5°C per minute (step 4). In the last step (step 5), the temperature of 50°C is held for 15 minutes. This profile of the RVA analysis corresponds to the one employed in WO 9634968.

After the programme has ended, the stirrer is removed and the beaker covered. The gelatinized starch is now available for the texture analysis after 24 hours.

30 The profile of the RVA analysis contains parameters which are shown for the comparison of different measurements and substances. In the context of the present invention, the following terms are to be understood as follows:

1. Maximum viscosity (RVA Max)

The maximum viscosity is understood as meaning the highest viscosity value, measured in RVUs, obtained in step 2 or 3 of the temperature profile.

5 2. Minimum viscosity (RVA Min)

The minimum viscosity is understood as meaning the lowest viscosity value, measured in RVUs, observed in the temperature profile after the maximum viscosity. Normally, this takes place in step 3 of the temperature profile.

10 3. Final viscosity (RVA Fin)

The final viscosity is understood as meaning the viscosity value, measured in RVUs, observed at the end of the measurement.

4. Setback (RVA Set)

What is known as the "setback" is calculated by subtracting the value of 15 the final viscosity from that of the minimum occurring after the maximum viscosity in the curve.

5. Gelatinization temperature (RVA T)

The gelatinization temperature is understood as meaning the point in time of the temperature profile where, for the first time, the viscosity increases 20 drastically for a brief period.

e) Analysis of the side-chain distribution of the amylopectin by means of ion-exchange chromatography

25 To separate amylose and amylopectin, 200 mg of starch are dissolved in 50 ml reaction vessels, using 12 ml of 90% (v/v) DMSO in H₂O. After addition of 3 volumes of ethanol, the precipitate is separated by centrifugation for 10 minutes at about 1800 g at room temperature (RT). The pellet is then washed with 30 ml of ethanol, dried and 30 dissolved in 40 ml of 1% (w/v) NaCl solution at 75°C. After the solution has cooled to 30°C, approximately 90 mg of thymol are added slowly,

and this solution is incubated for at least 60 h at 30°C. The solution is then centrifuged for 30 minutes at 2000 g (RT). The supernatant is then treated with 3 volumes of ethanol, and the amylopectin which settles out is separated by centrifugation for 5 minutes at 2000 g (RT).

5 The pellet (amylopectin) is then washed with ethanol and dried using acetone. By addition of DMSO to the pellet, one obtains a 1% solution, of which 200 µl are treated with 345 µl of water, 10 µl of 0.5 M sodium acetate (pH 3.5) and 5 µl of isoamylase (dilution 1:10; Megazyme) and incubated for about 16 hours at 37°C. A 1:5 aqueous dilution of this
10 digest is subsequently filtered through a 0.2 µm filter, and 100 µl of the filtrate are analysed by ion chromatography (HPAEC-PAD, Dionex). Separation was performed using a PA-100 column (with suitable precolumn), while detection was performed amperometrically. The elution conditions were as follows:

15 Solution A - 0.15M NaOH

Solution B – 1 M sodium acetate in 0.15M NaOH

t (min)	Solution A (%)	Solution B (%)
5	0	100
35	30	70
45	32	68
60	100	0
70	100	0
72	0	100
80	0	100
Stop		

20 Table 1: Composition of the elution buffer for the side chain analysis of the amylopectin at different times during the HPEAC-PAD Dionex analysis. Between the times stated, the composition of the elution buffer changes in each case linearly.

The determination of the relative amount of short side chains in the total of all side chains is carried out via the determination of the percentage of a particular side chain in the total of all side chains. The total of all side chains is determined via the determination of the total area under the peaks which represent the polymerization degrees of DP6 to 26 in the HPCL chromatogram.

5 The percentage of a particular side chain in the total of all side chains is determined via the determination of the ratio of the area under the peak which represents this side chain in the HPLC chromatogram to the total area. The programme Chromelion 6.20 Version 6.20 from 10 Dionex, USA, was used for determining the peak areas.

f) Granule size determination

15 Starch was extracted from potato tubers by standard methods (see Examples).

The granule size determination was then carried out using a photosedimentometer of type "Lumosed FS1" from Retsch GmbH, Germany, using the software V.2.3. The software settings were as follows:

20	Substance data:	Calibration No. 0
		Density [kg/m ³] 1500
15	Sedimentation fluid:	Type Water
		Viscosity [Pa s] 0.001
		Density [kg/m ³] 1000
25		Addition -
		Recordings 5 min
		Cut-off [μm] 250
		Passage [%] 100
		Measuring range 4.34 -
30	117.39 μm	Calibration N

Temperature 20°C

The granule size distribution was determined in aqueous solution and was carried out following the manufacturer's instructions and on the basis of the literature by, for example, H. Pitsch,
5 Korngrößenbestimmung [granule size determination]; LABO-1988/3
Fachzeitschrift für Labortechnik, Darmstadt.

g) Scanning electron micrographs (SEM)

To study the surface of the starch samples, the latter were dusted onto
10 the sample holder using a conductive adhesive. To avoid charging, the sample holders were finally sputtered with a 4 nm Pt coating. The starch samples were studied using the field emission scanning electron microscope JSM 6330 F (Jeol) at an accelerating voltage of 5 kV.

15 h) Determination of the activity of the SSIII, BEI and BEII proteins

These were carried out as specified in the examples.

Examples**Generation of the expression vector ME5/6**

pGSV71 is a derivative of the plasmid pGSV7, which is derived from the intermediary vector pGSV1. pGSV1 is a derivative of pGSC1700, whose construction has been described by Cornelissen and Vanderwiele (Nucleic Acid Research 17, (1989), 19-25). pGSV1 was obtained from pGSC1700 by deleting the carbenicillin resistance gene and deleting the T-DNA sequences of the TL-DNA region of the plasmid pTiB6S3.

5 pGSV7 contains the replication origin of the plasmid pBR322 (Bolivar et al., Gene 2, (1977), 95-113) and the replication origin of the *Pseudomonas* plasmid pVS1 (Itoh et al., Plasmid 11, (1984), 206). pGSV7 additionally contains the selectable marker gene *aadA*, from the *Klebsiella pneumoniae* transposon Tn1331, which confers resistance to the 10 antibiotics spectinomycin and streptomycin (Tolmasky, Plasmid 24 (3), (1990), 218-226; Tolmasky and Crosa, Plasmid 29(1), (1993), 31-40)

The plasmid pGSV71 was obtained by cloning a chimeric *bar* gene between the border regions of pGSV7. The chimeric *bar* gene contains the cauliflower mosaic virus promoter sequence for transcriptional initiation 15 (Odell et al., Nature 313, (1985), 180), the *Streptomyces hygroscopicus* *bar* gene (Thompson et al., Embo J. 6, (1987), 2519-2523) and the 3'-untranslated region of the pTiT37 T-DNA nopalatin synthase gene for transcriptional termination and for polyadenylation. The *bar* gene confers 20 tolerance to the herbicide glufosinate-ammonium.

25 The T-DNA contains the right border sequence of the TL-DNA from the plasmid pTiB6S3 (Gielen et al., EMBO J. 3, (1984), 835-846) at position 198-222. A polylinker sequence is located between nucleotide 223-249. The nucleotides 250-1634 contain the cauliflower mosaic virus p35S3 ..

20 .. The nucleotides 250-1634 contain the cauliflower mosaic virus p35S3 promoter region (Odell et al., see above). The coding sequence of the 30 *Streptomyces hygroscopicus* phosphinothricin resistance gene (*bar*) (Thompson et al. 1987, see above) is arranged between the nucleotides

1635-2186. The two terminal codons at the 5' end of the *bar* wild-type gene were replaced by the codons ATG and GAC. A polylinker sequence is located between the nucleotides 2187-2205. The 260 bp *Tagl* fragment of the untranslated 3' end of the nopaline synthase gene (3'nos) from the T-DNA of the plasmid pTiT37 (Depicker et al., J. Mol. Appl. Genet. 1, (1982), 561-573) is located between the nucleotides 2206 and 2465. The nucleotides 2466-2519 contain a polylinker sequence. The left border sequence of the pTiB6S3 TL-DNA (Gielen et al., EMBO J. 3, (1984), 835-846) is located between the nucleotides 2520-2544.

10 The vector pGSV71 was then cut using the enzyme *PstI* and made blunt-ended. The B33 promoter and the *ocs* cassette was then excised from the vector pB33-Kan in the form of an *EcoRI-HindIII* fragment, made blunt-ended and inserted into the vector pGSV71 which had been cut with *PstI* and made blunt-ended. The resulting vector was used as starting vector

15 for the construction of ME5/6: An oligonucleotide containing the cleavage sites *EcoRI*, *PacI*, *SpeI*, *SrfI*, *SpeI*, *NotI*, *PacI* and *EcoRI* was introduced into the *PstI* cleavage site of the vector ME4/6 located between the B33 promoter and the *ocs* element, duplicating the *PstI* cleavage site. The resulting expression vector was termed ME5/6.

20

Description of the vector pSK-Pac:

pSK-Pac is a derivative of pSK-Bluescript (Stratagene, USA) in which a *PacI* cleavage site was introduced at each flank of the multiple cloning site (MCS).

25

Generation of transgenic potato plants with a reduced gene expression of a BEI, SSIII and BEII gene

30 To generate transgenic plants with a reduced activity of a BEI, an SSIII and a BEII protein, transgenic plants with a reduced activity of a BEI and

an SSIII protein were generated in a first step. To this end, the T-DNA of the plasmid pB33- α BEI- α SSIII-Kan was transferred into potato plants with the aid of agrobacteria as described by Rocha-Sosa et al. (EMBO J. 8, (1989), 23-29).

- 5 To construct the plasmid pB33- α BEI- α SSIII-Kan (see Fig 7), the expression vector pBin33-Kan was constructed in a first step. To this end, the promoter of the *Solanum tuberosum* patatin gene B33 (Rocha-Sosa et al., 1989, see above) was ligated in the form of a *Dra*I fragment (nucleotides -1512 - +14) into the *Ssfl*-cut vector pUC19 (Genbank Acc. No. M77789), whose ends have been made blunt-ended with the aid of T4 DNA polymerase. This gave rise to the plasmid pUC19-B33. The B33 promoter was excised from this plasmid using *Eco*RI and *Sma*I and ligated into the suitably cut vector pBinAR. This gave rise to the plant expression vector pBin33-Kan. The plasmid pBinAR is a derivative of the vector
- 10 plasmid pBin19 (Bevan, Nucl. Acid Research 12, (1984), 8711-8721) and was constructed by Höfgen and Willmitzer (Plant Sci. 66, (1990), 221-230). A 1631 bp *Hind*II fragment which contains a partial cDNA encoding the potato BEI enzyme (Kossmann et al., 1991, Mol. & Gen. Genetics 230(1-2):39-44) was then made blunt-ended and introduced into the vector
- 15 pBin33, which had previously been cut with *Sma*I, in antisense orientation with regard to the B33 promoter (promoter of the *Solanum tuberosum* patatin gene B33; Rocha-Sosa et al., 1989). The resulting plasmid was cut open using *Bam*HI. A 1363 bp *Bam*HI fragment containing a partial cDNA encoding the potato SSIII enzyme (Abel et al., 1996, loc.cit.) was
- 20 introduced into the cleavage site, again in antisense orientation with regard to the B33 promoter.
- 25

30 After the transformation, various lines of transgenic potato plants in whose tubers a markedly reduced activity of a BEI and SSIII protein was observed were identified. The plants resulting from this transformation were termed 038VL.

To detect the activity of soluble starch synthases (SSIII) by non-denaturing gel electrophoresis, tissue samples of potato tubers were digested in 50 mM Tris-HCl pH 7.6, 2 mM DTT, 2.5 mM EDTA, 10% glycerol and 0.4 mM PMSF. The electrophoresis was carried out in a MiniProtean II chamber 5 (BioRAD). The monomer concentration of the gels, which had a thickness of 1.5 mm, amounted to 7.5% (w/v), and 25 mM Tris-Glycin pH 8.4 acted as the gel and the running buffers. Identical amounts of protein extract were applied and separated for 2 h at 10 mA for each gel.

The activity gels were subsequently incubated in 50 mM Tricine-NaOH pH 10 8.5, 25 mM potassium acetate, 2 mM EDTA, 2 mM DTT, 1 mM ADP-glucose, 0.1% (w/v) amylopectin and 0.5 M sodium citrate. Glucans formed were stained with Lugol's solution.

The BEI activity was likewise detected with the aid of non-denaturing gel electrophoresis:

15 To isolate proteins from plants, the sample material was comminuted in liquid nitrogen using a pestle and mortar, taken up in extraction buffer (50 mM sodium citrate, pH 6.5; 1 mM EDTA, 4 mM DTT), centrifuged (10 min, 14,000 g, 4°C) and then employed directly in the protein concentration measurement following the method of Bradford. Then, 5 to 20 µg of total 20 protein extract (as required) were treated with 4x loading buffer (20% glycerol, 125 mM Tris HCl, pH 6.8) and applied to a BE activity gel. The running buffer (RB) was composed as follows: RB = 30.2 g Tris-base, pH 8.0, 144 g glycine per 1 l H₂O.

After running of the gel had ended, each of the gels was incubated 25 overnight at 37°C in 25 ml of "phosphorylase buffer" (25 ml 1M sodium citrate pH 7.0, 0.47 g glucose-1-phosphate, 12.5 mg AMP, 2.5 mg phosphorylase a/b from rabbit). The gels were stained using Lugol's solution.

30 More in-depth analyses demonstrated that isolated starches from lines 038VL008 and 038VL107, in which both the BEI and the SSIII protein

were reduced, showed the highest phosphate content of all independent transformants studied.

Plants of these lines were subsequently transformed with the plasmid pGSV71- α BEII-basta as described by Rocha-Sosa et al. (EMBO J. 8, 5 (1989), 23-29).

Plasmid pGSV71- α BEII-basta was constructed by screening a tuber-specific potato cDNA library with a DNA fragment amplified using RT-PCR (Primer: 5'-gggggtgtggcttggacta and 5'-cccttctccctccataatccca; Stratagene ProSTAR™ HF Single-Tube RT-PCR system) with total RNA from tubers 10 as template, following standard methods. In this manner, an approximately 1250 bp DNA fragment (SEQ ID No. 8) was isolated and then subcloned into the EcoRV cleavage site of the cloning vector pSK-Pac (see hereinabove) in the form of an EcoRV-SmaI fragment and subsequently ligated into the expression vector ME5/6 in antisense orientation relative to 15 the promoter in the form of a PacI fragment. This gave rise to the plasmid pGSV71- α BEII-basta (see Fig 6).

Tuber tissue samples of the independent transformants were obtained from the plants obtained by transformation with the plasmid pGSV71- α BEII-basta, which were referred to as 108CF and 110CF, and their amylose content was determined (see Methods). The starches from the independent lines whose tubers had the highest amylose content were used for a further analysis of the starch characteristics. To prove that in these plants not only the activity of a BEI and SSIII protein is reduced, but 25 also that the activity of a BEII protein is reduced, another analysis was carried out with the aid of non-denaturing gel electrophoresis. The analysis was carried out following the same method as already carried out above for the analysis of the reducing BEI activity, with the exception that the non-denaturing polyacrylamide gel contained 0.5% of maltodextrin (Beba, 30 15% strength maltodextrin solution for newborns, Nestle) in addition to the above-described composition. The dextrin addition made it possible to

show the different activities of the BEI and BEII proteins after incubation of the gels in "phosphorylase buffer" (25 ml 1M sodium citrate pH 7.0, 0.47 g glucose-1-phosphate, 12.5 mg AMP, 2.5 mg phosphorylase a/b from rabbit) overnight at 37°C, followed by staining with Lugol's solution in a 5 gel.

Potato starch extraction process

All tubers of one line (4 to 5 kg) are processed jointly in a commercially available juice extractor (Multipress automatic MP80, Braun). The starch-containing fruit water is collected in a 10-l bucket (ratio bucket height: bucket diameter = approx. 1.1) containing 200 ml of tap water together with a spoon-tipful (approx. 3-4 g) of sodium disulphite. The bucket is subsequently filled completely with tap water. After the starch has been allowed to settle for 2 hours, the supernatant is decanted off, the starch is 10 resuspended in 10 l of tap water and poured over a sieve with a mesh size of 125 µm. After 2 hours (starch has again settled at the bottom of the bucket), the aqueous supernatant is again decanted off. This wash step is 15 repeated 3 more times so that the starch is resuspended a total of 5 times in fresh tap water. Thereafter, the starches are dried at 37°C to a water content of 12-17% and homogenized using a pestle and mortar. The 20 starches are now available for analyses.

Example 2

25

Analysis of the starch from plants with reduced BEI, SSIII and BEII gene expression

The starch from various independent lines of the transformations 108CF and 110CF described in Example 1 were isolated from potato tubers. The 30 physico-chemical properties of this starch were subsequently analysed. The results of the characterization of the modified starches are shown in

Table 2 (Tab. 2) for an example of a selection of certain plant lines. The analyses were carried out by the methods described hereinabove.

Tables 2, 3 and 4 which follow summarize the results of the RVA analysis based on starch from wild-type plants:

RVA analytical method 1

	RVA Max (%)	RVA Min (%)	RVA Fin (%)	RVA Set (%)	RVA T (%)	Gel strength
cv. Desiree	100	100	100	100	100	100
038VL008	158.7	69.8	72.0	79.5	73.0	55.4
108CF041	59.6	89.9	227.5	693.7	150.2	532.3
038VL107	151.1	94.3	94.0	93.0	82.2	52.2
110CF003	106.4	158.6	265.0	625.7	151.5	737.1

Table 2: Parameters of the RVA analysis of starch isolated from wild-type plants (cv.

5 Desiree), plants with a reduced activity of an SSIII and a BEI protein (038VL008, 038VL107), and of plants with a reduced activity of an SSIII and of a BEI and of a BEII protein (108CF041, 110CF003) in per cent based on data of starch of the wild type. The RVA analysis was carried out as described in Analytical method 1.

10 RVA analytical method 2

	RVA Max (%)	RVA Min (%)	RVA Fin (%)	RVA Set (%)	RVA T (%)	Gel strength
cv. Desiree	100	100	100	100	100	100
038VL008	167.1	40.4	52.6	77.6	54.2	63.0
108CF041	44.5	82.5	187.5	402.7	137.4	412.2
038VL107	152.0	76.1	81.9	93.8	76.9	51.7
110CF003	92.4	172.2	n.d.	n.d.	139.0	795.0

Table 3: Parameters of the RVA analysis of starch isolated from wild-type plants (cv.

Desiree), plants with a reduced activity of an SSIII and a BEI protein (038VL008, 038VL107), and of plants with a reduced activity of an SSIII and of a BEI and of a BEII protein (108CF041, 110CF003) in per cent based on data of starch of the wild type. The 15 RVA analysis was carried out as described in Analytical method 2.

RVA analytical method 3

	RVA Max (%)	RVA Min (%)	RVA Fin (%)	RVA Set (%)	RVA T (%)	Gel strength
cv. Desiree	100	100	100	100	100	100
038VL008	n.d.	50.2	76.5	127.8	77.0	100.5

108CF041	74.7	291.0	n.d.	205.7	236.0	630.3
038VL107	n.d.	84.5	86.4	90.1	102.3	58.1
110CF003	89.8	259.7	n.d.	n.d.	196.6	663.9

Table 4: Parameters of the RVA analysis of starch isolated from wild-type plants (cv. Desiree), plants with a reduced activity of an SSIII and a BEI protein (038VL008, 038VL107), and of plants with a reduced activity of an SSIII and of a BEI and of a BEII protein (108CF041, 110CF003) in per cent based on data of starch of the wild type. The 5 RVA analysis was carried out as described in Analytical method 3.

The following tables 5, 6 and 7 summarize the results of the RVA analysis.

The data do not refer to the wild type, but are the actual measurements:

10 RVA analytical method 1 (see also Fig. 1)

	RVA Max (RVU)	RVA Min (RVU)	RVA Fin (RVU)	RVA Set (RVU)	RVA T (RVU)	Gel strength
cv. Desiree	255.05	162.33	210.25	47.92	4.6	25.1
038VL008	404.83	113.25	151.33	38.08	3.36	13.9
108CF041	152.08	145.92	478.33	332.42	6.91	133.6
038VL107	385.5	153	197.58	44.58	3.78	13.1
110CF003	271.5	257.42	557.25	299.83	6.97	185

Table 5: Parameters of the RVA analysis of starch isolated from wild-type plants (cv. Desiree), plants with a reduced activity of an SSIII and a BEI protein (038VL008, 038VL107), and of plants with a reduced activity of an SSIII and of a BEI and of a BEII protein (108CF041, 110CF003) in RVUs. The RVA analysis was carried out as described 15 in Analytical method 1.

RVA analytical method 2 (see also Fig. 2)

	RVA Max (RVU)	RVA Min (RVU)	RVA Fin (RVU)	RVA Set (RVU)	RVA T (RVU)	Gel strength
cv. Desiree	212.17	113.75	169.25	55.5	28.78	23.8
038VL008	354.58	45.92	89	43.08	15.61	15
108CF041	94.33	93.83	317.33	223.5	39.53	98.1
038VL107	322.58	86.58	138.67	52.08	22.13	12.3
110CF003	196.08	195.92	n.d.	n.d.	39.99	189.2

Table 6: Parameters of the RVA analysis of starch isolated from wild-type plants (cv. Desiree), plants with a reduced activity of an SSIII and a BEI protein (038VL008, 038VL107), and of plants with a reduced activity of an SSIII and of a BEI and of a BEII protein (108CF041, 110CF003) in RVUs. The RVA analysis was carried out as described in Analytical method 2.

RVA analytical method 3 (see also Fig. 3)

	RVA Max (RVU)	RVA Min (RVU)	RVA Fin (RVU)	RVA Set (RVU)	RVA T (RVU)	Gel strength
Desiree	819.67	207.67	314.25	106.58	16.88	56.5
038VL008	n.d.	104.17	240.33	136.17	12.99	56.8
108CF041	612.33	604.25	823.5	219.25	39.83	356.1
038VL107	n.d.	175.42	271.5	96.08	17.27	32.8
110CF003	736.08	539.42	n.d.	n.d.	33.18	375.1

Table 7: Parameters of the RVA analysis of starch isolated from wild-type plants (cv. Desiree), plants with a reduced activity of an SSIII and a BEI protein (038VL008, 038VL107), and of plants with a reduced activity of an SSIII and of a BEI and of a BEII protein (108CF041, 110CF003) in RVUs. The RVA analysis was carried out as described in Analytical method 3.

Summary of the phosphate and amylose analyses:

No.	Genotype	Phosphate in C6 (%)	Total phosphate in (%)	Amylose (%)	Amylose (% WT)
1	cv. Desiree	100	100	22	100
2	038VL008	346.4	255.2	19.4	85.8
3	108CF041	557.3	427.6	36.8	162.8
4	038VL107	225.5	182.8	19.7	87.2
5	110CF003	446.4	348.3	34.6	153.1

Table 8: Phosphate and amylose contents of starch isolated from wild-type plants (cv. Desiree), plants with a reduced activity of an SSIII and a BEI protein (038VL008, 038VL107) and of plants with a reduced activity of an SSIII and of a BEI and of a BEII protein (108CF041, 110CF003). The phosphate contents in the C6 position of the

glucose monomers and the total phosphate content of the starch are indicated in per cent based on starch from wild-type plants; amylose contents are indicated in per cent amylose based on the total amount of the starch, or in per cent based on the amylose content of starch from wild-type plants.

5

The analysis of the side-chain distribution of the amylopectin was carried out as described above. The table which follows is a summary of the contributions of the individual peak areas:

10

Glucose units	cv. Desiree	038VL 008	108CF 041	038VL 107	110CF 003
dp 6	1.52	4.16	1.88	2.39	0.86
dp 7	1.4	1.4	0.63	1.42	0.59
dp 8	1.23	0.77	0.33	0.99	0.38
dp 9	2.05	1.42	0.74	1.79	0.75
dp 10	3.55	2.8	1.74	3.33	1.77
dp 11	5.16	4.41	2.92	4.96	3.46
dp 12	6.25	5.77	4.47	6.22	5.17
dp 13	6.71	6.7	5.63	6.87	6.35
dp 14	6.75	7.06	6.35	6.99	7.38
dp 15	6.48	6.76	6.62	6.65	7.63
dp 16	6.07	5.99	6.34	6.11	7.13
dp 17	5.6	5.21	5.81	5.49	6.3
dp 18	5.28	4.78	5.87	5.11	5.98
dp 19	4.99	4.74	6.17	4.94	5.91
dp 20	4.76	4.65	6.07	4.78	5.64
dp 21	4.5	4.46	5.65	4.5	5.26
dp 22	4.16	4.12	5.07	4.2	4.7
dp 23	3.77	3.68	4.59	3.78	4.19
dp 24	3.44	3.36	4.24	3.42	3.75
dp 25	3.08	3.09	3.86	3.07	3.49
dp 26	2.73	2.8	3.36	2.77	3.03
dp 27	2.39	2.58	2.95	2.37	2.65
dp 28	2.07	2.26	2.39	2.01	2.1
dp 29	1.67	1.87	1.87	1.71	1.69
dp 30	1.38	1.58	1.54	1.35	1.3
dp 31	1.07	1.28	1.02	1.04	0.87
dp 32	0.79	0.96	0.7	0.75	0.6
dp 33	0.57	0.69	0.6	0.51	0.51

dp 34	0.36	0.43	0.39	0.32	0.34
dp 35	0.22	0.22	0.19	0.17	0.2
Total	100	100	99.99	100.01	99.98

Table 9: The table shows a summary of the contributions of the individual peak areas of the HPAEC chromatogram to the total peak area of wild-type plants (cv. Desiree), of 038VL008 and 038VL107 plants (potato plants with reduced activity of a BEI protein and of an SSIII protein) and of selected lines of the transformations 108CF and 110CF (potato plants with a reduced activity of an SSIII protein and of a BEI protein and of a BEII protein). The number of glucose monomers in the individual side chains is shown as dp.

The peak chain length, whose value is the mean of the two chain lengths (given in DP) which contribute most to the total area under the peaks of the HPAEC chromatogram, is – in the case of debranched amylopectin – of wild-type plants at DP= 13, in the case of 038VL plants likewise at DP= 13 and in the case of the 108CF and 110CF plants, on average, at 15.

If the peak chain length of the transgenic plants is compared with the peak chain length of amylopectin of wild-type plants, the following values result for the peak chain length ratio (PCL ratio):

$$\text{PCL ratio for 038VL} = 13 / 13 = 1$$

$$\text{PCL ratio for 108/110CF} = 15 / 13 = 1.15$$

In addition, the starch granule morphology was analysed using a scanning electron microscope (SEM).

The surface of the starch granules of 108/110CF plants appears coated or raised with pore formation.

Moreover, the granule size determination was carried out using a "Lumosed"-type photosedimentometer from Retsch GmbH, Germany. The mean granule size of untreated starch samples was determined (Table 3).

Mean granule size [μ m]

Sample	Mean granule size
cv. Desiree	29.7
038VL008	21.5
108CF041	20.8
038VL107	22.9
110CF003	20.7

5 **Table 10:** Mean granule size values of starch isolated from wild-type plants (cv. Desiree),
 plants with a reduced activity of an SSIII and of a BEI protein (038VL008, 038VL107),
 and of plants with a reduced activity of an SSIII and of a BEI and of a BEII protein
 (108CF041, 110CF003).

10

Example 3

Analysis of the amylopectin side chain distribution by means of gel permeation chromatography

To separate amylose and amylopectin, 100 mg of starch are dissolved in
 15 6 ml of 90% strength (v/v) DMSO with constant stirring. After addition of 3
 volumes of ethanol, the precipitate is separated off by centrifugation for 10
 minutes at 1 800 g at room temperature. The pellet is subsequently
 washed with 30 ml of ethanol, dried and dissolved in 10 ml of 1% strength
 (w/v) NaCl solution at 60°C. After cooling the solution to 30°C,
 20 approximately 50 mg of thymol are added slowly, and this solution is
 incubated for 2 to 3 days at 30°C. The solution is subsequently
 centrifuged for 30 minutes at 2 000 g at room temperature. The
 supernatant is treated with three volumes of ethanol, and the amylopectin
 which precipitates is separated off by centrifugation for 5 minutes at
 25 2 000 g at room temperature. The pellet (amylopectin) is washed with

10 ml of 70% strength (v/v) ethanol, centrifuged for 10 minutes at 2 000 g at room temperature and then dried using acetone.

10 mg of amylopectin are subsequently stirred for 10 minutes at 70°C in 250 µl of 90% strength (v/v) DMSO. 375 µl of water at a temperature of 5 80°C are added to the solution until dissolution is complete.

200 µl of this solution are treated with 300 µl of a 16.6 mM sodium acetate solution pH 3.5 and 2 µl of isoamylase (0.24 µl/µl, Megazyme, Sydney, Australia) and the mixture is incubated for 15 hours at 37°C.

A 1:4 dilution of this aqueous isoamylase reaction mixture with DMSO, 10 comprising 90 mM sodium nitrate, is subsequently filtered through a 0.2 µm filter, and 24 µl of the filtrate is analysed chromatographically. Separation was carried out with two columns connected in series, first a Gram PSS3000 (Polymer Standards Service, with suitable precolumn), followed by a Gram PSS100. Detection was by means of refraction index 15 detector (RI 71, Shodex). The column was equilibrated with DMSO comprising 90 mM sodium nitrate. It was eluted with DMSO comprising 90 mM sodium nitrate at a flow rate of 0.7 ml/min over a period of 1 hour.

To correlate the elution volume with the molecular mass, the column used 20 was calibrated with dextran standards. The dextrans used, their molecular mass and the elution volumes are shown in Fig 9. Using the resulting calibration graph, the elution diagram was shown as a molecular weight distribution (Fig 10).

The chromatograms obtained were further evaluated using the program 25 Wingpc Version 6 from Polymer Standards Service GmbH, Mainz, Germany.

The total area under the line of the GPC chromatogram was divided into 30 individual segments, each of which represent groups of side chains of different lengths. The chosen segments contained glucan chains with the following degree of polymerization (DP = number of glucose monomers within one side chain): DP≤11, DP12-18, DP19-24, DP25-30, DP31-36, DP37-42, DP43-48, DP49-55, DP56-61 and DP62-123. To determine the

molecular weight of the individual side chains, a molecular weight of 162 was assumed for glucose. The total area under the line in the GPC chromatogram was then set as 100%, and the percentage of the areas of the individual segments was calculated based on the percentage of the 5 total area. Results obtained from this analysis are shown in Table 11.

	Wild type	08CF041 c
DP≥ 11	100%	40%
DP12-18	100%	50%
DP19-24	100%	69%
DP25-30	100%	91%
DP31-36	100%	111%
DP37-42	100%	116%
DP43-48	100%	110%
DP49-55	100%	107%
DP56-61	100%	109%
DP62-123	100%	157%

Table 11: Side chain profiles DP 12 to 18, DP 19 to 24, DP 25 to 30, DP 31 to 36, DP 37 to 42, DP 43-48, DP 49 to 55, DP 56 to 61 and DP 62 to 123 for amylopectin isolated 10 from wild-type plants (cv. Desiree) and from plants with a reduced activity of an SSIII and of a BEI and of a BEII protein (108CF041). The percentages indicate the modification of the individual side chain profiles based on amylopectin isolated from wild-type plants.

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NR. 7086 S. 7/9 4840
S. 85/07

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BAVERBIOSCIENCE GMBH

BCS 02-5002-PCT

Patent Claims

1. Plant cell which is genetically modified, the genetic modification leading to the reduction of the activity of one or more SSII proteins occurring endogenously in the plant cell and to the reduction of the activity of one or more BEI proteins which occur endogenously in the plant cell and to the reduction of the activity of one or more BEII proteins which occur endogenously in the plant cell in comparison to corresponding plant cells, of wild-type plants, which have not been genetically modified, whereas the genetically modified plant cells synthesize a modified starch, which after gelatinization of a 6% suspension in water forms a gel with a gel strength that is increased by at least 300% in comparison with the gel strength of starch extracted from corresponding plant cells, of wild-type plants, which have not been genetically modified.
2. Plant cell according to Claim 1, wherein the genetic modification is the introduction of one or more foreign nucleic acid molecules whose presence and/or expression leads to the reduction of the activity of one or more SSII and BEI and BEII proteins occurring in the plant cell in comparison with corresponding plant cells, of wild-type plants, which have not been genetically modified.
3. Plant containing plant cells according to one of Claims 1 or 2.
4. Method for generating a genetically modified plant, in which
 - a) a plant cell which synthesizes a modified starch, which starch after gelatinization of a 6% suspension in water forms a gel with a gel strength that is increased by at least 300% in comparison with the gel strength of starch extracted from corresponding plant cells, of wild-type plants, which have not been genetically modified, comprising the genetic modification of the plant cell, the genetic modification leading to the reduction of the activity of one or more SSII proteins which occur endogenously in the plant cell and to the reduction of the activity of one or more BEI proteins which occur endogenously in the plant cell and to the reduction of the activity of one or more BEII proteins which occur endogenously in the plant cell, in

Empf.zeit:27/09/2004 17:51

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Empf.nr.:908 P.005

18/04 '05 LUN 13:49 [TX/RX N° 7934]

BCS 02-5002-PCT

comparison with corresponding plant cells, of wild-type plants, which have not been genetically modified, is generated;

- b) a plant is regenerated from, or using, the plant cell generated in accordance with a); and,
- c) if appropriate, further plants are generated from the plant generated in accordance with step b).

5. Method for generating a transgenic plant according to Claim 4 which synthesizes a modified starch, in which

- a) a plant cell is genetically modified by the introduction of one or more foreign nucleic acid molecules whose presence and/or expression leads to the reduction of the activity of in each case at least one SSIII, BEI and BEII protein in comparison with corresponding wild-type plant cells which have not been genetically modified;
- b) a plant is regenerated from, or using, the cell generated in accordance with a); and
- c) if appropriate, further plants are generated from the plants generated in accordance with step b).

6. Plant according to Claim 3 or obtainable by the method according to one of Claims 4 or 5, which is a starch-storing plant.

7. Plant according to Claim 6, which is a potato plant.

8. Propagation material of plants according to one of Claims 3, 6 or 7, containing at least one plant cell according to one of Claims 1 or 2.

9. Use of one or more nucleic acid molecules which encode proteins with the enzymatic activity of at least one SSIII, at least one BEI and/or at least one BEII protein or their fragments for the generation of plant cells according to one of Claims 1 or 2 or of plants according to one of Claims 3 or 6 to 7.

Empf.zeit:27/09/2004 17:51

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18/04 '05 LUN 13:49 [TX/RX N° 7934]

10. Starch which can be obtained from plant cells according to one of Claims 1 or 2 or from a plant according to one of Claims 3, 6 or 7 or from propagation material according to Claim 8.

5

11. Starch according to Claim 10, which is a potato starch.

12. Method for producing a starch according to one of Claims 10 or 11, comprising the extraction of the starch from a plant according to one of Claims 3, 6 or 7 and/or from starch-storing parts of such a plant and/or from a plant cell according to one of Claims 1 or 2 and/or from propagation material according to Claim 8.

10

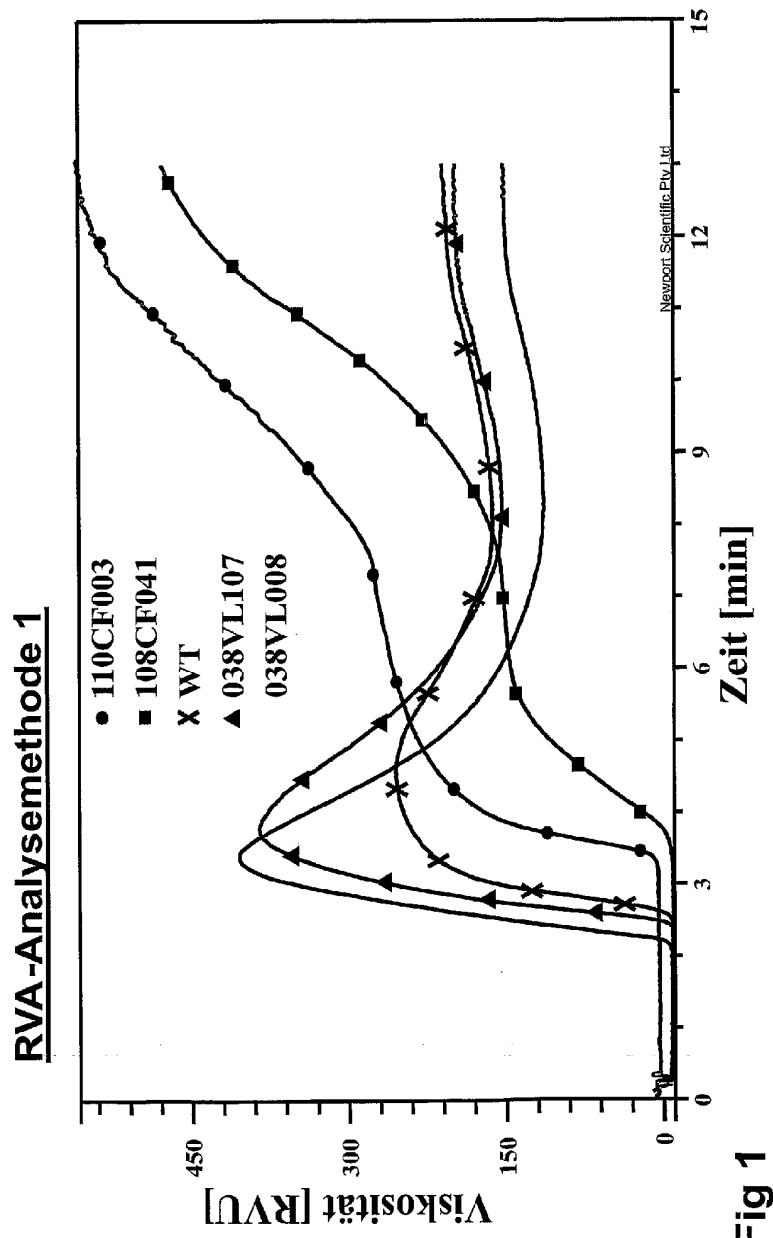
13. Starch according to one of Claims 10 or 11, obtainable by a method according to Claim 12.

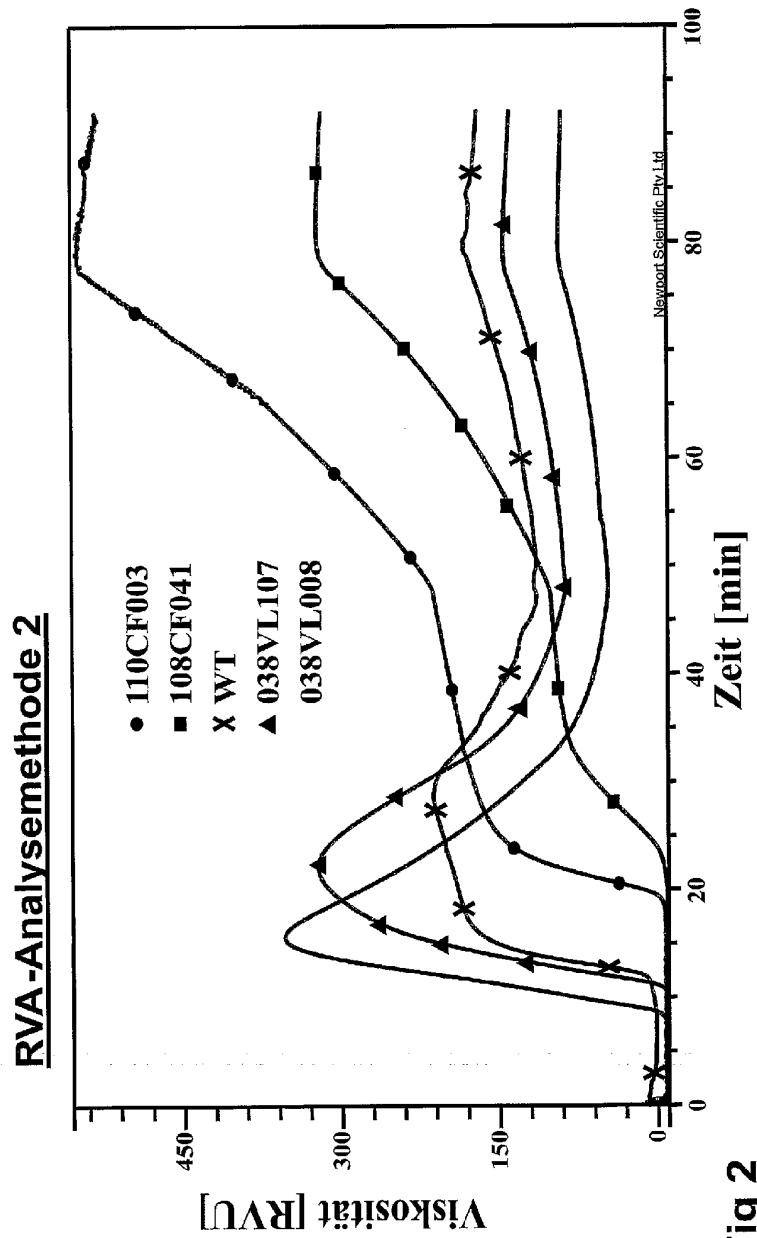
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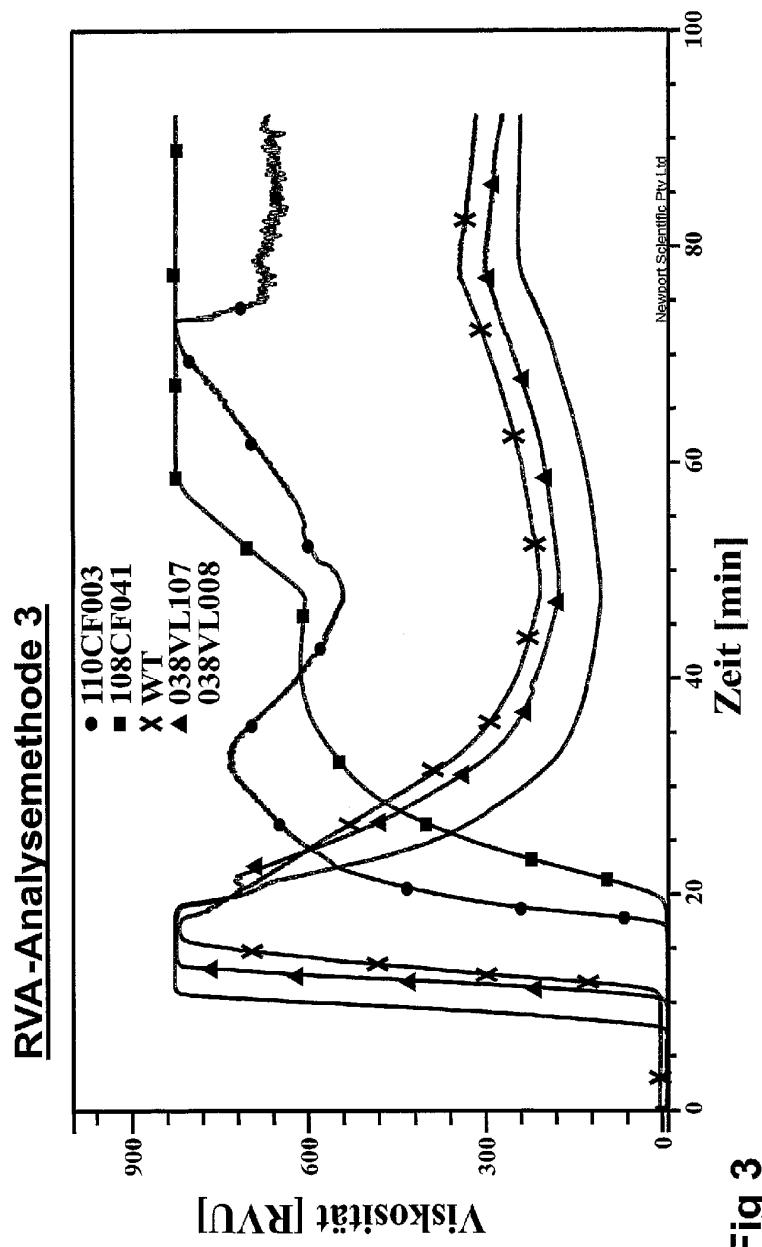
14. Method for modifying the starch of a plant, comprising the method for generating a plant according to one of Claims 3, 6 or 7 and obtaining starch from the plant or starch-containing parts thereof.

20

15. A plant cell according to Claim 1, substantially as hereinbefore described with reference to any one of the examples.

**Fig 1**

**Fig 2**

**Fig 3**

4/11

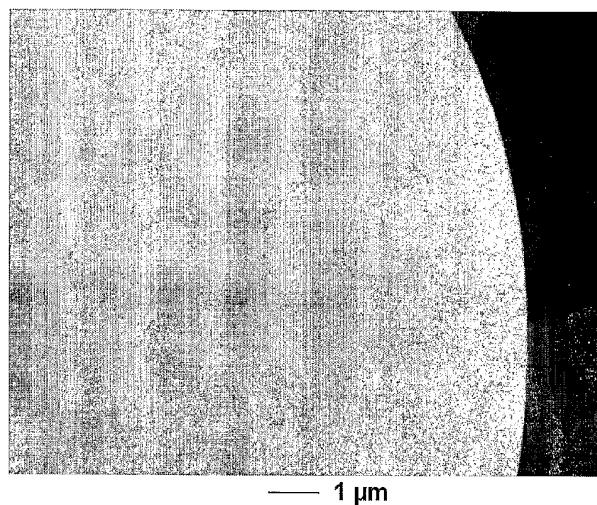


Fig 4

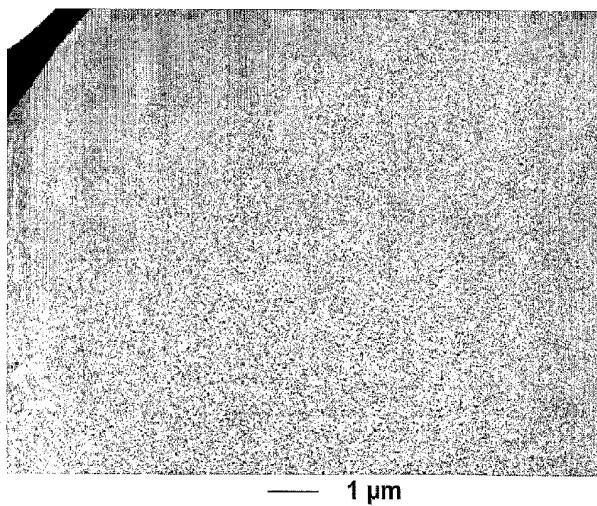


Fig 5

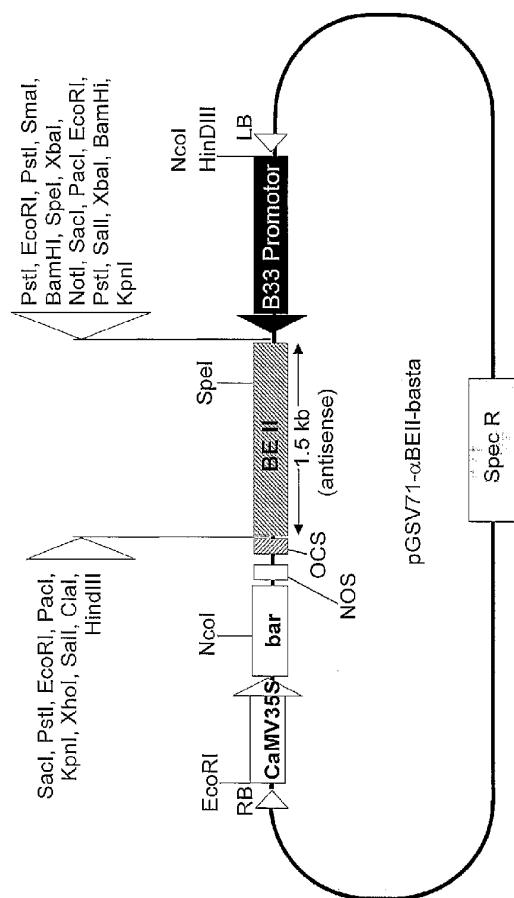


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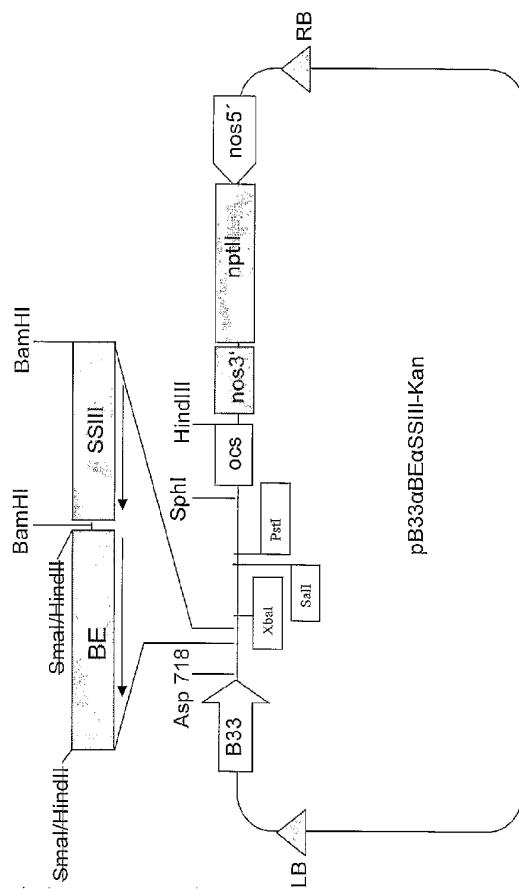


Fig 7

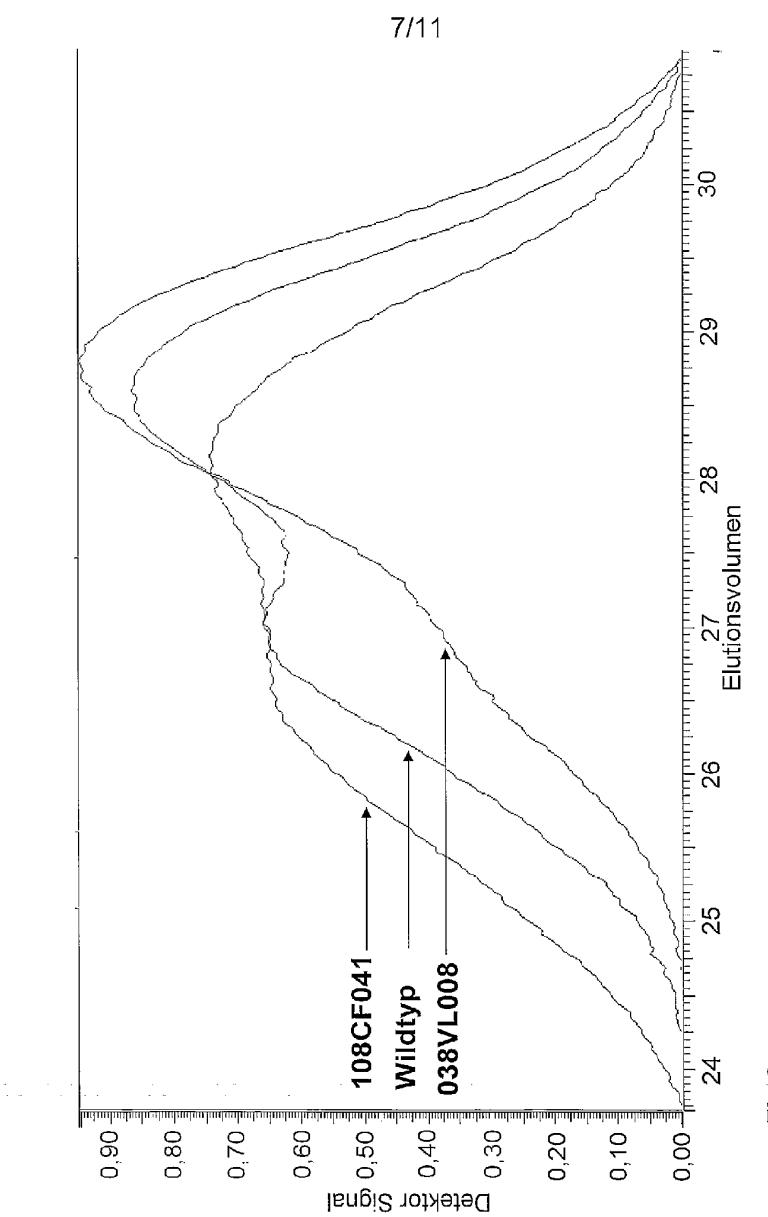
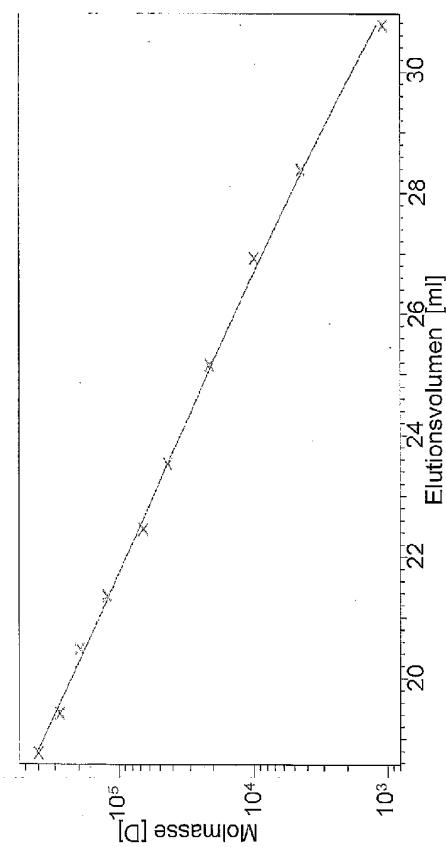


Fig 8

8/11



Kalibriertabelle:

Elutionsvolumen [ml]	Molmasse [D]	Probennahme
18.76	401300	Dextran T670
19.41	276500	Dextran T410
20.49	196300	Dextran T270
21.35	123600	Dextran T150
22.45	66700	Dextran T80
23.52	43500	Dextran T50
25.15	21400	Dextran T25
26.92	9890	Dextran T12
28.38	4440	Dextran T5
30.77	1080	Dextran T1

Fig 9

9/11

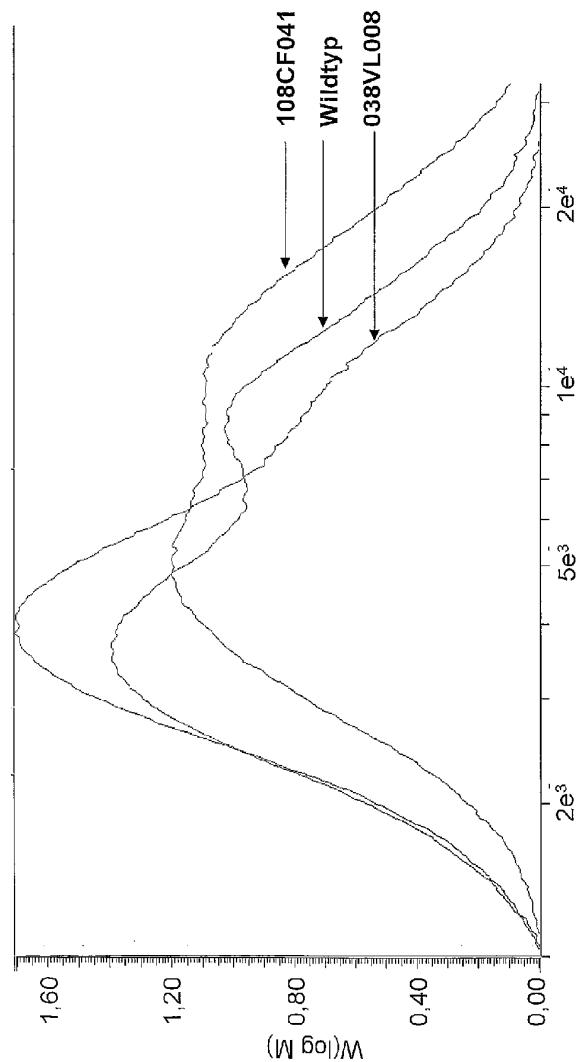


Fig 10

10/11

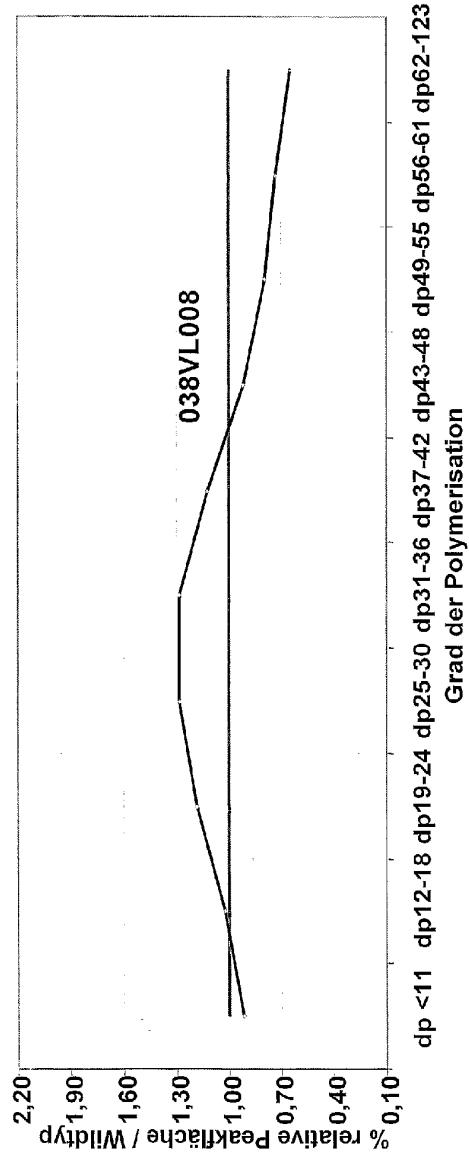


Fig 11

11/11

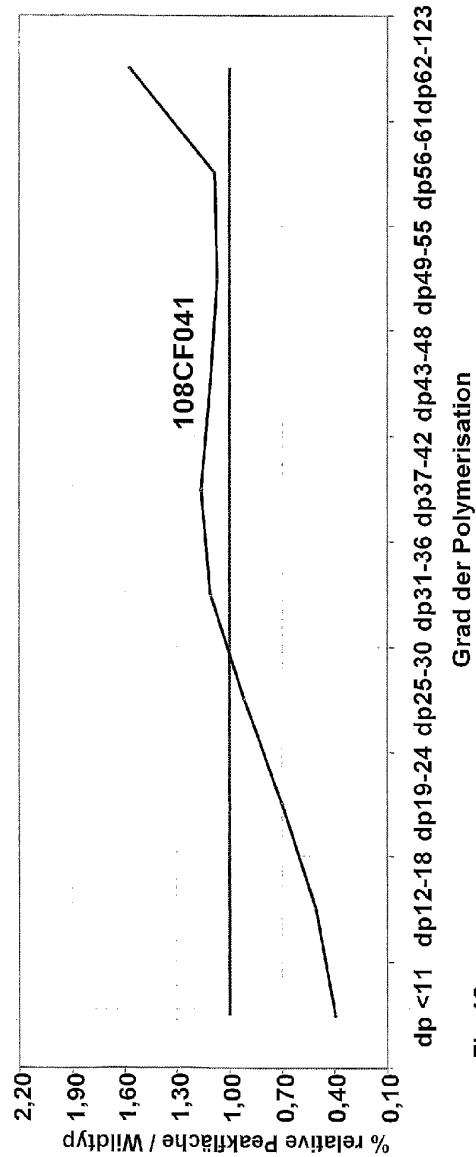


Fig 12

SEQUENCE LISTING

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<130> BCS 02 5002 - PCT

<150> EP 02028530.0

<151> 2002-12-19

<150> EP 03090275.3

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<301> Abel,G.J., Springer,F., Willmitzer,L. and Kossmann,J.

<302> Cloning and functional analysis of a cDNA encoding a novel
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<307> 1996

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3434
 Gly Gln Val Val Leu Leu Gly Ser Ala Pro Asp Pro Arg Val Gln
 1065 1070 1075

 aac gat ttt gtt aat ttg gca aat caa ttg cac tcc aaa tat aat
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 Asn Asp Phe Val Asn Leu Ala Asn Gln Leu His Ser Lys Tyr Asn
 1080 1085 1090

 gac cgc gca cga ctc tgt ct^a aca tat gac gag cca ctt tct cac
 3524
 Asp Arg Ala Arg Leu Cys Leu Thr Tyr Asp Glu Pro Leu Ser His
 1095 1100 1105

 ctg ata tat c^c ggt gct gat ttt att cta gtt cct tca ata ttt
 3569
 Leu Ile Tyr Ala Gly Ala Asp Phe Ile Leu Val Pro Ser Ile Phe
 1110 1115 1120

 gag cca tgt gga cta aca caa ctt acc gct atg aga tat ggt tca
 3614
 Glu Pro Cys Gly Leu Thr Gln Leu Thr Ala Met Arg Tyr Gly Ser
 1125 1130 1135

 att cca gtc gtg cgt aaa act gga gga ctt tat gat act gta ttt
 3659
 Ile Pro Val Val Arg Lys Thr Gly Gly Leu Tyr Asp Thr Val Phe
 1140 1145 1150

 gat gtt gac cat gac aaa gag aga gca caa cag tgt ggt ctt gaa
 3704
 Asp Val Asp His Asp Lys Glu Arg Ala Gln Gln Cys Gly Leu Glu
 1155 1160 1165

 cca aat gga ttc agc ttt gat gga gca gat gct ggc gga gtt gat
 3749
 Pro Asn Gly Phe Ser Phe Asp Gly Ala Asp Ala Gly Gly Val Asp

1170

1175

1180

tat gct ctg aat aga gct ctc tct gct tgg tac gat ggt cgg gat
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 Tyr Ala Leu Asn Arg Ala Leu Ser Ala Trp Tyr Asp Gly Arg Asp

1185

1190

1195

tgg ltc aac tct tta tgc aag cag gtc atg gaa caa gat tgg tct
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 Trp Phe Asn Ser Leu Cys Lys Gln Val Met Glu Gln Asp Trp Ser

1200

1205

1210

tgg aac cga cct gct ctt gat tal llg gag ctt tac cat gct gct
 3884
 Trp Asn Arg Pro Ala Leu Asp Tyr Leu Glu Leu Tyr His Ala Ala

1215

1220

1225

aga aag tta gaa tag ttatgttgc agatgttagc agaaaaalltc accagatctg
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 Arg Lys Leu Glu

1230

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 4059

caacatcatga aagagcttag cctctaataa tggatgtcatt gatgattatt tggatggaa
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<211> 1230

<212> PRT

<213> Solanum tuberosum

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35 40 45

Asp Gly Met Val Thr Gly Val Ser Phe Pro Phe Cys Ala Asn Leu Ser
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Gly Arg Arg Arg Arg Lys Val Ser Thr Thr Arg Ser Gln Gly Ser Ser
65 70 75 80

Pro Lys Gly Phe Val Pro Arg Lys Pro Ser Gly Met Ser Thr Gln Arg
85 90 95

Lys Val Gln Lys Ser Asn Gly Asp Lys Glu Ser Gln Ser Thr Ser Thr
100 105 110

Ser Lys Glu Ser Glu Ile Ser Asn Gln Lys Thr Val Glu Ala Arg Val
115 120 125

Glu Thr Ser Asp Asp Asp Thr Lys Val Val Val Arg Asp His Lys Phe
130 135 140

Leu Glu Asp Glu Asp Glu Ile Asn Gly Ser Thr Lys Ser Ile Ser Met
145 150 155 160

Ser Pro Val Arg Val Ser Ser Gln Phe Val Glu Ser Glu Glu Thr Gly
165 170 175

Gly Asp Asp Lys Asp Ala Val Lys Leu Asn Lys Ser Lys Arg Ser Glu
180 185 190

Glu Ser Asp Phe Leu Ile Asp Ser Val Ile Arg Glu Gln Ser Gly Ser
 195 200 205

Gln Gly Glu Thr Asn Ala Ser Ser Lys Gly Ser His Ala Val Gly Thr
 210 215 220

Lys Leu Tyr Glu Ile Leu Gln Val Asp Val Glu Pro Gln Gln Leu Lys
 225 230 235 240

Glu Asn Asn Ala Gly Asn Val Glu Tyr Lys Gly Pro Val Ala Ser Lys
 245 250 255

Leu Leu Glu Ile Thr Lys Ala Ser Asp Val Glu His Thr Glu Ser Asn
 260 265 270

Glu Ile Asp Asp Leu Asp Thr Asn Ser Phe Phe Lys Ser Asp Leu Ile
 275 280 285

Glu Glu Asp Glu Pro Leu Ala Ala Gly Thr Val Glu Thr Gly Asp Ser
 290 295 300

Ser Leu Asn Leu Arg Leu Glu Met Glu Ala Asn Leu Arg Arg Gln Ala
 305 310 315 320

Ile Glu Arg Leu Ala Glu Glu Asn Leu Leu Gln Gly Ile Arg Leu Phe
 325 330 335

Cys Phe Pro Glu Val Val Lys Pro Asp Glu Asp Val Glu Ile Phe Leu
 340 345 350

Asn Arg Gly Leu Ser Thr Leu Lys Asn Glu Ser Asp Val Leu Ile Met
 355 360 365

Gly Ala Phe Asn Glu Trp Arg Tyr Arg Ser Phe Thr Thr Arg Leu Thr
 370 375 380

Glu Thr His Leu Asn Gly Asp Trp Trp Ser Cys Lys Ile His Val Pro
 385 390 395 400

Lys Glu Ala Tyr Arg Ala Asp Phe Val Phe Phe Asn Gly Gln Asp Val
405 410 415

Tyr Asp Asn Asn Asp Gly Asn Asp Phe Ser Ile Thr Val Lys Gly Gly
420 425 430

Met Gln Ile Ile Asp Phe Glu Asn Phe Leu Leu Glu Glu Lys Trp Arg
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Glu Gln Glu Lys Leu Ala Lys Glu Gln Ala Glu Arg Glu Arg Leu Ala
450 455 460

Glu Glu Gln Arg Arg Ile Glu Ala Glu Lys Ala Glu Ile Glu Ala Asp
465 470 475 480

Arg Ala Gln Ala Lys Glu Glu Ala Ala Lys Lys Lys Lys Val Leu Arg
485 490 495

Glu Leu Met Val Lys Ala Thr Lys Thr Arg Asp Ile Thr Trp Tyr Ile
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Glu Pro Ser Glu Phe Lys Cys Glu Asp Lys Val Arg Leu Tyr Tyr Asn
515 520 525

Lys Ser Ser Gly Pro Leu Ser His Ala Lys Asp Leu Trp Ile His Gly
530 535 540

Gly Tyr Asn Asn Trp Lys Asp Gly Leu Ser Ile Val Lys Lys Leu Val
545 550 555 560

Lys Ser Glu Arg Ile Asp Gly Asp Trp Trp Tyr Thr Glu Val Val Ile
565 570 575

Pro Asp Gln Ala Leu Phe Leu Asp Trp Val Phe Ala Asp Gly Pro Pro
580 585 590

Lys His Ala Ile Ala Tyr Asp Asn Asn His Arg Gln Asp Phe His Ala
595 600 605

Ile Val Pro Asn His Ile Pro Glu Glu Leu Tyr Trp Val Glu Glu Glu
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His Gln Ile Phe Lys Thr Leu Gln Glu Glu Arg Arg Leu Arg Glu Ala
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Ala Met Arg Ala Lys Val Glu Lys Thr Ala Leu Leu Lys Thr Glu Thr
 645 650 655

Lys Glu Arg Thr Met Lys Ser Phe Leu Leu Ser Gln Lys His Val Val
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Tyr Thr Glu Pro Leu Asp Ile Gln Ala Gly Ser Ser Val Thr Val Tyr
 675 680 685

Tyr Asn Pro Ala Asn Thr Val Leu Asn Gly Lys Pro Glu Ile Trp Phe
 690 695 700

Arg Cys Ser Phe Asn Arg Trp Thr His Arg Leu Gly Pro Leu Pro Pro
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Gln Lys Met Ser Pro Ala Glu Asn Gly Thr His Val Arg Ala Thr Val
 725 730 735

Lys Val Pro Leu Asp Ala Tyr Met Met Asp Phe Val Phe Ser Glu Arg
 740 745 750

Glu Asp Gly Gly Ile Phe Asp Asn Lys Ser Gly Met Asp Tyr His Ile
 755 760 765

Pro Val Phe Gly Gly Val Ala Lys Glu Pro Pro Met His Ile Val His
 770 775 780

Ile Ala Val Glu Met Ala Pro Ile Ala Lys Val Gly Gly Leu Gly Asp
 785 790 795 800

Val Val Thr Ser Leu Ser Arg Ala Val Gln Asp Leu Asn His Asn Val
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Asp Ile Ile Leu Pro Lys Tyr Asp Cys Leu Lys Met Asn Asn Val Lys
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Asp Phe Arg Phe His Lys Asn Tyr Phe Trp Gly Gly Thr Glu Ile Lys
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Val Trp Phe Gly Lys Val Glu Gly Leu Ser Val Tyr Phe Leu Glu Pro
 850 855 860

Gln Asn Gly Leu Phe Ser Lys Gly Cys Val Tyr Gly Cys Ser Asn Asp
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Gly Glu Arg Phe Gly Phe Cys His Ala Ala Leu Glu Phe Leu Leu
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Gln Gly Gly Phe Ser Pro Asp Ile Ile His Cys His Asp Trp Ser Ser
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Ala Pro Val Ala Trp Leu Phe Lys Gln Tyr Thr His Tyr Gly Leu
 915 920 925

Ser Lys Ser Arg Ile Val Phe Thr Ile His Asn Leu Glu Phe Gly Ala
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Asp Leu Ile Gly Arg Ala Met Thr Asn Ala Asp Lys Ala Thr Thr Val
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Ser Pro Thr Tyr Ser Gln Glu Val Ser Gly Asn Pro Val Ile Ala Pro
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His Leu His Lys Phe His Gly Ile Val Asn Gly Ile Asp Pro Asp Ile
 980 985 990

Trp Asp Pro Leu Asn Asp Lys Phe Ile Pro Ile Pro Tyr Thr Ser Glu
 995 1000 1005

Asn Val Val Glu Gly Lys Thr Ala Ala Lys Glu Ala Leu Gln Arg
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Lys Leu Gly Leu Lys Gln Ala Asp Leu Pro Leu Val Gly Ile Ile
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Thr Arg Leu Thr His Gln Lys Gly Ile His Leu Ile Lys His Ala
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Ile Trp Arg Thr Leu Glu Arg Asn Gly Gln Val Val Leu Leu Gly
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Ser Ala Pro Asp Pro Arg Val Gln Asn Asp Phe Val Asn Leu Ala
 1070 1075 1080

Asn Gln Leu His Ser Lys Tyr Asn Asp Arg Ala Arg Leu Cys Leu
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Thr Tyr Asp Glu Pro Leu Ser His Leu Ile Tyr Ala Gly Ala Asp
 1100 1105 1110

Phe Ile Leu Val Pro Ser Ile Phe Glu Pro Cys Gly Leu Thr Gln
 1115 1120 1125

Leu Thr Ala Met Arg Tyr Gly Ser Ile Pro Val Val Arg Lys Thr
 1130 1135 1140

Gly Gly Leu Tyr Asp Thr Val Phe Asp Val Asp His Asp Lys Glu
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Arg Ala Gln Gln Cys Gly Leu Glu Pro Asn Gly Phe Ser Phe Asp
 1160 1165 1170

Gly Ala Asp Ala Gly Gly Val Asp Tyr Ala Leu Asn Arg Ala Leu
 1175 1180 1185

Ser Ala Trp Tyr Asp Gly Arg Asp Trp Phe Asn Ser Leu Cys Lys
 1190 1195 1200

Gln Val Met Glu Gln Asp Trp Ser Trp Asn Arg Pro Ala Leu Asp
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Tyr Leu Glu Leu Tyr His Ala Ala Arg Lys Leu Glu
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<211> 61

<212> PRT

<213> Solanum tuberosum

<400> 3

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Trp Ser Cys Lys Ile His Val Pro Lys Glu Ala Tyr Arg Ala Asp Phe
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Val Phe Phe Asn Gly Gln Asp Val Tyr Asp Asn Asn Asp Gly Asn Asp
 35 40 45

Phe Ser Ile Thr Val Lys Gly Gly Met Gln Ile Ile Asp
 50 55 60

<210> 4

<211> 1641

<212> DNA

<213> Solanum tuberosum

<400> 4

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<212> PRT

<213> Solanum tuberosum

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<308> Swiss Prot / P30924

<309> 1993-07-26

<400> 5

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 35 40 45

Arg Met Lys Arg Tyr Val Asp Gln Lys Met Leu Ile Glu Lys Tyr Glu
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Gly Pro Leu Glu Glu Phe Ala Gln Gly Tyr Leu Lys Phe Gly Phe Asn
 65 70 75 80

Arg Glu Asp Gly Cys Ile Val Tyr Arg Glu Trp Ala Pro Ala Ala Gln
 85 90 95

Glu Ala Glu Val Ile Gly Asp Phe Asn Gly Arg Asn Gly Ser Asn His
 100 105 110

Met Met Glu Lys Asp Gln Phe Gly Val Trp Ser Ile Arg Ile Pro Asp
 115 120 125

Val Asp Ser Lys Pro Val Ile Pro His Asn Ser Arg Val Lys Phe Arg
 130 135 140

Phe Lys His Gly Asn Gly Val Trp Val Asp Arg Ile Pro Ala Trp Ile
 145 150 155 160

Lys Tyr Ala Thr Ala Asp Ala Thr Lys Phe Ala Ala Pro Tyr Asp Gly
 165 170 175

Val Tyr Trp Asp Pro Pro Pro Ser Glu Arg Tyr His Phe Lys Tyr Pro
 180 185 190

Arg Pro Pro Lys Pro Arg Ala Pro Arg Ile Tyr Glu Ala His Val Gly
 195 200 205

Met Ser Ser Ser Glu Pro Arg Val Asn Ser Tyr Arg Glu Phe Ala Asp
 210 215 220

Asp Val Leu Pro Arg Ile Lys Ala Asn Asn Tyr Asn Thr Val Gln Leu
 225 230 235 240

Met Ala Ile Met Glu His Ser Tyr Tyr Gly Ser Phe Gly Tyr His Val
 245 250 255

Thr Asn Phe Phe Ala Val Ser Asn Arg Tyr Gly Asn Pro Glu Asp Leu
 260 265 270

Lys Tyr Leu Ile Asp Lys Ala His Ser Leu Gly Leu Gln Val Leu Val
 275 280 285

Asp Val Val His Ser His Ala Ser Asn Asn Val Thr Asp Gly Leu Asn
 290 295 300

Gly Phe Asp Ile Gly Gln Gly Ser Gln Glu Ser Tyr Phe His Ala Gly
 305 310 315 320

Glu Arg Gly Tyr His Lys Leu Trp Asp Ser Arg Leu Phe Asn Tyr Ala
 325 330 335

Asn Trp Glu Val Leu Arg Phe Leu Leu Ser Asn Leu Arg Trp Trp Leu
 340 345 350

Glu Glu Tyr Asn Phe Asp Gly Phe Arg Phe Asp Gly Ile Thr Ser Met
 355 360 365

Leu Tyr Val His His Gly Ile Asn Met Gly Phe Thr Gly Asn Tyr Asn
 370 375 380

Glu Tyr Phe Ser Glu Ala Thr Asp Val Asp Ala Val Val Tyr Leu Met
 385 390 395 400

Leu Ala Asn Asn Leu Ile His Lys Ile Phe Pro Asp Ala Thr Val Ile
 405 410 415

Ala Glu Asp Val Ser Gly Met Pro Gly Leu Ser Arg Pro Val Ser Glu
 420 425 430

Gly Gly Ile Gly Phe Asp Tyr Arg Leu Ala Met Ala Ile Pro Asp Lys
 435 440 445

Trp Ile Asp Tyr Leu Lys Asn Lys Asn Asp Glu Asp Trp Ser Met Lys
450 455 460

Glu Val Thr Ser Ser Leu Thr Asn Arg Arg Tyr Thr Glu Lys Cys Ile
465 470 475 480

Ala Tyr Ala Glu Ser His Asp Cln Ser Ile Val Gly Asp Lys Thr Ile
485 490 495

Ala Phe Leu Leu Met Asn Lys Glu Met Tyr Ser Gly Met Ser Cys Leu
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Thr Asp Ala Ser Pro Val Val Asp Ala Gly Ile Ala Leu Asp Lys Met
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His Gly
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<210> 6

<211> 2649

<212> DNA

<213> Solanum tuberosum

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<308> EMBL / AJ011890

<309> 1999-04-07

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<302> Improvements in or relating to plant starch composition

<308> EMBL / A58164

<309> 1998-03-05

<310> WO 96 34968

<311> 1996-05-03

<312> 1996-11-07

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<212> PRT
<213> Solanum tuberosum

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35 40 45

Glu Lys Ser Ser Tyr Asn Ser Glu Phe Arg Pro Ser Thr Val Ala Ala
50 55 60

Ser Gly Lys Val Leu Val Pro Gly Thr Gln Ser Asp Ser Ser Ser
65 70 75 80

Ser Thr Asp Gln Phe Glu Phe Thr Glu Thr Ser Pro Glu Asn Ser Pro
85 90 95

Ala Ser Thr Asp Val Asp Ser Ser Thr Met Glu His Ala Ser Gln Ile
100 105 110

Lys Thr Glu Asn Asp Asp Val Glu Pro Ser Ser Asp Leu Thr Gly Ser
115 120 125

Val Glu Glu Leu Asp Phe Ala Ser Ser Leu Gln Leu Gln Glu Gly Gly
130 135 140

Lys Leu Glu Glu Ser Lys Thr Leu Asn Thr Ser Glu Glu Thr Ile Ile
145 150 155 160

Asp Glu Ser Asp Arg Ile Arg Glu Arg Gly Ile Pro Pro Pro Gly Leu
165 170 175

Gly Gln Lys Ile Tyr Glu Ile Asp Pro Leu Leu Thr Asn Tyr Arg Gln
180 185 190

His Leu Asp Tyr Arg Tyr Ser Gln Tyr Lys Lys Leu Arg Glu Ala Ile

195

200

205

Asp Lys Tyr Glu Gly Gly Leu Glu Ala Phe Ser Arg Gly Tyr Glu Lys
 210 215 220

Met Gly Phe Thr Arg Ser Ala Thr Gly Ile Thr Tyr Arg Glu Trp Ala
 225 230 235 240

Ieu Gly Ala Gln Ser Ala Ala Leu Ile Gly Asp Phe Asn Asn Trp Asp
 245 250 255

Ala Asn Ala Asp Ile Met Thr Arg Asn Glu Phe Gly Val Trp Glu Ile
 260 265 270

Phe Leu Pro Asn Asn Val Asp Gly Ser Pro Ala Ile Pro His Gly Ser
 275 280 285

Arg Val Lys Ile Arg Met Asp Thr Pro Ser Gly Val Lys Asp Ser Ile
 290 295 300

Pro Ala Trp Ile Asn Tyr Ser Leu Gln Leu Pro Asp Glu Ile Pro Tyr
 305 310 315 320

Asn Gly Ile His Tyr Asp Pro Pro Glu Glu Glu Arg Tyr Ile Phe Gln
 325 330 335

His Pro Arg Pro Lys Pro Lys Ser Leu Arg Ile Tyr Glu Ser His
 340 345 350

Ile Gly Met Ser Ser Pro Glu Pro Lys Ile Asn Ser Tyr Val Asn Phe
 355 360 365

Arg Asp Glu Val Leu Pro Arg Ile Lys Lys Leu Gly Tyr Asn Ala Leu
 370 375 380

Gln Ile Met Ala Ile Gln Glu His Ser Tyr Tyr Ala Ser Phe Gly Tyr
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His Val Thr Asn Phe Phe Ala Pro Ser Ser Arg Phe Gly Thr Pro Asp
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405

410

415

Asp Leu Lys Ser Leu Ile Asp Lys Ala His Glu Leu Gly Ile Val Val
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Leu Met Asp Ile Val His Ser His Ala Ser Asn Asn Thr Leu Asp Gly
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Leu Asn Met Phe Asp Cys Thr Asp Ser Cys Tyr Phe His Ser Gly Ala
450 455 460

Arg Gly Tyr His Trp Met Trp Asp Ser Arg Leu Phe Asn Tyr Gly Asn
465 470 475 480

Trp Glu Val Leu Arg Tyr Leu Leu Ser Asn Ala Arg Trp Trp Leu Asp
485 490 495

Ala Phe Lys Phe Asp Gly Phe Arg Phe Asp Gly Val Thr Ser Met Met
500 505 510

Tyr Ile His His Gly Leu Ser Val Gly Phe Thr Gly Asn Tyr Glu Glu
515 520 525

Tyr Phe Gly Leu Ala Thr Asp Val Asp Ala Val Val Tyr Leu Met Leu
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Val Asn Asp Leu Ile His Gly Leu Phe Pro Asp Ala Ile Thr Ile Gly
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Glu Asp Val Ser Gly Met Pro Thr Phe Cys Ile Pro Val Gln Glu Gly
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Gly Val Gly Phe Asp Tyr Arg Leu His Met Ala Ile Ala Asp Lys Arg
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Ile Glu Leu Leu Lys Lys Arg Asp Glu Asp Trp Arg Val Gly Asp Ile
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Val His Thr Leu Thr Asn Arg Arg Trp Ser Glu Lys Cys Val Ser Tyr

610

615

620

Ala Glu Ser His Asp Gln Ala Leu Val Gly Asp Lys Thr Ile Ala Phe
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Trp Leu Met Asp Lys Asp Met Tyr Asp Phe Met Ala Leu Asp Arg Pro
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Ser Thr Ser Leu Ile Asp Arg Gly Ile Ala Leu His Lys Met Ile Arg
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Leu Val Thr Met Gly Leu Gly Gly Glu Gly Tyr Leu Asn Phe Met Gly
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Asn Glu Phe Gly His Pro Glu Trp Ile Asp Phe Pro Arg Ala Glu Gln
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His Leu Ser Asp Gly Ser Val Ile Pro Gly Asn Gln Phe Arg Tyr Asp
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Lys Cys Arg Arg Arg Phe Asp Leu Gly Asp Ala Glu Tyr Leu Arg Tyr
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Arg Gly Leu Gln Glu Phe Asp Arg Pro Met Gln Tyr Leu Glu Asp Lys
 740 745 750

Tyr Glu Phe Met Thr Ser Glu His Gln Phe Ile Ser Arg Lys Asp Glu
 755 760 765

Gly Asp Arg Met Ile Val Phe Glu Lys Gly Asn Leu Val Phe Val Phe
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Asn Phe His Trp Thr Lys Ser Tyr Ser Asp Tyr Arg Ile Ala Cys Leu
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Lys Pro Gly Lys Tyr Pro Val Ala Leu Asp Ser Asp Asp Pro Leu Phe
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Gly Gly Phe Gly Arg Ile Asp His Asn Ala Glu Tyr Phe Thr Phe Glu

820	825	830
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Gly Trp Tyr Asp Asp Arg Pro Arg Ser Ile Met Val Tyr Ala Pro Cys	835	840	845
---	-----	-----	-----

Lys Thr Ala Val Val Tyr Ala Leu Val Asp Lys Glu Glu Glu Glu	850	855	860
---	-----	-----	-----

Glu Glu Glu Glu Val Ala Ala Val Glu Glu Val Val Val Glu	865	870	875	880
---	-----	-----	-----	-----

Glu Glu

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