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(54) Title: PLANTS WHICH SYNTHESIZE A MODIFIED STARCH, METHODS FOR PRODUCING THE PLANTS, THEIR USE, AND THE MODIFIED STARCH

(54) Bezeichnung: PFLANZEN, DIE EINE MODIFIZIERTE STÄRKE SYNTHEZISIEREN, VERFAHREN ZUR HERSTELLUNG DER PFLANZEN, IHRE VERWENDUNG SOWIE DIE MODIFIZIERTE STÄRKE

## (57) Abstract

The invention relates to recombinant nucleic acid molecules containing two or more nucleotide sequences which code enzymes involved in the metabolism of starch. The invention also relates to methods for producing transgenic plant cells and plants which synthesize a starch that is modified with regard to the phosphate content and side-chain structure thereof. In addition, the invention relates to vectors and host cells which contain the inventive nucleic acid molecules, to the plant cells and plants produced using the inventive methods, to the starch which is synthesized by the inventive plant cells and plants, and to methods for producing this starch.

## (57) Zusammenfassung

Die vorliegende Erfindung betrifft rekombinante Nukleinsäuremoleküle, die zwei oder mehr Nukleotidsequenzen enthalten, die am Stärkemetabolismus beteiligte Enzyme kodieren, Verfahren zur Herstellung transgener Pflanzenzellen und Pflanzen, die eine Stärke synthetisieren, die bezüglich ihres Phosphatgehalts und ihrer Seitenkettenstruktur verändert ist. Des Weiteren betrifft die vorliegende Erfindung Vektoren und Wirtszellen, welche die erfindungsgemäßen Nukleinsäuremoleküle enthalten, die aus den erfindungsgemäßen Verfahren hervorgehenden Pflanzenzellen und Pflanzen, die von den erfindungsgemäßen Pflanzenzellen und Pflanzen synthetisierte Stärke sowie Verfahren zur Herstellung dieser Stärke.

**Abstract of the disclosure**

Plants synthesizing a modified starch, the generation of the plants, their use, and the modified starch.

The present invention relates to recombinant nucleic acid molecules which contain two or more nucleotide sequences which encode enzymes which participate in the starch metabolism, methods for generating transgenic plant cells and plants which synthesize starch which is modified with regard to its phosphate content and its side-chain structure. The present invention furthermore relates to vectors and host cells which contain the nucleic acid molecules according to the invention, the plant cells and plants which originate from the methods according to the invention, to the starch synthesized by the plant cells and plants according to the invention, and to processes for the preparation of this starch.

Plants synthesizing a modified starch, process for the generation of the plants, their use, and the modified starch.

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The present invention relates to recombinant nucleic acid molecules which contain two or more nucleotide sequences which encode enzymes which participate in the starch metabolism, to processes for generating transgenic plant cells, and plants which synthesize starch which is modified with regard to their phosphate content and their side-chain structure. The present invention furthermore relates to vectors and host cells which contain the nucleic acid molecules according to the invention, the plant cells and plants which originate from the processes according to the invention, to the starch synthesized by the plant cells and plants according to the invention, and to processes for the preparation of this starch.

Bearing in mind the increasing importance of plant constituents as renewable resources, biotechnology research attempts to adapt plant raw materials to the demands of the processing industry. Thus, to make possible the use of renewable resources in as many fields of application as possible, it is necessary to make available a great variety of materials.

Not only oils, fats and proteins, but also polysaccharides, constitute important renewable resources from plants. A pivotal position in the polysaccharides is taken up not only by cellulose, but also by starch, which is one of the most important storage substances in higher plants. Not only corn, rice and wheat, but also potato, plays an important role, in particular in starch production.

The polysaccharide starch is a polymer of chemically uniform units, the glucose molecules. However, it is of a highly complex mixture of different forms of molecules which differ with regard to their degree of polymerization and the occurrence of branchings in the glucose chains. Starch is therefore no uniform raw material. In particular, we differentiate between amylose starch,

an essentially unbranched polymer of  $\alpha$ -1,4-glycosidically linked glucose molecules, and amylopectin starch which, in turn, constitutes a complex mixture of differently branched glucose chains. Other branchings are generated by the occurrence of additional  $\alpha$ -1,6-glycosidic linkages. In typical

5 plants used for starch production such as, for example, corn or potatoes, the synthesized starch consists to approx. 25% of amylose starch and approx. 75% of amylopectin starch.

10 The molecular structure of the starch, which is largely determined by the degree of branching, the amylose/amylopectin ratio, the average length and distribution of the side chains and the presence of phosphate groups is of prime importance for important functional properties of the starch and its aqueous solutions, respectively. Important functional properties which must be mentioned are, for example, solubility, retrogradation behavior, film-forming

15 properties, viscosity, color stability, the gelatination properties and the binding and adhesion properties. Also, starch granule size may be of importance for various applications. The production of high-amylose starches is also of particular interest for certain applications. Furthermore, a modified starch contained in plant cells may advantageously alter the behavior of the plant

20 cells under certain conditions. For example, a reduced starch degradation during the storage of starch-containing organs such as, for example, seeds or tubers, before their processing, for example for starch extraction, is feasible. It is also of interest to produce modified starches which make plant cells or plant organs which contain this starch better suited for processing, for example in

25 the preparation of foodstuffs such as popcorn or cornflakes from corn or of potato chips, potato crisps or potato powder from potatoes. Of particular interest is the improvement of the starches regarding a reduced cold sweetening, i.e. a reduced liberation of reducing sugars (in particular glucose) upon prolonged storage at low temperatures. Potatoes especially are

30 frequently stored at temperatures from 4 to 8°C to minimize starch degradation during storage. The reducing sugars liberated during this process,

in particular glucose, result in undesired browning reactions in the production of potato chips or potato crisps (so-called Maillard reactions).

The starch which can be isolated from plants is frequently adapted to  
5 particular industrial purposes with the aid of chemical modifications which, as a rule, require time and money. It seems therefore desirable to find possibilities of generating plants which synthesize a starch whose properties already meet the specific demands of the processing industry and thus combine economical and ecological advantages.

10 A possibility of providing such plants is, in addition to breeding measures, the direct genetic alteration of the starch metabolism of starch-producing plants by genetic engineering methods. However, a prerequisite therefor is the identification and characterization of the enzymes which participate in starch  
15 synthesis modification and starch degradation (starch metabolism) and isolation of the corresponding DNA sequences which encode these enzymes.

The biochemical pathways which lead to the synthesis of starch are essentially known. In plant cells, starch synthesis takes place in the plastids.  
20 In photosynthetically active tissues, these plastids are the chloroplasts, in photosynthetically inactive, starch-storing tissues the amyloplasts.

Important enzymes which participate in starch synthesis are, for example, the branching enzymes, ADP glucose pyrophosphorylases, granule-bound starch  
25 synthases, soluble starch synthases, debranching enzymes, disproportioning enzymes, plastid starch phosphorlyases and the R1 enzymes (R1 proteins).

It is an object of the present invention to provide other, or alternative, genetic engineering methods for modifying the starch metabolism in starch-  
30 synthesizing plants (for example rye, barley, oats, corn, wheat, sorghum and millet, sago, rice, peas, marrowfat peas, cassava, potatoes, tomatoes, oilseed

rape, soybeans, hemp, flax, sunflowers, cowpeas, mung beans, beans, bananas or arrowroot) or suitable nucleic acid molecules by means of which plant cells can be transformed to allow the synthesis of altered advantageous starch varieties.

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Such altered starch varieties exhibit, for example, modifications regarding their degree of branching, the amylose/amylopectin ratio, the phosphate content, the starch granule size and/or the average length and distribution of the side chains (i.e. the side chain structure).

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It is a further object of the invention to provide methods which allow the generation of transgenic plants which synthesize an altered (modified) starch variety.

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Surprisingly, transgenic plants or plant cells which have been transformed with the nucleic acid molecules according to the invention or to be used according to the invention synthesize a starch which is altered in the particular manner according to the invention with regard to its physicochemical properties and/or its side chain structure. In contrast, known starches which are synthesized by transgenic plants exhibit no alterations according to the invention.

These objects are achieved according to the invention by providing the use forms specified in the claims.

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The invention therefore relates to a recombinant nucleic acid molecule (nucleotide sequence) comprising  
a) at least one nucleotide sequence (polynucleotide or nucleic acid molecule) encoding a protein with the function of a soluble starch synthase III or fragments of said nucleotide sequence and b) one or more nucleotide sequences which encode a protein selected from amongst Group A, which consists of proteins which have the function of branching enzymes (BE I, IIa

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and IIb), ADP glucose pyrophosphorylases, granule-bound starch synthases, soluble starch synthases I, II or other, debranching enzymes, disproportioning enzymes, plastid starch phosphorylases, R1 enzymes, amylases and glucosidases or fragments thereof – preferably soluble starch synthases II, 5 soluble starch synthases I and/or branching enzymes and fragments thereof – and nucleic acid molecules which hybridize with one of said nucleotide sequences or fragments thereof, preferable a deoxyribonucleic acid molecule or ribonucleic acid molecule, especially preferably a cDNA molecule. Especially preferred is a nucleic acid molecule which hybridizes specifically with one of said 10 nucleotide sequences or fragments thereof.

In particular, the invention relates to a recombinant nucleic acid molecule, comprising

- a) at least one nucleotide sequence encoding a protein which has the function of a soluble starch synthase III or fragments of said nucleotide sequence 15 and
- b) one or more nucleotide sequences which encode a protein having the function of a branching enzyme.

The invention also relates to transgenic plants or plant cells transformed with the said recombinant nucleic acid molecules and a modified starch obtained 20 from the said transgenic plants or plant cells.

The invention also relates to a process for the preparation of a transgenic plant cell, in which

- a) at least one nucleotide sequence encoding a protein with the function of a soluble starch synthase III or fragments of said nucleotide sequence 25 and
- b) one or more nucleotide sequences which encode a protein having the function of a branching enzyme or fragments thereof are integrated into the genome of a plant cell simultaneously or sequentially,  
wherein said nucleotide sequences inhibit the synthesis of endogenous 30 soluble starch synthase III and endogenous branching enzyme.

The invention further relates to a vector comprising the said recombinant nucleic acid molecules.

Nucleotide sequences which are suitable according to the invention and which encode a protein which has the function of a soluble starch synthase III are disclosed, for example, in EP-A-0779363. The term "nucleotide sequence 5 encoding a protein which has the function of a soluble starch synthase III" is to be understood as meaning for the purposes of the present invention in particular those sequences whose encoding region has a length of 3000-4500 bp, preferably 3200-4250 bp, especially preferably 3400-4000 bp and whose homology to the entire encoding region of a nucleic acid encoding a protein which 10 has the function of a starch synthase amounts to at least 70%, preferably at least 80%, especially preferably at least 90% and very especially preferably at least 95%.

Nucleotide sequences which are suitable according to the invention and which encode a protein from amongst Group A are, for example, soluble starch 15 synthases (Type I, II or other) or granule-bound starch synthase isoforms (for example Hergersberg, 1988, PhD thesis, University of Cologne; Abel, 1995, PhD thesis, FU Berlin; Abel et al., 1996. Plant Journal 10(6):981-991; Visser et al., 1989, Plant Sci. 64:185-192; van der Leij et al., 1991, Mol. Gen. Genet. 228:240-248; EP-A-0779363; WO 92/11376; WO 96/15248, in which SSSB is a soluble 20 starch synthase I and GBSSII is a soluble starch synthase II;

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WO 97/26362; WO 97/44472; WO 97/45545; Delrue et al., 1992, *J. Bacteriol.* 174: 3612-3620; Baba et al., 1993, *Plant Physiol.* 103:565-573; Dry et al., 1992, *The Plant Journal* 2,2: 193-202 or else in the EMBL database entries X74160; X58453; X88789); branching enzyme isoforms (branching enzymes I, 5 IIa, IIb), debranching enzyme isoforms (debranching enzymes, isoamylases, pullulanases) or disproportioning enzyme isoforms, described, for example, in WO 92/14827; WO 95/07335; WO 95/09922; WO 96/19581; WO 97/22703; WO 97/32985; WO 97/42328; Takaha et al., 1993, *J. Biol. Chem.* 268: 10 1391-1396 or else in the EMBL database entry X83969, and those for ADP glucose pyrophosphorylases, plastid starch phosphorylase isoforms and R1 enzymes (R1 proteins), described, for example, in EP-A-0368506; EP-A-0455316; WO 94/28146; DE 19653176.4; WO 97/11188; Brisson et al., 1989, *The Plant Cell* 1:559-566; Buchner et al., 1996, *Planta* 199:64-73; Camirand et al., 1989, *Plant Physiol.* 89(4 Suppl.) 61; Bhatt & Knowler, *J. Exp. Botany* 41 (Suppl.) 5-7; Lin et al., 1991, *Plant Physiol.* 95:1250-1253; Sonnewald et al., 1995, *Plant Mol. Biol.* 27:567-576; DDBJ No. D23280; Lorberth et al., 1998, *Nature Biotechnology* 16:473-477 and also for amylases and glucosidases.

15 20 The nucleotide sequences which are suitably employed in accordance with the invention are of prokaryotic or eukaryotic origin, preferably of bacterial, fungal or plant origin.

25 The term "fragment" denotes, for the purposes of the present invention, portions of the nucleic acid molecules according to the invention or to be used in accordance with the invention which have a length of at least 15 bp, preferably at least 150 bp, especially preferably at least 500 bp, but which generally do not exceed a length of 5000 bp, preferably 2500 bp.

30 The term "hybridization" denotes, for the purposes of the present invention, a hybridization under conventional hybridization conditions, preferably under

stringent conditions as they are described, for example, by Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). In particular, the term “fragment” comprises biologically active molecules.

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Especially preferably, a “specific hybridization” takes place under the following highly stringent conditions:

Hybridization buffer: 2 x SSC; 10 x Denhardt solution (Ficoll 400 + PEG + BSA; ratio 1:1:1); 0.1% SDS; 5 mM EDTA; 50 mM  $\text{Na}_2\text{HPO}_4$ ; 250  $\mu\text{g}/\text{ml}$

10 herring sperm DNA; 50  $\mu$ g/ml tRNA; or 0.25 M sodium phosphate buffer pH 7.2; 1 mM EDTA; 7% SDS at a

Hybridization temperature of  $T = 55$  to  $68^{\circ}\text{C}$ ,

Wash buffer : 0.2 x SSC; 0.1% SDS and

Wash temperature:  $T = 40$  to  $68^{\circ}\text{C}$ .

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The molecules which hybridize with the nucleic acid molecules according to the invention or to be used in accordance with the invention also encompass fragments, derivatives and allelic variants of the nucleic acid molecules according to the invention or to be used in accordance with the invention.

20 "Fragments" are to be understood as meaning not only portions of the nucleic acid molecules which are long enough to encode a functionally active portion of the proteins described. The term "derivative" means, within the context of the present invention, that the sequences of these molecules differ from the sequences of the nucleic acid molecules according to the invention or to be

25 used in accordance with the invention in one or more positions and exhibit a high degree of homology to these sequences. Homology means a sequential identity of at least 60%, preferably over 70%, and especially preferably over 85%, in particular over 90% and very especially preferably over 95%. The

30 to be used in accordance with the invention may have originated by means of one or more deletions, substitutions, insertions (addition) or recombinations.

Furthermore, homology means that a functional and/or structural equivalence exists between the nucleic acid molecules in question and the proteins encoded by them. The nucleic acid molecules which are homologous to the molecules according to the invention or to be used in accordance with the invention and which constitute derivatives of these molecules are, as a rule, variations of these molecules which constitute modifications which exert the same, a virtually identical or a similar biological function. They may be naturally occurring variations, for example sequences from other plant species, or mutations, it being possible for these mutations to have occurred naturally or to have been introduced by directed mutagenesis. The variations may further be synthetic sequences. The allelic variants may be naturally occurring variants or else synthetic variants or variants generated by recombinant DNA technology.

15 The nucleic acid molecules according to the invention or to be used in accordance with the invention may be DNA molecules, in particular cDNA molecules, or, if appropriate, the combination of genomic molecules. The nucleic acid molecules according to the invention or to be used in accordance with the invention may furthermore be RNA molecules. The nucleic acid molecules according to the invention or to be used in accordance with the invention or fragments thereof may have been obtained, for example, from natural sources, generated by means of recombinant technology or generated by synthesis.

20 To express the nucleic acid molecules according to the invention or to be used in accordance with the invention in sense or antisense orientation in plant cells, they are linked with regulatory DNA elements which ensure transcription in plant cells. These include, in particular, promoters. In general, any promoter which is active in plant cells is suitable for expression. The promoter may have been chosen in such a way that expression is constitutive or only in a particular tissue, at a particular point in time of plant development

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or at a point in time determined by external factors which can be, for example, chemically or biologically inducible. Relative to the transformed plant, the promoter can be homologous or heterologous, as can the nucleotide sequence. Examples of suitable promoters are the cauliflower mosaic virus

5 35S RNA promoter and the ubiquitin promoter from corn for constitutive expression, the patatin promoter B33 (Rocha-Sosa et al., 1989, EMBO J. 8:23-29) for tuber-specific expression in potatoes or a promoter which ensures expression only in photosynthetically active tissues, for example the ST-LS1 promoter (Stockhaus et al., 1987, Proc. Natl. Acad. Sci. USA 84: 7943-7947; 10 Stockhaus et al., 1989, EMBO J. 8: 2445-2451), the Ca/b promoter (e.g. US 5656496, US 5639952, Bansal et al., 1992, Proc. Natl. Acad. Sci. USA, 89, 3654-3658) and the Rubisco SSU promoter (see, for example, US 5034322, US 4962028) or for an endosperm-specific expression of the glutelin promoter (Leisy et al., Plant Mol. Biol. 14, (1990), 41-50; Zheng et al., 15 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 HMG promoter from wheat, the USP promoter, the phaseolin promoter or promoters of zein genes from corn (Pedersen et al., Cell 29, (1982), 1015-1026; Quattroccio et al., Plant Mol. Biol. 15 (1990), 81-93).

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A termination sequence which terminates the nucleic acid molecule according to the invention may serve to correctly end transcription and to add to the transcript a poly-A tail, which is considered to have a function in stabilizing the transcripts. Such elements have been described in the literature (cf. Gielen et 25 al., 1989, EMBO J. 8:23-29) and are, as a rule, exchangeable as desired.

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The nucleic acid molecules according to the invention or to be used in accordance with the invention can be used for generating transgenic plant cells and plants which show an increase and/or reduction in the activity of the soluble starch synthase III and of at least one further enzyme of starch metabolism. To this end, the nucleic acid molecules according to the invention

or to be used in accordance with the invention are introduced into suitable vectors, provided with the regulatory nucleic acid sequences which are necessary for efficient transcription in plant cells, and introduced into plant cells. On the one hand, there is the possibility of using the nucleic acid

5 molecules according to the invention or to be used in accordance with the invention for inhibiting the synthesis of the endogenous soluble starch synthase III and/or of at least one further protein from amongst Group A in the cells. This may be achieved with the aid of antisense constructs, in-vivo mutagenesis, a cosuppression effect which occurs, or with the aid of suitably

10 constructed ribozymes. On the other hand, the nucleic acid molecules according to the invention or to be used in accordance with the invention can be used for expressing the soluble starch synthase III and/or at least one further protein from amongst Group A in cells of transgenic plants and thus lead to an increased activity, in the cells, of the enzymes which have been

15 expressed in each case.

In addition, there exists the possibility of using the nucleic acid molecules according to the invention or to be used in accordance with the invention for inhibiting the synthesis of the endogenous soluble starch synthase III and the

20 overexpression of at least one further protein from amongst Group A in the cells. Finally, the nucleic acid molecules according to the invention or to be used in accordance with the invention may also be used for expressing the soluble starch synthase III and for inhibiting at least one further protein from amongst Group A in cells of transgenic plants. The two last-mentioned

25 embodiments of the invention thus lead, in the cells, to a simultaneous inhibition and increase in activity of the enzymes which are inhibited or expressed, respectively.

30 The invention furthermore relates to a vector comprising a nucleic acid molecule according to the invention.

The term "vector" encompasses plasmids, cosmids, viruses, bacteriophages and other vectors conventionally used in genetic engineering which contain the nucleic acid molecules according to the invention and which are suitable for transforming cells. Such vectors are preferably suitable for transforming

5 plant cells. Especially preferably, they permit integration of the nucleic acid molecules according to the invention, if appropriate together with flanking regulatory regions, into the genome of the plant cell. Examples are binary vectors such as pBinAR or pBinB33, which can be employed in agrobacteria-mediated gene transfer.

10 In a preferred embodiment, the vector according to the invention is distinguished by the fact that the nucleotide sequence encoding a protein which has the function of a soluble starch synthase III or fragments thereof is present in sense or in antisense orientation.

15 In a further preferred embodiment, the vector according to the invention is distinguished by the fact that the nucleotide sequence which encodes one or more proteins selected from amongst Group A or fragments thereof is present in sense or in antisense orientation.

20 In yet a further preferred embodiment, the vector according to the invention is distinguished by the fact that the nucleotide sequence which encodes a plurality of proteins selected from amongst Group A or fragments thereof is present partly in sense and partly in antisense orientation.

25 Very especially preferably, the vector according to the invention comprises one or more regulatory elements which ensure transcription and synthesis of an RNA in a prokaryotic or eukaryotic cell.

30 In addition, it is possible to introduce, by means of customary techniques of molecular biology (see, for example, Sambrook et al., 1989, Molecular

Cloning, A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbour, NY), various mutations into the DNA sequences according to the invention or to be used in accordance with the invention, which leads to the synthesis of proteins with biological properties which may

5 be altered. On the one hand, it is possible to generate deletion mutants in which sequences are generated, by progressive deletions from the 5' or from the 3' end of the encoding DNA sequences which lead to the synthesis of analogously truncated proteins. For example, such deletions at the 5' end of the DNA sequence allow the targeted production of enzymes which, due to

10 the removal of the relevant transit or signal sequences, are no longer localized in their original (homologous) compartment, but in the cytosol, or which, due to the addition of other signal sequences, are localized in one or more other (heterologous) compartments (for example plastid, vacuole, mitochondrion, apoplast).

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On the other hand, it is also feasible to introduce point mutations in positions where an altered amino acid sequence affects, for example, the enzyme activity or the regulation of the enzyme. Thus, it is possible to generate, for example mutants which have an altered  $K_M$  or  $k_{cat}$  value or which are no longer 20 subject to the regulatory mechanisms normally present in the cell, for example via allosteric regulation or covalent modification.

25 For the genetic engineering manipulation in prokaryotic cells, the DNA sequences according to the invention or to be used in accordance with the invention or fragments of these sequences can be introduced into plasmids which permit mutagenesis or an altered sequence by the recombination of DNA sequences. Base exchanges may be performed or natural or synthetic sequences may be added, with the aid of standard methods in molecular biology (cf. Sambrook et al., 1989, loc.cit.). To link the DNA portions to each 30 other, adaptors or linkers may be attached to the portions. Furthermore, manipulations which provide suitable restriction cleavage sites or which

remove excessive DNA or restriction cleavage sites which are no longer needed may be employed. Where insertions, deletions or substitutions are suitable, in-vitro mutagenesis, primer repair, restriction or ligation may be employed. The analytical methods which are generally carried out are

5 sequence analysis, restriction analysis and, if appropriate, other methods of biochemistry and molecular biology.

The invention furthermore relates to a transgenic host cell with altered starch metabolism, in particular to prokaryotic or eukaryotic cells, preferably bacterial 10 or plant (monocotyledonous or dicotyledonous) cells (for example of *E.coli*, *Agrobacterium*, *Solanaceae*, *Poideae*, rye, barley, oats, corn, wheat, sorghum and millet, sago, rice, peas, marrowfat peas, cassava, potatoes, tomatoes, oilseed rape, soybeans, hemp, flax, sunflowers, cowpeas, mung 15 beans, beans, bananas or arrowroot) which contains one or more nucleic acid molecules according to the invention or one or more vectors according to the invention or which is derived from such a cell.

Yet another subject of the invention is a transgenic host cell with altered starch metabolism, in particular prokaryotic or eukaryotic cells, preferably bacterial or plant cells (for example of *E.coli*, *Agrobacterium*, *Solanaceae*, *Poideae*, rye, barley, oats, corn, wheat, sorghum and millet, sago, rice, peas, marrowfat peas, cassava, potatoes, tomatoes, oilseed rape, soybeans, hemp, flax, sunflowers, cowpeas, mung beans, beans, bananas or arrowroot) which 20 a) contains at least one nucleotide sequence encoding a protein which has the function of a soluble starch synthase III or fragments thereof and b) one or more nucleotide sequences which encode a protein selected from amongst Group A or fragments thereof or nucleotide sequences which hybridize with these nucleic acid molecules, or which is derived from such a cell.

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30 The host cells according to the invention cannot only be generated by using the nucleic acid molecules according to the invention, but also by means of

successive transformation (for example by so-called "supertransformation"), by employing a plurality of portions of the nucleotide sequence according to the invention or a plurality of vectors comprising portions of the nucleotide sequence according to the invention which encode a protein which has the

5 function of a soluble starch synthase III or fragments thereof and, in addition, one or more proteins selected from the group A consisting of branching enzymes, ADP glucose pyrophosphorylases, granule-bound starch synthases, soluble starch synthases I, II or other, debranching enzymes, disproportioning enzymes, plastid starch phosphorylases, amylases, glucosidases, R1

10 enzymes, fragments thereof - preferably soluble starch synthases II, soluble starch synthases I and/or branching enzymes and also fragments thereof - and nucleic acid molecules which hybridize with one of said nucleotide sequences or fragments thereof, in a plurality of cell transformation steps.

Here, the cell is transformed with a) at least one nucleic acid molecule which

15 encodes a protein which has the function of a soluble starch synthase III, its fragment or with a vector comprising said nucleic acid molecule, and b) transformed simultaneously or in succession with one or more nucleic acid molecules which encode a protein which is selected from group A, consisting of branching enzymes, ADP glucose pyrophosphorylases, granule-bound

20 starch synthases, soluble starch synthases I, II or other, debranching enzymes, disproportioning enzymes, amylases, glucosidases, plastid starch phosphorylases and R1 enzymes, or fragments thereof - preferably soluble starch synthases II, soluble starch synthases I and/or branching enzymes and their fragments - and nucleic acid molecules which hybridize with one of said

25 nucleotide sequences or fragments thereof, or one or more vectors comprising one or more of said nucleic acid molecules in any desired sequence.

A further subject of the invention is a process for the production of a

30 transgenic host cell, bacterial cell, plant cell or plant, which synthesizes a modified starch, wherein a) at least one nucleic acid molecule which encodes

a protein which has the function of a soluble starch synthase III or fragments thereof and b) one or more nucleic acid molecules which encode a protein which is selected from group A, consisting of branching enzymes, ADP glucose pyrophosphorylases, granule-bound starch synthases, soluble starch synthases I, II or other, debranching enzymes, disproportioning enzymes, amylases, glucosidases, plastid starch phosphorylases and R1 enzymes or fragments thereof - preferably soluble starch synthases II, soluble starch synthases I and/or branching enzymes and fragments thereof - and nucleic acid molecules which hybridize with one of said nucleotide sequences or fragments thereof are integrated into the genome of a cell and a whole plant is optionally regenerated from the transgenic plant cell.

A further embodiment of the present invention relates to a method of generating a transgenic host cell, bacterial cell, plant cell or plant which synthesizes a modified starch, which comprises integrating one or more nucleic acid molecules according to the invention or one or more vectors according to the invention into the genome of a cell and optionally regenerating a whole plant from the transgenic plant cell.

20 Providing the nucleic acid molecules according to the invention makes it possible to engage in the starch metabolism of plants, with the aid of genetic engineering methods, and to alter it in such a way that the result is the synthesis of a modified starch which is altered relative to starch synthesized in wild-type plants with regard to, for example, structure, water content, protein content, lipid content, fiber content, ash/phosphate content, amylose/amyllopectin ratio, molecular mass distribution, degree of branching, granule size, granule shape and crystallization, or else in its physico-chemical properties such as flowing and absorption behavior, gelatinization temperature, viscosity, thickening capacity, solubility, gel structure, 25 transparency, thermal stability, shear stability, stability to acids, tendency to undergo retrogradation, gelling, freeze-thaw-stability, complex formation, 30

iodine binding, film formation, adhesion power, enzyme stability, digestibility or reactivity.

There is also the possibility of increasing the yield in suitably genetically

5 engineered plants by increasing the activity of proteins which are involved in the starch metabolism, for example by overexpressing suitable nucleic acid molecules, or by providing mutants which are no longer subject to the cell's regulatory mechanisms and/or which exhibit different temperature dependencies relating to their activity. A particularly pronounced increase in

10 yield may be the result of increasing the activity of one or more proteins which are involved in the starch metabolism in specific cells of the starch-storing tissues of transformed plants such as, for example, in the tuber in the case of potatoes or in the endosperm of corn or wheat. The economic importance and the advantages of these possibilities of engaging in the starch metabolism are

15 enormous.

When expressing the nucleic acid molecules according to the invention or to be used in accordance with the invention in plants, it is possible, in principle, for the synthesized protein to be localized in any desired compartment of the

20 plant cell. To achieve location in a particular compartment (cytosol, vacuole, apoplast, plastids, mitochondria), the transit or signal sequence which ensures location must, if necessary, be deleted (removed) and the remaining encoding region must, if necessary, be linked to DNA sequences which ensure location in the particular compartment. Such sequences are known (see, for example,

25 Braun et al., EMBO J. 11 (1992), 3219-3227; Wolter et al., Proc. Natl. Acad. Sci. USA 85 (1988), 846-850; Sonnewald et al., Plant J. 1 (1991), 95-106). Localization in certain parts of plants or tissues can be ensured, for example, by specific promoters which are well known to the person skilled in the art.

30 The generation of plant cells with a reduced activity of a protein involved in the starch metabolism can be achieved, for example, by expressing a suitable

antisense RNA, a sense RNA for achieving a cosuppression effect, in-vivo mutagenesis or by expressing a suitably constructed ribozyme which specifically cleaves transcripts which encode one of the proteins involved in the starch metabolism, using a nucleic acid molecule according to the

5 invention, preferably by expressing an antisense transcript.

To this end, it is possible to use, on the one hand, a DNA molecule which encompasses all of the sequence which encodes a protein involved in the starch metabolism inclusive of any flanking sequences which may be present,

10 as well as DNA molecules which only encompass portions of the encoding sequence, these portions having a minimum length of 15 bp, preferably of at least 100-500 bp, in particular over 500 bp. As a rule, DNA molecules are used which are shorter than 5000 bp, preferably shorter than 2500 bp.

15 It is also possible to use DNA sequences which exhibit a high degree of homology to the sequences of the DNA molecules according to the invention, but are not fully identical with them. The minimum homology should exceed approx. 65%. The use of sequences with a homology of approximately 75 to 85% and, in particular, approximately 85 to 95%, is preferred.

20

The expression of ribozymes for reducing the activity of specific proteins in cells is known to those skilled in the art and described, for example, in EP-B1-0 321 201. The expression of ribozymes in plant cells was described, for example, by Feyter et al. (Mol. Gen. Genet. 250 (1996), 329-338).

25

Proteins involved in the starch metabolism may also be reduced in the plant cells according to the invention by so-called "in-vivo mutagenesis", where a hybrid RNA-DNA-oligonucleotide ("chimeroplast") is introduced into cells by means of transforming them (Kipp P.B. et al., Poster Session at the "5<sup>th</sup>

30 International Congress of Plant Molecular Biology, 21-27, 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 95/15972; Kren et al., Hepatology 25 (1997), 1462-1468; Cole-Strauss et al., Science 273 (1996), 1386-1389).

5

A portion of the DNA component of the RNA-DNA-oligonucleotide used for this purpose is homologous to a nucleic acid sequence of an endogenous protein, but exhibits a mutation in comparison with the nucleic acid sequence of the endogenous protein or contains a heterologous region enclosed by the homologous regions.

10

Base pairing of the homologous regions of the RNA-DNA-oligonucleotide and of the endogenous nucleic acid molecule followed by homologous recombination allows the mutation or heterologous region contained in the

15

DNA component of the RNA-DNA-oligonucleotide to be transferred into the genome of a plant cell. This leads to a reduced activity of the protein involved in the starch metabolism.

20

Alternatively, the enzyme activities which are involved, in the plant cells, in the starch metabolism may be reduced by a cosuppression effect. This method is described, for example, by Jorgensen (Trends Biotechnol. 8 (1990), 340-344), Niebel et al., (Curr. Top. Microbiol. Immunol. 197 (1995), 91-103), 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).

25

To inhibit the synthesis, in the transformed plants, of a plurality of enzymes involved in starch biosynthesis, it is possible to use DNA molecules for transformation which simultaneously contain, in antisense orientation and under the control of a suitable promoter, a plurality of regions which encode the relevant enzymes. Each sequence may be under the control of its

30

promoter, or, alternatively, the sequences can be transcribed by a joint promoter as a fusion, so that the synthesis of the proteins in question is inhibited to approximately the same or to a different extent. As regards the length of the individual encoding regions which are used in such a construct,

5 what has already been said above for the generation of antisense constructs also applies here. In principle, there is no upper limit for the number of antisense fragments transcribed starting from a promoter in such a DNA molecule. However, the resulting transcript should not, as a rule, exceed a length of 25 kb, preferably 15 kb.

10

The nucleic acid molecules according to the invention or to be used in accordance with the invention therefore make it possible to transform plant cells and simultaneously to inhibit the synthesis of a plurality of enzymes.

15 Moreover, it is possible to introduce the nucleic acid molecules according to the invention or to be used in accordance with the invention into traditional

mutants which are deficient or defect with regard to one or more starch biosynthesis genes (Shannon and Garwood, 1984, in Whistler, BeMiller and Paschall, Starch:Chemistry and Technology, Academic Press, London, 2nd

20 Edition: 25-86). These defects can relate, for example, to the following

proteins: globule-bound starch synthase (GBSS I) and soluble starch synthases (SS I, II, III and others), branching enzymes (BE I, IIa and IIb), debranching enzymes (R-enzymes, isoamylases, pullulanases), disproportioning enzymes and plastid starch phosphorylases.

25

The present invention thus also relates to transgenic plant cells obtainable by a process according to the invention which have been transformed with a vector or nucleic acid molecule according to the invention or to be used in accordance with the invention, and to transgenic plant cells or plants derived

30 from such transformed cells. The cells according to the invention contain one or more nucleic acid molecules according to the invention or to be used in

accordance with the invention, these preferably being linked to one or more regulatory DNA elements (for example promoter, enhancer, terminator) which ensure the transcription in plant cells, in particular a promoter. The cells according to the invention can be distinguished from naturally occurring plant

5 cells, *inter alia*, by the fact that they contain a nucleic acid molecule according to the invention which does not occur naturally in these cells, or by the fact that such a molecule exists integrated at a location in the cell's genome where it does not occur otherwise, i.e. in a different genomic environment.

Furthermore, the transgenic plant cells according to the invention can be

10 distinguished from naturally occurring plant cells by the fact that they contain at least one copy of a nucleic acid molecule according to the invention stably integrated into their genome, if appropriate in addition to copies of such a molecule which occur naturally in the cells or nucleic acid molecules to be used in accordance with the invention. If the nucleic acid molecule(s)

15 introduced into the cells is (are) additional copies to molecules which already occur naturally in the cells, then the plant cells according to the invention can be distinguished from naturally occurring plant cells in particular by the fact that this additional copy, or these additional copies, is, or are, localized at sites in the genome at which it does not occur naturally, or they do not occur

20 naturally. This can be checked, for example, with the aid of a Southern blot analysis.

Preferred plant cells according to the invention are those in which the enzyme activity of individual enzymes which are involved in the starch metabolism is

25 increased and/or reduced by in each case at least 10%, especially preferably at least 30% and very especially preferably by at least 50%.

Moreover, the plant cells according to the invention can be distinguished from naturally occurring plant cells preferably by at least one of the following

30 features: if the nucleic acid molecule which has been introduced is heterologous relative to the plant cell, the transgenic plant cells exhibit

transcripts of the nucleic acid molecules according to the invention which have been introduced. These can be detected by, for example, northern blot analysis. For example, the plant cells according to the invention contain one or more proteins encoded by a nucleic acid molecule according to the invention

5 or to be used in accordance with the invention which has been introduced.

This can be detected by, for example, immunological methods, in particular by a western blot analysis.

If the nucleic acid molecule according to the invention or to be used in

10 accordance with the invention which has been introduced is homologous relative to the plant cell, the cells according to the invention can be

distinguished from naturally occurring cells, for example, on the basis of the additional expression of nucleic acid molecules according to the invention or to be used in accordance with the invention. For example, the transgenic plant

15 cells contain more or fewer transcripts of the nucleic acid molecules according to the invention or to be used in accordance with the invention. This can be detected by, for example, northern blot analysis. "More" or "fewer" in this context means preferably at least 10% more or fewer, preferably at least 20% more or fewer and especially preferably at least 50% more or fewer transcripts

20 than corresponding untransformed cells. Furthermore, the cells preferably exhibit a corresponding increase or decrease in the content of protein encoded by the nucleic acid molecules which have been introduced (at least 10%, 20% or 50%).

25 The transgenic plant cells can be regenerated to entire plants by techniques known to those skilled in the art. The plants obtainable by regenerating the transgenic plant cells according to the invention, and processes for the generation of transgenic plants by regenerating entire plants from the plant cells according to the invention, are also subject matter of the present

30 invention. Another subject of the invention are plants which contain the transgenic plant cells according to the invention. In principle, the transgenic

plants can be plants of any species, i.e. not only monocotyledonous, but also dicotyledonous plants. The plants are preferably useful plants and starch-storing plants such as, for example, cereal species (rye, barley, oats, corn, wheat, sorghum and millet, etc.), sago, rice, peas, marrowfat peas, cassava,

5 potatoes, tomatoes, oilseed rape, soybeans, hemp, flax, sunflowers, cowpeas, mung beans or arrowroot.

The invention also relates to propagation material of the plants according to the invention, for example fruits, seeds, tubers, root stocks, seedlings,

10 cuttings, calli, protoplasts, cell cultures and the like.

Altering the enzymatic activities of the enzymes involved in the starch metabolism results in the synthesis, in the plants generated by the process according to the invention, of a starch with an altered structure.

15

A large number of cloning vectors is available for preparing the introduction of foreign genes into higher plants, vectors which contain a replication signal for *E.coli* and a marker gene for the selection of transformed bacterial cells.

Examples of such vectors are pBR322, pUC series, M13mp series,

20 pACYC184 and the like. The desired sequences can be introduced into the vector at a suitable restriction cleavage site. The plasmid obtained is used for transforming *E.coli* cells. Transformed *E.coli* cells are grown in a suitable medium, and then harvested and lysed. The plasmid is recovered. The analytical methods for characterizing the plasmid DNA obtained are generally 25 restriction analyses, gel electrophoreses and other methods of biochemistry and molecular biology (Sambrook et al. loc.cit.). After each manipulation, the plasmid DNA can be cleaved and DNA fragments obtained linked with other DNA sequences. Each plasmid DNA sequence can be cloned into the same or other plasmids.

30

A large number of techniques is 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 transformants, protoplast fusion by means of polyethylene glycol (PEG), injection, DNA electroporation, the introduction of DNA by means of the

5 biolistic method, and other possibilities (Gene Transfer to Plants. pp. 24-29, eds.: Potrykus, I. and Spangenberg, G., Springer Verlag Berlin Heidelberg 1995).

10 The injection and electroporation of DNA into plant cells require no particular aspects of the plasmids or the DNA used. Simple plasmids such as, for example, pUC derivatives can be used. However, if entire plants are to be regenerated from such transformed cells, the presence of a selectable marker gene is required.

15 Depending on the method of introducing desired genes into the plant cell, further DNA sequences may be required. If, for example, the Ti or Ri plasmid is used for transforming the plant cell, at least the right border, but frequently the right and left border of the Ti and Ri plasmid T-DNA must be linked to the genes to be introduced as a flanking region.

20 If agrobacteria are used for the transformation, the DNA to be introduced must be cloned into specific plasmids, either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the agrobacterial Ti or Ri plasmid by homologous recombination owing to sequences which are homologous to sequences in the T-DNA. The former also contains the *vir* region, which is required for the T-DNA transfer. Intermediate vectors cannot replicate in agrobacteria. The intermediate vector can be transferred to *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors are capable of replication in *E.coli* and in 25 agrobacteria. They contain a selection marker gene and a *linker* or *polylinker* which are framed the by the left and right T-DNA border region. They can be 30

transformed directly into the agrobacteria (Holsters et al. (1978) Mol. Gen. Genet. 163:181-187). The agrobacterium which acts as the host cell should contain a plasmid carrying a *vir* region. The *vir* region is required for transferring the T-DNA into the plant cell. Additional T-DNA may be present.

5 The agrobacterium thus transformed is used for transforming plant cells.

The use of T-DNA for transforming plant cells has been researched intensively and been described in EP 120516; Hoekema, In: The Binary Plant Vector System Offsetdrukkerij Kanters B.V., Alblasserdam (1985), Chapter V;

10 Fraley et al., Crit. Rev. Plant. Sci., 4: 1-46 and An et al. (1985) EMBO J. 4: 277-287.

To transfer the DNA into the plant cell, plant explants can expediently be cocultured with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*.

15 Entire plants can then be regenerated from the infected plant material (for example leaf section, stalk sections, roots, but also protoplasts, or plant cells grown in suspension culture) in a suitable medium which can contain, for example, antibiotics or biocides for selecting transformed cells. The resulting plants can then be examined for the presence of the DNA which has been  
20 introduced. Other possibilities of introducing foreign DNA using the biolistic method or by protoplast transformation are known (cf., for example, Willmitzer, L, 1993 Transgenic plants. In: Biotechnology, A Multi-Volume Comprehensive Treatise (H.J. Rehm, G. Reed, A. Pühler, P. Stadler, eds.), Vol. 2, 627-659, VCH Weinheim-New York-Basel-Cambridge).

25

While the transformation of dicotyledonous plants via Ti-plasmid vector systems with the aid of *Agrobacterium tumefaciens* is well established, more recent work suggests that even monocotyledonous plants are indeed accessible to transformation by means of agrobacterium-based vectors (Chan et al., Plant Mol. Biol. 22 (1993), 491-506; Hiei et al., Plant J. 6 (1994), 271-282).

Alternative systems for the transformation of monocotyledonous plants are the transformation by means of the biolistic method, protoplast transformation, the electroporation of partially permeabilized cells, and the introduction of DNA by means of glass fibers.

5

Specifically different methods have been described in the literature for the transformation of corn (cf., for example, WO95/06128, EP 0 513 849; EP 0 465 875). EP 292 435 describes a method with the aid of which fertile plants can be obtained starting from a mucus-free, friable, granular corn callus. In this context, Shillito et al. (Bio/Technology 7 (1989), 581) have observed that the capability of regenerating fertile plants requires starting from callus suspension cultures from which a dividing protoplast culture with the capability of regenerating plants can be made. Prioli and Söndahl (Bio/Technology 7 (1989), 589) also describe the regeneration and obtaining of fertile corn plants from corn protoplasts.

Once the introduced DNA is integrated into the genome of the plant cell, it is, as a rule, stable therein and is also retained in the progeny of the originally transformed cell. It normally contains a selection marker which mediates the transformed plant cells resistance to a biocide or an antibiotic such as kanamycin, G 418, bleomycin, hygromycin or phosphinothricin and the like. The individual marker chosen should therefore allow the selection of transformed cells over cells which lack the introduced DNA.

20 25 Within the plant, the transformed cells grow in the customary manner (see, also, McCormick et al. (1986) Plant Cell Reports 5:81-84). The resulting plants can be grown normally and hybridized with plants which have the same transformed germplasm or other germplasm. The resulting hybrids have the corresponding phenotypic features.

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Two or more generations should be grown to ensure that the phenotypic

feature is stably retained and inherited. Also, seeds or the like should be harvested to ensure that the phenotype in question or other features have been retained.

- 5 Due to the expression of a nucleic acid molecule according to the invention, the transgenic plant cells and plants according to the invention synthesize a starch whose, for example, physico-chemical properties are altered in comparison with starch synthesized in wild-type plants.
- 10 Yet a further subject of the invention is the starch obtainable from a plant cell and a plant according to the invention, its propagation material or according to a process according to the invention.

Yet another subject of the invention is a process for the production of starch in 15 a manner known per se, in which host cells, plant cells, plants, plant parts according to the invention or propagation material is processed or integrated into the process.

- 20 In a preferred embodiment, the starch according to the invention is distinguished in that its phosphate content is reduced by at least 30%, preferably at least 50%, especially preferably at least 70% and very especially preferably at least 90% in comparison with a starch obtainable from an untransformed cell or plant (i.e. the wild type) and in that its glucan content (cf. Fraction 3 in Example No. 13) after isoamylase treatment in the exclusion 25 volume of an HPLC column system composed of 2 TSK-gel 2000SW columns connected in series and one TSK-gel 3000SW column in 10 mM sodium acetate, pH 3.0 (at a flow rate of 0.35 ml/min as shown in Example No. 13) is increased by at least 50%, preferably at least 150%, especially preferably at least 300% and very especially preferably at least 500%.

30

In a further embodiment, the starch according to the invention is distinguished

in that its phosphate content is increased by at least 10%, preferably at least 30%, and especially preferably at least 50% in comparison with a starch obtainable from an untransformed cell or plant (i.e. the wild type) and in that its glucan content (cf. Fraction 3 in Example No. 13) after isoamylase

5 treatment in the exclusion volume of an HPLC column system composed of 2 TSK-gel 2000SW columns connected in series and one TSK-gel 3000SW column in 10 mM sodium acetate, pH 3.0 (at a flow rate of 0.35 ml/min as shown in Example No. 13) is increased by at least 50%, preferably at least 150%, especially preferably at least 300% and very especially preferably at 10 least 500%.

Processes for extracting the starch from plants or starch-storing plant organs are known to those skilled in the art. Processes for extracting starch from corn kernels are described, for example, by Eckhoff et al. (Cereal Chem. 73 (1996)

15 54-57). As a rule, the extraction of maize starch on an industrial scale is performed by wet milling. Moreover, processes for extracting the starch from a variety of starch-storing plants are described, for example in "Starch: Chemistry and Technology (Editors: Whistler, BeMiller and Paschall (1994), 2nd Edition, Academic Press Inc. London Ltd; ISBN 0-12-746270-8; see, for 20 example, Chapter XII, pages 412-468; corn and sorghum starches: production; by Watson; Chapter XIII, pages 469-479: tapioca, arrowroot and sago starches: production; by Corbishley and Miller; Chapter XIV, pages 479-490: potato starch: production and uses; by Mitch; Chapter XV, pages 491 to 506: wheat starch: production, modification and uses; by Knight and Oson; 25 and Chapter XVI, pages 507 to 528: rice starch: production and uses; by Rohmer and Klem). Devices normally used in processes for extracting starch from plant material are separators, decanters, hydrocyclones, spray driers and fluidized-bed driers.

30 A further embodiment of the present invention also includes the use of the starch according to the invention in industry, preferably for the production of

foodstuffs, packaging materials or disposable products.

The starch according to the invention can be chemically and/or physically modified by processes known to those skilled in the art and is suitable, in its 5 unmodified or modified form, for a variety of applications in the food or non-food sector.

In principle, the possible uses of the starch according to the invention can be divided into two important sectors. One sector encompasses the hydrolysis 10 products of the starch, mainly glucose and glucose units, which are obtained by enzymatic or chemical methods. They are used as starting material for other chemical modifications and processes such as fermentation. What may be important here is the simplicity and inexpensive design of a hydrolysis process as is currently performed essentially enzymatically using 15 amylglucosidase. What would be feasible is a financial saving by using less enzyme. This could be caused by altering the structure of the starch, for example increasing the surface area of the granule, better degradability by a lower degree of branching, or a sterical structure which limits the accessibility for the enzymes employed.

20

The other sector in which the starch according to the invention can be used as a so-called native starch, due to its polymeric structure, can be divided into two further fields of application:

25 1. The food industry

Starch is a traditional additive to a large number of foodstuffs in which its function is essentially to bind aqueous additives or to cause an increased viscosity or else increased gelling. Important characteristics are the flowing characteristics, the sorptive characteristics, the swelling temperature, the 30 gelatinization temperature, the viscosity, the thickening power, starch solubility, transparency, gel structure, thermal stability, shear stability, stability

to acids, the tendency to undergo retrogradation, the film-forming capacity, the freeze-thaw-stability, digestibility and the ability of forming complexes with, for example, inorganic or organic ions.

5      2. The non-food industry

In this important sector, starch is employed as auxiliary for various preparation processes or as an additive in products. When using starch as an auxiliary, mention must be made, in particular, of the paper and board industry. Starch acts mainly for retardation purposes (retaining solids), binding filler particles and fine, as a stiffener and for dehydration. Moreover, the advantageous properties of the starch regarding stiffness, hardness, sound, touch, luster, smoothness, bonding strength and the surfaces.

10     2.1. Paper and board industry

15     Within the papermaking process, four fields of application must be distinguished, i.e. surface, coating, mass and spraying. With 80% of the consumption, the surface starch accounts usually for the greatest starch quantity, 8% are used as coating starch, 7% as mass starch and 5% as spraying starch.

20     The demands on starch with regard to surface treatment are essentially high whiteness, an adapted viscosity, highly-stable viscosity, good film formation and low dust formation. When used for coating, the solids content, a suitable viscosity, a high binding capacity and a high pigment affinity play an important role. Of importance when used as additive to the mass is rapid, uniform, loss-free distribution, high mechanical strength and complete retention in paper cloth. If the starch is used in the spraying sector, again, an adapted solids content, high viscosity and a high binding capacity are of importance.

25

2.2. The adhesives industry

30     An important field of application for starches is in the adhesives industry, where the potential uses can be divided into four subsections: the use as a

pure starch paste, the use in starch pastes which have been treated with specialty chemicals, the use of starch as additive to synthetic resins and polymer dispersions, and the use of starches as extenders for synthetic adhesives. 90% of the starch-based adhesives are employed in the sectors

5 production of corrugated board, production of paper sacks and bags, production of composite materials for paper and aluminum, production of boxboard and gumming adhesive for envelopes, stamps and the like.

### 2.3. Textile industry and textile care products industry

10 An important field of application for starches as auxiliaries and additives is the sector production of textiles and textile care products. The following four fields of application must be distinguished within the textile industry: the use of starch as sizing agent, i.e. as auxiliary for smoothing and strengthening the burning behavior as a protection from the tensile forces applied during

15 weaving, and for increasing abrasion resistance during weaving, starch as a textile finishing agent, in particular after quality-reducing pretreatments such as bleaching, dyeing and the like, starch as thickener in the preparation of dye pastes for preventing bleeding, and starch as additive to warping agents for sewing yarns.

20

### 2.4. Construction materials industry

The fourth field of application is the use of starches as additives in construction materials. An example is the production of gypsum plasterboards, where the starch which is admixed to the gypsum slurry

25 gelatinizes with the water, diffuses to the surface of the plaster core and there binds the board to the core. Other fields of application are the admixture to rendering and mineral fibers. In the case of ready-mixed concrete, starch products are employed for delaying binding.

30 2.5. Soil stabilization

A limited market for starch products is the production of soil stabilizers, which

are employed for the temporary protection of the soil particles from water when the soil is disturbed artificially. According to present knowledge, product combinations of starch and polymer emulsions equal the previously employed products with regard to their erosion- and crust-reducing effect, but are

5 markedly less expensive.

#### 2.6. Use in crop protection products and fertilizers

One field of application for using starch is in crop protection products for altering the specific properties of the products. Thus, starches are employed

10 for improving the wettability of crop protection products and fertilizers, for the metered release of the active ingredients, for converting liquid active ingredients, volatile active ingredients and/or active ingredients with an offensive odor into microcrystalline, stable, shapable substances, for mixing incompatible compounds and for extending the duration of action by reducing

15 decomposition.

#### 2.7. Pharmaceuticals, medicine and the cosmetics industry

Another field of application is the sector of the pharmaceuticals, medicine and

cosmetics industry. In the pharmaceuticals industry, starches are employed as

20 binders for tablets or for diluting the binder in capsules. Moreover, starches are used as tablet disintegrants, since they absorb fluids after swallowing and swell within a short time to such an extent that the active ingredient is liberated. Medicinal lubricating powders and wound powders are starch-based

for reasons of quality. In the cosmetics sector, starches are employed, for

25 example, as carriers of powder additives such as fragrances and salicylic acid. A relatively large field of application for starch is toothpaste.

#### 2.8. Addition of starch to coal and briquettes

A field of application for starch is as an additive to coal and briquettes. With

30 an addition of starch, coal can be agglomerated, or briquetted, in terms of high quantity, thus preventing early decomposition of the briquettes. In the case of

barbeque coal, the starch addition amounts to between 4 and 6%, in the case of calorized coal to between 0.1 and 0.5%. Moreover, starches are gaining importance as binders since the emission of noxious substances can be markedly reduced when starches are added to coal and briquettes.

5

#### 2.9. Ore slick and coal silt preparation

Furthermore, starch can be employed as flocculant in the ore slick and coal silt preparation.

10 2.10. Foundry auxiliary

A further field of application is as an additive to foundry auxiliaries. Various casting processes require cores made with sands treated with binders. The binder which is predominantly employed nowadays is bentonite, which is treated with modified starches, in most cases swellable starches. The purpose 15 of adding starch is to increase flowability and to improve the binding power. In addition, the swellable starches can meet other demands of production engineering, such as being cold-water-dispersible, rehydratable and readily miscible with sand and having high water binding capacity.

20 2.11. Use in the rubber industry

In the rubber industry, starch is employed for improving the technical and visual quality. The reasons are the improvement of the surface luster, the improvement of handle and of appearance, and to this end starch is scattered to the tacky gummed surfaces of rubber materials prior to cold curing, and 25 also the improvement of the rubber's printability.

#### 2.12. Production of leather substitutes

Modified starches may furthermore also be sold for the production of leather substitutes.

30

### 2.13. Starch in synthetic polymers

In the polymer sector, the following fields of application can be envisaged: the use of starch degradation products in the processing process (the starch is only a filler, there is no direct bond between the synthetic polymer and the starch) or, alternatively, the use of starch degradation products in the production of polymers (starch and polymer form a stable bond).

The use of starch as a pure filler is not competitive in comparison with other substances such as talc. However, this is different when the specific properties of starch make an impact and thus markedly alter the spectrum of characteristics of the end products. An example of this is the use of starch products in the processing of thermoplasts, such as polyethylene. Here, the starch and the synthetic polymer are combined by coexpression in a ratio of 1:1 to give a masterbatch, from which various products are produced together with granulated polyethylene, using conventional process techniques. By using starch in polyethylene films, an increased substance permeability in the case of hollow bodies, an improved permeability for water vapor, an improved antistatic behavior, an improved antiblock behavior and an improved printability with aqueous inks can be achieved. The current disadvantages relate to the insufficient transparency, the reduced tensile strength and a reduced elasticity.

Another possibility is the use of starch in polyurethane foams. By adapting the starch derivatives and by process-engineering optimization, it is possible to control the reaction between synthetic polymers and the starches' hydroxyl groups in a directed manner. This results in polyurethane films which have the following spectrum of properties, owing to the use of starch: a reduced heat extension coefficient, a reduced shrinking behavior, an improved pressure-tension behavior, an increase in permeability for water vapor without altering the uptake of water, a reduced flammability and a reduced ultimate tensile strength, no drop formation of combustible parts, freedom from halogens and

reduced aging. Disadvantages which still exist are a reduced printability and a reduced impact strength.

Product development is currently no longer restricted to films. Solid polymer  
5 products such as pots, slabs and dishes which contain a starch content of  
over 50% may also be prepared. Moreover, starch/polymer mixtures are  
considered advantageous since their biodegradability is much higher.

Starch graft polymers have become exceedingly important owing to their  
10 extremely high water binding capacity. They are products with a starch  
backbone and a side chain of a synthetic monomer, grafted on following the  
principle of the free-radical chain mechanism. The currently available starch  
graft polymers are distinguished by a better binding and retention capacity of  
up to 1000 g of water per g of starch combined with high viscosity. The fields  
15 of application for these superabsorbers have extended greatly in recent years  
and are, in the hygiene sector, the products diapers and pads and, in the  
agricultural sector, for example seed coatings.

What is decisive for the application of novel, genetically engineered starches  
20 are, on the one hand, structure, water content, protein content, lipid content,  
fiber content, ash/phosphate content, amylose/amylopectin ratio, molecular  
mass distribution, degree of branching, granule size and granule shape and  
crystallization, and, on the other hand, also the characteristics which affect the  
following features: flowing and sorption behavior, gelatinization temperature,  
25 viscosity, thickening power, solubility, gel structure, transparency, heat  
stability, shear stability, stability to acids, tendency to undergo retrogradation,  
gel formation, freeze-thaw stability, complex formation, iodine binding, film  
formation, adhesive power, enzyme stability, digestibility and reactivity.

30 The production of modified starches by means of genetic engineering  
methods can, on the one hand, alter the properties, for example of the starch

derived from the plant in such a way that other modifications by means of chemical or physical alterations are no longer required. On the other hand, starches which have been altered by genetic engineering methods may be subjected to further chemical modification, which leads to further

5     improvements in quality for some of the above-described fields of application. These chemical modifications are known in principle. They are, in particular, modifications by heat and pressure treatment, treatment with organic or inorganic acids, enzymatic treatment, oxidations or esterifications, which lead, for example, to the formation of phosphate starches, nitrate starches, sulfate

10    starches, xanthate starches, acetate starches and citrate starches. Moreover, mono- or polyhydric alcohols in the presence of strong acids may be employed for preparing starch ethers, resulting in starch alkyl ethers, o-allyl ethers, hydroxyalkyl ethers, O-carboxymethyl ethers, N-containing starch ethers, P-containing starch ethers, S-containing starch ethers, crosslinked

15    starches or starch graft polymers.

A use of the starches according to the invention is in industrial application, preferably for foodstuffs or the preparation of packaging materials and disposable articles.

20    The examples which follow serve to illustrate the invention and constitute in no way a restriction.

Abbreviations:

25	BE	branching enzyme
	bp	base pair
	GBSS	granule bound starch synthase
	IPTG	isopropyl- $\beta$ -D-thiogalactopyranoside
	SS	soluble starch synthase
30	PMSF	phenylmethylsulfonyl fluoride

## Media and solutions used in the examples:

	20 x SSC	175.3 g NaCl 88.2 g sodium citrate
5		to 1000 ml with double-distilled H <sub>2</sub> O pH 7.0 with 10 N NaOH
10	Buffer A	50 mM Tris-HCl pH 8.0 2.5 mM DTT 2 mM EDTA 0.4 mM PMSF 10% glycerol 0.1 % sodium dithionite
15	Buffer B	50 mM Tris-HCl pH 7.6 2.5 mM DTT 2 mM EDTA
20	Buffer C	0.5 M sodium citrate pH 7.6 50 mM Tris-HCl pH 7.6 2.5 mM DTT 2 mM EDTA
25	10 x TBS	0.2 M Tris-HCl pH 7.5 5.0 M NaCl
	10 x TBST	10 x TBS 0.1% (v/v) Tween 20
30	Elution buffer	25 mM Tris pH 8.3 250 mM glycine

Dialysis buffer	50 mM Tris-HCl pH 7.0 50 mM NaCl 2 mM EDTA 14.7 mM $\beta$ -mercaptoethanol
5	0.5 mM PMSF
Protein buffer	50 mM sodium phosphate buffer pH 7.2 10 mM EDTA 0.5 mM PMSF
10	14.7 mM beta-mercaptoethanol

Description of the figures:

Fig. 1 represents a schematic RVA temperature profile (viscosity and temperature vs. Time [min]), this with the viscosimetric parameters 15 T=gelatinization temperature, temperature at the beginning of gelatinization; Max specifies the maximum viscosity; Min specifies the minimum viscosity; Fin specifies the viscosity at the end of the measurement; Set is the difference (D) of Min and Fin (setback).

20 Fig. 2 shows the side-chain distribution of the amylopectin samples determined on the right by means of HPAEC-PAC (voltage [mV] vs. Time [min]) and, on the left, determined by gel permeation chromatography (current [nC] vs. Time [min]).  
25 A = control (wild-type No. 1); B = (asSSII, No. 7); C = (asSSIII, No. 8); D = (asSSII asSSIII, No. 13); E = (asSSII asSSIII, No. 14)

The numbers given in brackets in the description of the figures relate to the numbers of the starch samples described in Tables 1 and 2.

30 The following methods were used in the examples:

**1. Cloning method**

The vector pBluescript II SK (Stratagene) was used for cloning into *E.coli*

5 For the transformation of plants, the gene constructions were cloned into the binary vector pBinAR Hyg (Höfgen & Willmitzer, 1990, Plant Sci. 66:221-230) and pBinB33-Hyg.

**2. Bacterial strains and plasmids**

10

The *E.coli* strain DH5a (Bethesda Research Laboratories, Gaithersburgh, USA) was used for the Bluescript vector p Bluescript II KS (Stratagene) and for the pBinAR Hyg and pBinB33 Hyg constructs. The *E.coli* strain XL1-Blue was used for the *in vivo* excision.

15

**pBinAR**

The plasmid pBinAR is a derivative of the binary vector plasmid pBin19 (Bevan, 1984), which was constructed as follows:

A 529 bp fragment encompassing the nucleotides 6909-7437 of the

20 cauliflower mosaic virus 35S promoter was isolated from plasmid pDH51 as an EcoRI/Kpnl fragment (Pietrzak et al., 1986), ligated between the EcoRI and Kpnl cleavage sites of the pUC18 polylinker and was termed plasmid pUC18-35S. With the aid of the restriction endonucleases HindIII and Pvull, a 192 bp fragment was isolated from plasmid pAGV40 (Herrera-Estrella et al., 1983), which encompasses DNA of the Ti plasmid pTiACH5 (Gielen et al, 1984) (nucleotides 11749-11939). After the Pvull cleavage site had been provided with SphI linkers, the fragment was ligated between the SphI and HindIII cleavage sites of pUC18-35S, and this was termed plasmid pA7. Moreover, the entire polylinker comprising the 35S promoter and the ocs 25 terminator was excised with EcoRI and HindIII and ligated into the suitably cleaved pBin19. This gave rise to the plant expression vector pBinAR (Höfgen 30

and Willmitzer, 1990).

#### pBinB33

The promoter of the *Solanum tuberosum* patatin gene B33 (Rocha-Sosa et al., 1989) was ligated, as a DraI fragment (nucleotides -1512 - +14) into the Sst I- cleaved vector pUC19, which had 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 with EcoRI and SmaI and ligated into the suitably cleaved vector pBinAR. This gave rise to the plant expression vector pBinB33.

#### pBinAR-Hyg

Starting from plasmid pA7 (cf. description of vector pBinAR), the EcoRI-HindIII fragment comprising the 35S promoter, the ocs terminator and the polylinker portion between 35S promoter and ocs terminator was introduced into the suitably cleaved plasmid pBin-Hyg.

#### pBinB33-Hyg

Starting from plasmid pBinB33, the EcoRI-HindIII fragment comprising the B33 promoter, part of the polylinker and the ocs terminator was cleaved out and ligated into the suitably cleaved vector pBin-Hyg. This gave rise to the plant expression vector pBinB33-Hyg.

### 3. Transformation of *Agrobacterium tumefaciens*

The DNA was transferred by direct transformation following the method of Höfgen&Willmitzer (1988, Nucleic Acids Res. 16:9877). The plasmid DNA of transformed agrobacteria was isolated following the method of Birnboim&Doly (1979, Nucleic Acids Res. 7:1513-1523), subjected to suitable restriction cleavage, and then analyzed by gel electrophoresis.

#### 4. Transformation of potatoes

The plasmids were transformed into the potato plants (*Solanum tuberosum* L.cv. Desiree, Vereinigte Saatzuchten eG, Ebstorf) with the aid of the 5 *Agrobacterium tumefaciens* strain C58C1 (Dietze et al. (1995) in Gene Transfer to Plants. pp. 24-29, eds.: Potrykus, I. and Spangenberg, G., Springer Verlag, Deblaere et al., 1985, Nucl. Acids Res. 13:4777-4788).

Ten small leaves of a sterile potato culture which had been wounded with a 10 scalpel were placed into 10 ml MS medium (Murashige&Skoog (1962) Physiol. Plant. 15: 473) supplemented with 2% sucrose and containing 50 ml of an *Agrobacterium tumefaciens* overnight culture grown under selection conditions. After the culture had been shaken gently for 3-5 minutes, it was incubated for 2 more days in the dark. For callus induction, the leaves were 15 then placed on MS medium supplemented with 1.6% glucose, 5 mg/l naphthylacetic acid, 0.2 mg/l benzylaminopurine, 250 mg/l claforan, 50 mg/l canamycin, and 0.80% Bacto agar. After the leaves had been incubated for one week at 25°C and 3000 Lux, they were placed on MS medium supplemented with 1.6% glucose, 1.4 mg/l zeatin ribose, 20 mg/l 20 naphthylacetic acid, 20 mg/l gibberellic acid, 250 mg/l claforan, 50 mg/l canamycin, and 0.80% Bacto agar, to induce shoots.

#### 5. Plant culture regime

25 Potato plants were kept in the greenhouse under the following regime:  
 Light period                            16 h at 25,000 Lux and 22°C  
 Dark period                            8 hours at 15°C  
 Atmospheric humidity                60 %

## 6. Radiolabeling of DNA fragments

The DNA fragments were radiolabeled with the aid of a DNA Random Primer Labeling Kit by Boehringer Mannheim (Germany) following the manufacturer's 5 instructions.

## 7. Determination of starch synthase activity

Determination of starch synthase activity was done by determining the 10 incorporation of  $^{14}$  C Glucose from ADP[ $^{14}$  C glucose] into a methanol/KCl-insoluble product as described by Denyer & Smith, 1992, *Planta* 186:609-617.

## 8. Detection of soluble starch synthases in the native gel

15 To detect the activity of soluble starch synthases by non-denaturing gel electrophoresis, tissue samples of potato tubers were hydrolyzed 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 (BioRAD). The monomer concentration of the 1.5-mm-thick gels was 7.5% (w/v), and 20 25 mM Tris-glycine pH 8.4 was used as gel buffer and running buffer. Identical amounts of protein extract were applied and separated for 2 hours at 10 mA per gel.

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

## 9. Starch analysis

30 The starch formed by the transgenic potato plants was characterized by the following methods:

a) Determination of the amylose/amylopectin ratio in starch from potato plants

Starch was isolated from potato plants by standard methods, and the 5 amylose:amylopectin ratio was determined by the method described by Hovenkamp-Hermelink et al. (Potato Research 31 (1988) 241-246).

b) Determination of the phosphate content

10 In potato starch, some glucose units may be phosphorylated on the carbon atoms at positions C2, C3 and C6. To determine the degree of phosphorylation at the C6-position of the glucose, 100 mg of starch were hydrolyzed for 4 hours at 95°C in 1 ml of 0.7 M HCl (Nielsen et. al. (1994) Plant Physiol. 105: 111-117). After neutralization with 0.7 M KOH, 50 ml of the 15 hydrolysate were subjected to a visual-enzymatic test to determine glucose-6-phosphate. The change in absorption of the test batch (100 mM imidazole/HCl; 10 mM MgCl<sub>2</sub>; 0.4 mM NAD; 2 units of *Leuconostoc mesenteroides*, glucose-6-phosphate dehydrogenase; 30°C) was monitored at 334 nm.

20

The total phosphate was determined as described by Ames, 1996, Methods in Enzymology VIII, 115-118.

c) Analysis of the amylopectin side chains

25

To analyze distribution and length of the side chains in the starch samples, 1 ml of a 0.1% starch solution was digested with 0.4 U of isoamylase (Megazyme International Ireland Ltd., Bray, Ireland) overnight at 37°C in 100 mM sodium citrate buffer, pH 3.5.

30

The rest of the analysis was carried out as described by Tomlinson et al.,

(1997), Plant J. 11:31-47, unless otherwise specified.

d) Granule size determination

5 The granule size was determined using a "Lumosed" photosedimentometer by Retsch GmbH, Germany. To this end, 0.2 g of starch were suspended in approx. 150 ml of water and immediately measured. The program supplied by the manufacturer calculated the mean diameter of the starch granules, assuming an average starch density of 1.5 g/l.

10

e) Gelatinization properties

The gelatinization or viscosity properties of the starch were recorded using a Viskograph E by Brabender OHG, Germany, or using a Rapid Visco Analyzer,

15 Newport Scientific Pty Ltd, Investment Support Group, Warriewood NSW 2102, Australia. When using the Viskograph E, a suspension of 20 g of starch in 450 ml of water was subjected to the following heating program: heating at 3°/min from 50°C to 96°C, keep constant for 30 minutes, cool at 3°/min to 30°C, and again keep constant for 30 minutes. The temperature profile gave 20 characteristic gelatinization properties.

When measuring using the Rapid Visco Analyzer (RVA), a suspension of 2 g of starch in 25 ml of water was subjected to the following heating program: suspend for 60 seconds at 50°C, heat at 12°/min from 50°C to 95°C, keep

25 constant for 2.5 minutes, cool at 12°C/min to 50°C, and again keep constant for 2 minutes. The RVA temperature profile gave the viscometric parameters of the test starches for the maximum viscosity (Max), the end viscosity (Fin), the gelatinization temperature (T), the minimum viscosity occurring after the maximum viscosity (Min), and the difference between minimum and end 30 viscosity (setback, Set) (cf. Table 1 and Fig. 1).

## f) Determination of the gel strength

To determine the gel strength by means of a Texture Analyzer, 2 g of starch were gelatinized in 25 ml of water (cf. RVA measurement) and then stored for

5 24 hours in a sealed container at 25°C with the exclusion of air. The samples were mounted underneath the probe (circular stamp) of a Texture Analyzer TA-XT2 (Stable Micro Systems), and the gel strength was determined with the following parameter settings:

10	Test speed	0.5 mm
	Penetration depth	7 mm
	Contact area (of the stamp)	113 mm <sup>2</sup>
	Pressure/contact area	2 g

## 15 10. Determination of glucose, fructose and sucrose

To determine the glucose, fructose and sucrose content, small tuber portions (approx. diameter 10 mm) of potato tubers were frozen in liquid nitrogen and then extracted for 30 minutes at 80°C in 0.5 ml of 10 mM HEPES, pH 7.5;

20 80% (vol./vol.) ethanol. The supernatant, which contains the solubles: was removed and the volume was determined. The supernatant was used for determining the amount of soluble sugars. The quantitative determination of soluble glucose, fructose and sucrose was carried out in a batch with the following composition

25	100,0	mM imidazole/HCl, pH 6.9
	1.5	mM MgCl <sub>2</sub>
	0.5	mM NADP <sup>+</sup>
	1.3	mM ATP
	10-50	µl sample
30	1.0	unit yeast glucose-6-phosphate dehydrogenase

The batch was incubated for 5 minutes at room temperature. The sugars were subsequently determined photometrically by measuring the absorption at 340 nm after the successive addition of

- 1.0 unit yeast hexokinase (to determine glucose),
- 5 1.0 unit yeast phosphoglucoisomerase (to determine fructose) and
- 1.0 unit yeast invertase (to determine sucrose)

#### 11. Determination of the water uptake capacity (WUC)

- 10 To determine the water uptake capacity, the solubles of the starch which had swelled at 70°C were removed by centrifugation (10 min at 10,000 x g) and the residue was then weighed. The water uptake capacity of the starch was based on the initial starch quantity corrected by the soluble matter.
- 15 
$$\text{WUC (g/g)} = (\text{residue} - (\text{initial quantity-solubles})) / (\text{initial quantity-solubles})$$

Use examples:

#### Example 1: Preparation of plasmid p35S $\alpha$ SSI-Hyg

- 20 A 1831 bp Asp718/XbaI fragment containing a partial cDNA encoding the potato SS I (Abel, G., (1995), PhD thesis, Freie Universität Berlin) was introduced between the Asp718 and XbaI cleavage site of the vector pBinAR-Hyg in antisense orientation relative to the 35S promoter.

#### Example 2: Preparation of plasmid p35S-SSI-Kan

- 25 A 2384 bp EcoRI fragment containing cDNA encoding potato SS I (Abel 1995, loc.cit.) was made blunt-ended and introduced into the vector pBinAR, which
- 30 had previously been cut with SmaI, in sense orientation relative to the 35S promoter.

**Example 3: Preparation of plasmid p35SaSSII-Kan**

A 1959 bp Smal/Asp718 fragment containing a partial cDNA encoding potato SS II (Abel, 1995, termed GBSS II therein) was made blunt-ended and

5 introduced into the Smal cleavage site of the vector pBinAR in antisense orientation relative to the 35S promoter.

**Example 4: Preparation of plasmid pB33-SSII-Hyg**

10 A 2619 bp Smal/Sall fragment containing a cDNA encoding the potato SS II (Abel, 1995, loc.cit.) was introduced into the vector pBinB33-Hyg, which had previously been cut with Smal and Sall in sense orientation relative to the B33 promoter.

15 Example 5: Preparation of plasmid p35SaSSIII-Hyg

A 4212 bp Asp718/XbaI fragment containing a cDNA encoding the potato SS III (Abel et al., 1996, Plant J. 10(6):981-991) was inserted between the Asp718 and the XbaI cleavage site of the vector pBinAR-Hyg in antisense

20 orientation relative to the 35S promoter.

**Example 6: Preparation of plasmid p35S-SSIII-Kan**

A 4191 bp EcoRI fragment containing a cDNA encoding the potato SS III

25 (Abel et al., 1996, loc.cit.) was made blunt-ended and introduced into the Smal cleavage site of the vector pBinAR in sense orientation relative to the 35S promoter.

**Example 7: Preparation of plasmid pB33aBEαSSIII-Kan**

30

A 1650 bp HindII fragment which contains a partial cDNA encoding the potato

BE enzyme (Kossmann et al., 1991, Mol. & Gen. Genetics 230(1-2):39-44) was made blunt-ended and introduced into the vector pBinB33 which had been precut with SmaI in antisense orientation relative to the B33 promoter. The resulting plasmid was cut open with BamHI. A 1362 bp BamHI fragment containing a partial cDNA encoding the potato SS III enzyme (Abel et al., 1996, loc.cit.) was introduced into the cleavage site, again in antisense orientation relative to the B33 promoter.

Example 8: Preparation of plasmid p35SaSSII- $\alpha$ SSIII-Kan

A 1546 bp EcoRV/HincII fragment containing a partial cDNA encoding for the potato SS II (Abel, 1995, loc.cit.) was cloned into the vector pBluescript II KS which had been cut with EcoRV/HincII, then excised via an Asp718/BamHI digest and introduced in antisense orientation relative to the 35S promoter into the vector pBinAR which had been digested in the same manner. Then, a 1356 bp BamHI fragment containing a partial cDNA encoding the potato SS III (Abel et al., 1996, loc.cit.) was introduced into the BamHI cleavage site of the vector pBinAR-SSII, again in antisense orientation relative to the 35S promoter.

20

Example 9: Preparation of plasmid pB33 $\alpha$ SSI $\alpha$ SSIII-Kan

A 2384 bp EcoRI fragment containing a cDNA encoding the potato SS I (Abel, 1995, loc.cit.) was made blunt-ended and cloned into the SmaI-cleavage site of the pBinB33 vector in antisense orientation relative to the B33 promoter. A 1362 bp BamHI fragment containing a partial cDNA encoding the potato SS III (Abel et al., 1996, loc.cit.) was introduced into the BamHI cleavage site of the resulting vector, again in antisense orientation relative to the B33 promoter.

30

**Example 10: Preparation of plasmid p35SaSSII-Hyg**

A 1959 bp SmaI/Asp718 fragment containing a partial cDNA encoding for the SS II (Abel, 1995, loc.cit.) was made blunt-ended and introduced into the

5 SmaI cleavage site of the pBinAR-Hyg vector in antisense orientation relative to the 35S promoter.

**Example 10B: Preparation of plasmid pB33aR1-Hyg**

10 A 1.9 kB fragment of R1 from *S. tuberosum* (WO 97/11188) was obtained by digestion with Asp718 from the vector pBluescript. The fragment was cloned into the Asp718 cleavage site behind the B33 promoter in antisense orientation of the vector pB33-Binar-Hyg. This vector contains a hygromycin resistance.

15

**Example 11: Introduction of the plasmids into the genome of potato cells**

The plasmids given in Examples 1 to 10 were transferred into agrobacteria, either individually and/or in succession, with the aid of which potato cells were

20 transformed as described above. Subsequently, entire plants were regenerated from the transformed plant cells.

Transgenic plant cells of the genotype asSSI-asSSII-asSSIII were generated by transformation with the plasmid p35SaSSI-Hyg described in Ex. No. 1 and

25 subsequent retransformation with the plasmid p35SaSSII-aSSIII-Kan described in Ex. No. 8.

Transgenic plant cells of the genotype asSSII-asSSI-asSSIII were generated by transformation with the plasmid p35SaSSII-Hyg described in Ex. No. 10

30 and subsequent retransformation with the plasmid pB33aSSIaSSIII-Kan described in Ex. No. 9.

As a result of the transformation, the transgenic potato plants synthesized altered starch varieties.

**Example 12: Physico-chemical characterization of the modified starches**

The starch formed by the transgenic plants generated in accordance with Example 11 differs, for example, from starch synthesized by wild-type plants (potato) with regard to its phosphate or amylose content and the viscosities and gelatinization properties which were determined by RVA. The results of the physico-chemical characterization of the modified starches are shown in Table 1. In the antisense constructs, the enzyme activities of the suppressed soluble starch synthases were reduced by up to 85% relative to the untransformed control plants.

**Table 1: Properties of the modified starches**

No.	Genotype	Phosphate (%)	Amylose (%)	RVA Max (%)	RVA Min (%)	RVA Fin (%)	RVA Set (%)	RVA T (%)	Gel strength (%)
1	Désirée (wild type)	100	100	100	100	100	100	100	100
2	asSSI	100	100	113	100	100	114	100	112
3	oeSSI	140	100	118	152	111	45	100	106
4	oeSSI	91	100	87	178	131	55	100	335
5	oeSSI	127	100	100	157	121	63	100	313
6	cosSSI	100	100	106	100	100	100	100	127
7	asSSII	55	118	76	91	95	113	98	151
8	asSSIII	197	123	82	75	76	79	95	84
9	oeSSIII	100		100	100	88	87	100	68
10	cosSSIII	210		100	60	70	74	95	83
11	asBE	170	91	124	94	90	76	100	91

No.	Genotype	Phosphate (%)	Amylose (%)	RVA Max (%)	RVA Min (%)	RVA Fin (%)	RVA Set (%)	RVA T (%)	Gel strength (%)
12	asBE-asSSIII	292		128	69	75	97	95	100
13	asSSII-asSSIII	31	124	30	77	107	229	93	212
14	asSSII-asSSIII	39	110	45	88	113	216	94	189
15	asSSI-asSSIII	115							
15b	asSSI-asSSIII	86	100	82	74	96	168	100	100
16	asSSII-asSSIII-asSSI								
17	asSSI-asSSII-asSSIII	54	115	60	141	105	133	97	105
18	asSSII-asSSI-asSSIII								
19	asBE-asSSIII-oeSSI	370	85	131	55	60	84	93	66
20	asBE-asSSIII-asR1	125	136						
21	oeSSIII-oeSSII	105	100	127	122	126	136	94	189

Key: SSI = starch synthase isoform I; SSII = starch synthase isoform II;  
 SSIII = starch synthase isoform III; BE = branching enzyme;  
 as = antisense; oe = overexpressed (sense); cos = cosuppressed (sense);  
 Rapid Visco Analyzer- (RVA) data: max designates the maximum viscosity;  
 min the minimum viscosity; fin the viscosity at the end of the  
 measurement; set is the difference (D) of min and fin (Setback) and T is  
 the gelatinization temperature. The percentages are based on the wild type  
 (= 100%).

#### Example 13: Characterization of the side chains of the modified starches

The glucan chains were separated after removing the amylose by means of thymol precipitation (Tomlinson et al. loc. cit.) using a high performance anion exchanger chromatography system with an amperometric detector (HPEAC-PAD, Dionex). The samples (10 mg/ml amylopectin) were dissolved in 40%

DMSO and 1/10 part by volume of 100 mM sodium acetate pH 3.5 and 0.4 U of isoamylase (Megazyme) were added. After incubation, 10  $\mu$ l of the sample were applied to the column system and eluted as described by Tomlinson et al. (loc. cit.).

5

The results of the HPEAC-PAD analysis regarding length and distribution of the side chains of the starch samples Nos. 1, 7, 8, 13 and 14 (cf. Tables 1 and 2) are shown in Fig. 2.

10 Another HPLC system for detecting the side-chain distribution consisted of 3 columns connected in series (2 TSK-Gel 2000SW and one TSK-Gel 3000SW, TosoHaas, Stuttgart, Germany) as described by Hizukuri ((1986) Carbohydr. Res. 147:342-347). 100  $\mu$ l of the prepared sample were applied to the column system. The eluent used was 10 mM sodium acetate pH 3.0 at a flow rate of  
15 0.35 ml/min. The glucans were detected by means of a refraction index detector (Gynkotek), and the chain lengths of the eluted linear glucans were determined by mass spectrometry and iodometry (Hizukuri (1986) loc. cit.).

20 The results of the gel-chromatographic HPLC analysis regarding length and distribution of the side chains of starch samples Nos. 1, 7, 8, 13 and 14 (cf. Tables 1 and 2) are shown in Fig. 2.

25 Table 2 shows the percentages of various side-chain fractions of the starches which have been analyzed. Fraction 1 represents the percentage of the A and B1 chains (Hizukuri (1986) loc. cit.), Fraction 2 represents the percentage of the B2, B3 and B4 chains (Hizukuri (1986) loc. cit.) and Fraction 3 shows the percentage of the high-molecular glucan molecules which elute in the elution volume.

30

Table 2: Distribution of the amylopectin side chains of the modified starch

No.	Genotype	Fraction 1 (%)	Fraction 2 (%)	Fraction 3 (%)
1	Désirée (wild type)	58.7	40.3	1.0
7	asSSII	62.6	36.5	0.9
8	asSSIII	72.4	26.3	1.3
13	asSSIIasSSIII	66.9	27.5	5.6
14	asSSIIasSSIII	61.5	35.1	3.4

Comprises/comprising and grammatical variations thereof when used in  
 5 this specification are to be taken to specify the presence of stated features,  
 integers, steps or components or groups thereof, but do not preclude the  
 presence or addition of one or more other features, integers, steps, components  
 or groups thereof.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A process for the preparation of a transgenic plant cell, in which
  - a) at least one nucleotide sequence encoding a protein with the function of a soluble starch synthase III or fragments of said nucleotide sequence and
    - b) one or more nucleotide sequences which encode a protein having the function of a branching enzyme or fragments thereof are integrated into the genome of a plant cell simultaneously or sequentially, wherein said nucleotide sequences inhibit the synthesis of endogenous soluble starch synthase III and endogenous branching enzyme.
2. A transgenic plant cell, comprising
  - a) at least one nucleotide sequence encoding a protein which has the function of a soluble starch syntase III or fragments of said nucleotide sequence and
    - b) one or more nucleotide sequences which encode a protein having the function of a branching enzyme or fragments thereof, wherein said nucleotide sequences inhibit the synthesis of endogenous soluble starch synthase III and endogenous branching enzyme.
3. A recombinant nucleic acid molecule, comprising
  - a) at least one nucleotide sequence encoding a protein which has the function of a soluble starch synthase III or fragments of said nucleotide sequence and
    - b) one or more nucleotide sequences which encode a protein having function of a branching enzyme.
4. A nucleic acid molecule as claimed in claim 3, which is a deoxyribonucleic acid molecule.
5. A nucleic acid molecule as claimed in claim 4, which is a cDNA molecule.

6. A nucleic acid molecule as claimed in claim 3, which is a ribonucleic acid molecule.

7. A vector comprising a nucleic acid molecule as claimed in any one of claims 3-6.

8. A vector comprising a nucleic acid molecule as claimed in any one of claims 3-6, wherein the nucleotide sequence encoding a protein which has the function of a soluble starch synthase or fragments thereof is present in sense or antisense orientation.

9. A vector comprising a nucleic acid molecule as claimed in any one of claims 3-6, wherein the nucleotide sequence encoding one or more proteins having the function of a branching enzyme or fragments thereof is present in sense or antisense orientation.

10. A vector comprising a nucleic acid molecule as claimed in any one of claims 3-6, which is linked to one or more regulatory elements which ensure the transcription and synthesis of an RNA in a eukaryotic cell.

11. The process as claimed in claim 1, in which one or more nucleic acid molecules as claimed in any one of claims 3-6 or one or more vectors as claimed in any one of claims 7-10 are integrated into the genome of a plant cell.

12. The transgenic plant cell as claimed in claim 2, wherein the plant cell comprises one or more nucleic acid molecules as claimed in any one of claims 3-6 or one or more vectors as claimed in any one of claims 7-10.

13. A method of generating a transgenic plant which synthesizes a modified starch, in which an entire plant is regenerated from a plant cell as claimed in any one of claims 2 and 12.

14. A plant comprising one or more cells as claimed in any one of claims 2 and 12.

15. The plant as claimed in claim 14, which is a starch-storing plant.
16. The plant as claimed in claim 15, which is a wheat, corn or rice plant.
17. The plant as claimed in claim 14, which is a potato plant.
18. Propagation material of a plant as claimed in any one of claims 14-17.
19. A process for the preparation of starch, in which starch is obtained from cells as claimed in one or more of claims 2 and 12, plants as claimed in one or more of claims 14-17 or propagation material as claimed in claim 18.
20. A starch obtained from a cell as claimed in any one of claims 2 and 12, a plant as claimed in any one of claims 14-17 or from propagation material as claimed in claim 18.
21. The starch as claimed in claim 20, which has in comparison with a starch obtained from an untransformed cell or plant, a phosphate content which is increased by at least 30%.
22. The use of a starch as claimed in claims 20 or 21 in industry, preferably for the production of foodstuffs, packaging materials or disposable articles.
23. The use of cells as claimed in one or more of claims 2 and 12, plants as claimed in one or more of claims 14-17 or propagation material as claimed in claim 18 for the production of starch.

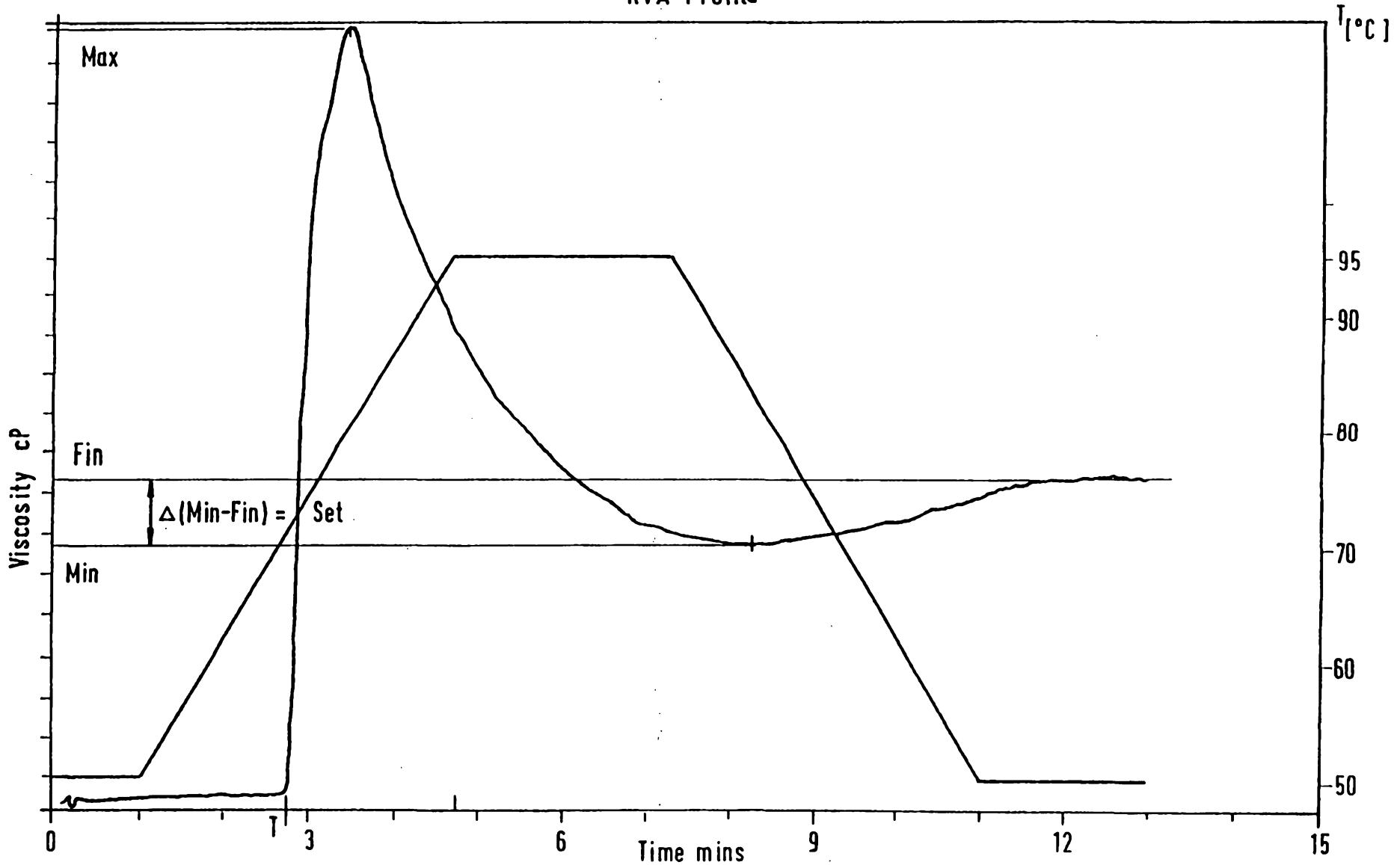
DATED this 18th day of February 2004

**BAYER CROPSCIENCE GMBH**

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FIG.1

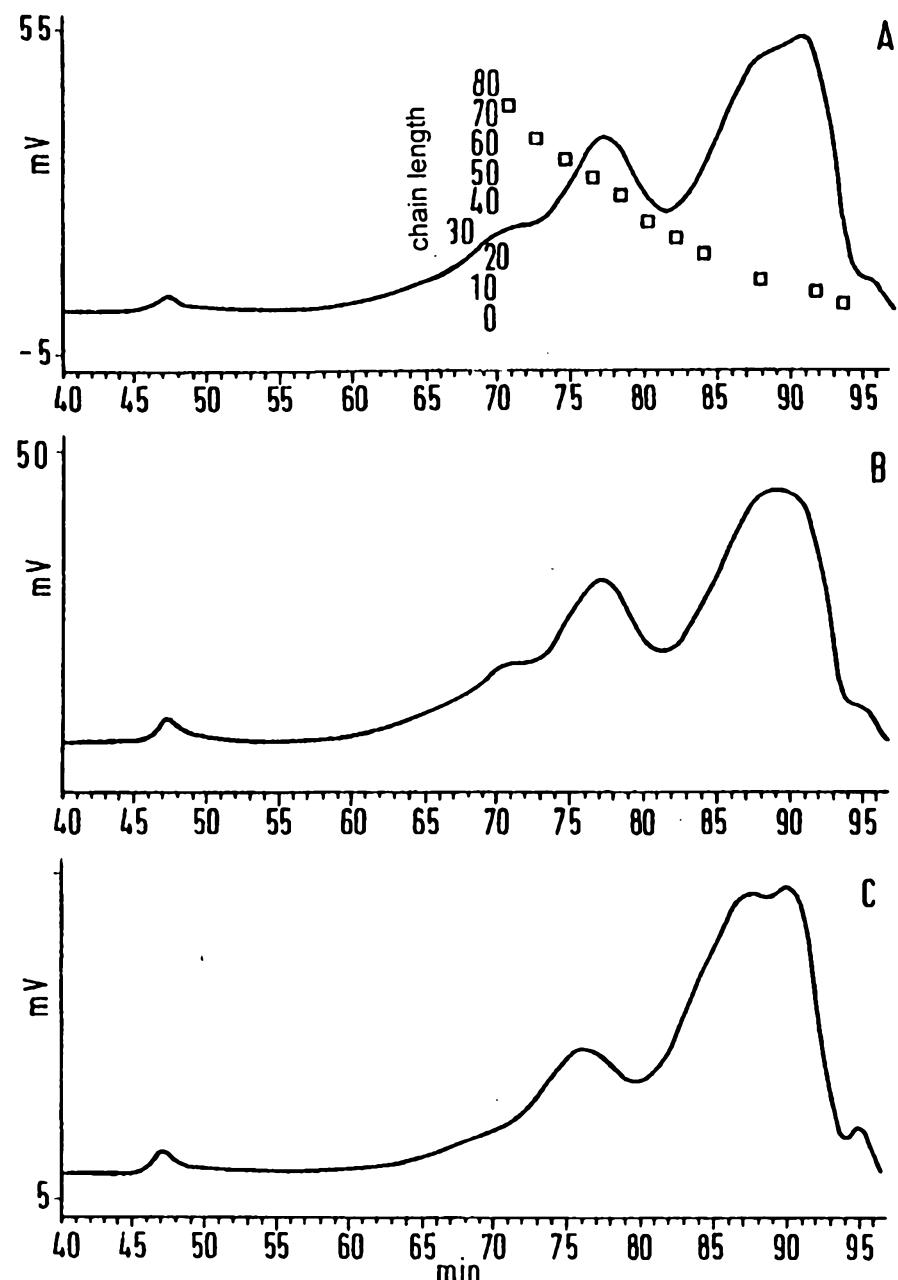
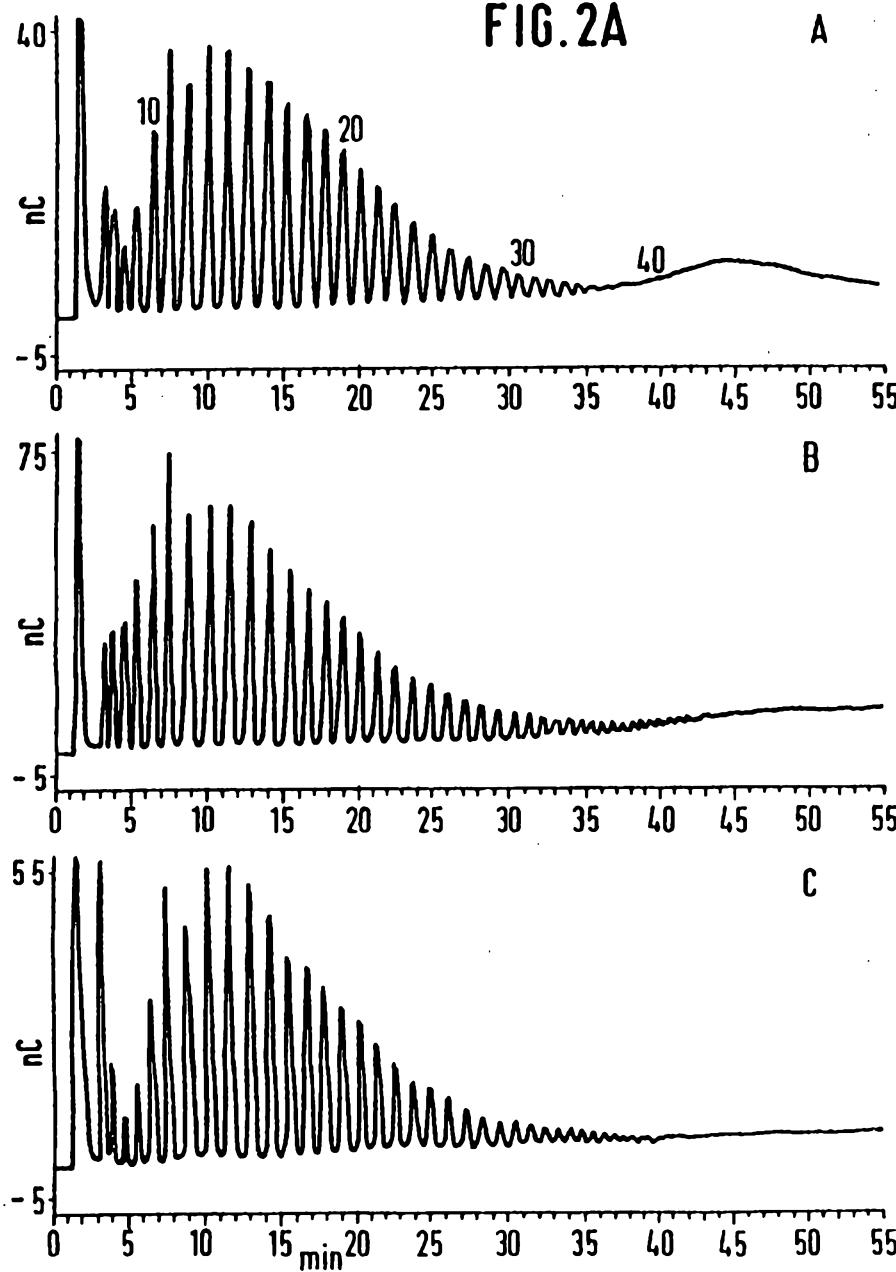
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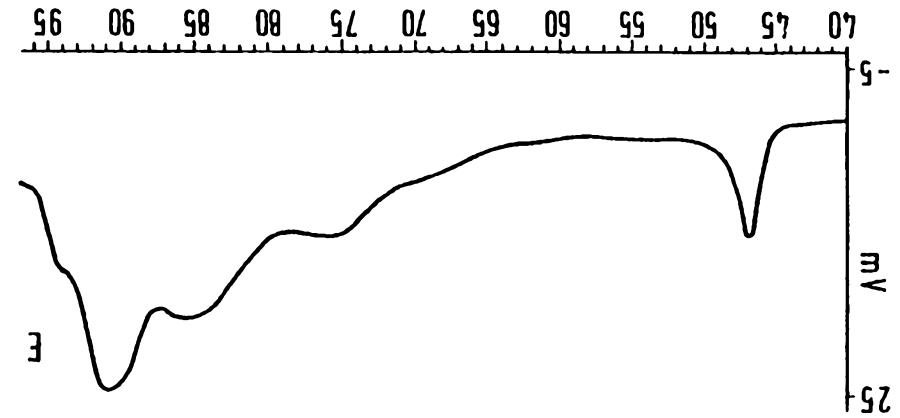
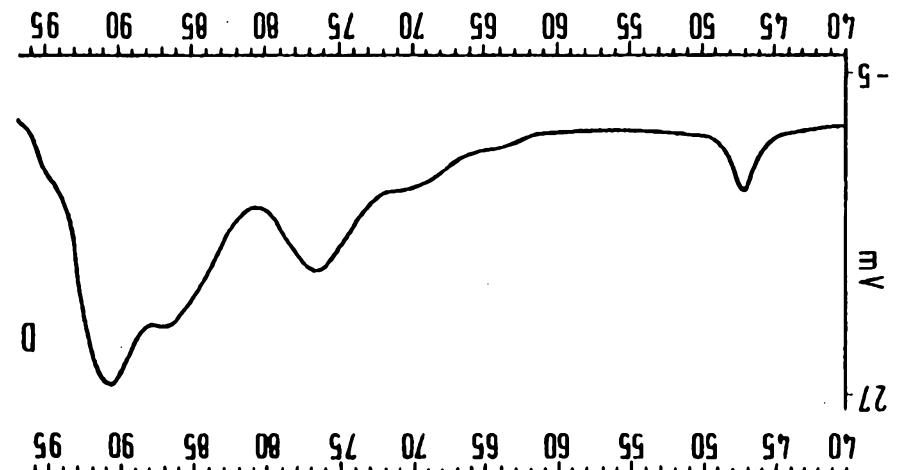
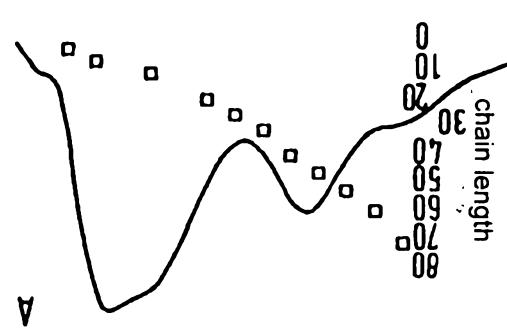


FIG. 2B

