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(54)【発明の名称】修飾型デンプンを合成する単子葉植物の細胞および植物体

(57)【要約】
本発明は、遺伝的修飾が、Ｒ１タンパク質の生物学的活性を有するタンパク質をコードする外因性核酸分子の導入を含む、遺伝的に修飾された単子葉植物の細胞および植物体に関する。本発明はさらに、これらを生産する手段および方法に関する。この種の植物細胞および植物体は、対応する遺伝的に修飾されていない単子葉植物体のデンプンと比較して、リン酸含量が高いこと、および／またはリン酸化パターンが修飾されていること、および／またはＲＶＡプロファイルにおける最終粘性が高いこと、および／またはＤＳＣ解析におけるピーク温度が低いこと、および／またはテクスチャー解析におけるゲル強度が高いことを特徴とする修飾型デンプンを合成する。したがって本発明は、本発明の植物細胞および植物体から合成されるデンプン、ならびに該デンプンを生産する方法にも関する。本発明はさらに、このような修飾型デンプンを含む小麦粉、ならびにこのような小麦粉および／またはデンプンを含む食品およびパン製品に関する。

【特許請求の範囲】
【請求項１】
遺伝的修飾が少なくとも１種の外因性核酸分子の導入を含み、外因性核酸分子が以下からなる群より選択される、遺伝的に修飾された単子葉植物細胞：
（ａ）配列番号：１に記載されたヌクレオチド配列のコード領域を含む核酸分子；（ｂ）配列番号：２に記載されたアミノ酸配列を有するジャガイモ（Ｓｏｌａｎｕｍ ｔｕｂｅｒｏｓｕｍ）のＲ１タンパク質をコードする核酸分子；
（ｃ）配列番号：１に記載されたヌクレオチド配列の誘導体を構成する核酸分子；および（ｄ）（ａ）、（ｂ）、または（ｃ）に記載された核酸分子の断片を構成する核酸分子。
【請求項２】
対応する遺伝的に修飾されていない野生型植物細胞と比較して、Ｒ１タンパク質の高い生物学的活性を示す、請求項１記載の植物細胞。
【請求項３】
対応する遺伝的に修飾されていない野生型植物細胞のデンプンと比較して、グルコース単量体のＣ６位におけるリン酸含量が高いデンプンを合成する、請求項１または２のいずれか一項記載の植物細胞。
【請求項４】
対応する遺伝的に修飾されていない野生型植物細胞のデンプンと比較して、最終粘性が高いデンプンおよび／またはピーク温度が低いデンプンを合成する、請求項１～３のいずれか一項記載の植物細胞。
【請求項５】
膨着後に、対応する遺伝的に修飾されていない野生型植物細胞のデンプンのゲルと比較して、ゲル強度が高いゲルを形成するデンプンを合成する、請求項１～４のいずれか一項記載の植物細胞。
【請求項６】
コムギ、イネ、オオムギ、オーツムギ、ミレット、ライムギ、およびトウモロコシからなる群より選択される植物体に起源を有する、請求項１～５のいずれか一項記載の植物細胞。
【請求項７】
コムギ植物体に起源を有する、請求項１～５のいずれか一項記載の植物細胞。
【請求項８】
グルコース単量体のＣ６位におけるリン酸含量が少なくとも０．１ ｎｍｏｌ Ｃ６ Ｐ mg^{-1} デンプンであるデンプンを合成する、請求項７記載の植物細胞。
【請求項９】
単子葉植物の細胞が遺伝的に修飾され、また遺伝的修飾が少なくとも１種の外因性核酸分子の導入を含む、請求項１～８のいずれか一項記載の植物細胞を生産する方法。
【請求項１０】
請求項１～８のいずれか一項記載の植物細胞を含む植物体。
【請求項１１】
デンプン貯蔵器官において、対応する遺伝的に修飾されていない野生型植物体のデンプン貯蔵器官に由来するデンプンと比較して、グルコース単量体のＣ６位におけるリン酸含量が高いデンプンが合成される、請求項１０記載の植物体。
【請求項１２】
イネ、コムギ、またはトウモロコシの植物体である、請求項１０～１１のいずれか一項記載の植物体。
【請求項１３】
（ａ）単子葉植物の細胞が遺伝的に修飾され（遺伝的修飾は少なくとも１種の外来製核酸分子の導入を含む）；
（ｂ）段階（ａ）の細胞から植物体が再生され、また任意選択で
（ｃ）段階（ｂ）で作製された植物体からさらに植物体が生産される、

請求項１０～１２のいずれか一項記載の植物体を生産する方法。
【請求項１４】
外因性核酸分子が、デンプン貯蔵組織におけるＲ１遺伝子の発現を器官特異的に促進するプロモーターの制御下にある、請求項１３記載の方法。
【請求項１５】
コムギ植物体に関する、請求項１３～１４のいずれか一項記載の方法。
【請求項１６】
請求項１～８のいずれか一項記載の植物細胞を含む、請求項１０～１２のいずれか一項記載の植物体の増殖材料。
【請求項１７】
請求項１０～１２のいずれか一項記載の単子葉植物体、または請求項１～８のいずれか一項記載の単子葉植物細胞の作製を目的とする、請求項１記載の核酸分子の使用。
【請求項１８】
請求項１～８のいずれか一項記載の植物細胞、または請求項１０～１２のいずれか一項記載の植物体から得られるデンプン。
【請求項１９】
対応する野生型植物体のデンプンと比較して、グルコース単量体のＣ６位におけるリン酸含量が高いこと、および／または最終粘性が高いこと、および／またはピーク温度が低いことを特徴とする、請求項１８記載のデンプン。
【請求項２０】
対応する野生型植物体のデンプンと比較して、グルコース単量体のＣ６位におけるリン酸含量が高いこと、および／または最終粘性が高いこと、および／またはピーク温度が低いことを特徴とするデンプン。
【請求項２１】
グルコース単量体のＣ６位におけるリン酸含量が少なくとも０．１ｎｍｏｌ　Ｃ６　Ｐ　ｍｇ$^{-1}$　デンプンであることを特徴とするデンプン。
【請求項２２】
最終粘性が少なくとも５０％高いこと、および／または少なくとも１．５℃低いピーク温度を有することを特徴とする、請求項２１記載のデンプン。
【請求項２３】
膨着後にデンプンが、グルコース単量体のＣ６位におけるリン酸含量が同じである対応する化学的にリン酸化されたデンプンのゲルと比較して、および／または対応する遺伝的に修飾されていない植物体のデンプンのゲルと比較して、高いゲル強度を示すゲルを形成することを特徴とする、請求項１８～２２のいずれか一項記載のデンプン。
【請求項２４】
デンプンのアミロース成分の総リン酸含量が、化学的にリン酸化されたデンプンのアミロース成分と比較して低いことを特徴とする、請求項１８～２３のいずれか一項記載のデンプン。
【請求項２５】
化学的にリン酸化されたデンプンと比較して、修飾されたリン酸化パターンを示すことを特徴とする、請求項１８～２４のいずれか一項記載のデンプン。
【請求項２６】
コムギのデンプンであることを特徴とする、請求項１８～２５のいずれか一項記載のデンプン。
【請求項２７】
請求項１０～１２のいずれか一項記載の植物体からのデンプンの抽出を含む、請求項１８～２６のいずれか一項記載のデンプンを生産する方法。
【請求項２８】
工業分野において、好ましくは食料品の製造を目的とする、請求項１８～２６のいずれか一項記載のデンプンの使用。

【請求項２９】
請求項１８～２６のいずれか一項記載のデンプンを含む小麦粉。
【請求項３０】
請求項１～８のいずれか一項記載の植物細胞から、または請求項１０～１２のいずれか一項記載の植物体から、または請求項１６記載の増殖材料から得られる小麦粉。
【請求項３１】
ベーキングミクスチャーの作製、および／または食品の生産を目的とする、請求項２９もしくは３０記載の小麦粉、または請求項１８～２６のいずれか一項記載のデンプンの使用。
【請求項３２】
請求項２９もしくは３０記載の小麦粉、または請求項１８～２６のいずれか一項記載のデンプンを含むベーキングミクスチャー
【請求項３３】
請求項２９もしくは３０記載の小麦粉を用いて、および／または請求項３２記載のベーキングミクスチャーを用いて、および／または請求項１８～２６のいずれか一項記載のデンプンを用いて生産された食品。
【発明の詳細な説明】
【０００１】
本発明は、遺伝的修飾が、Ｒ１タンパク質の生物学的活性を有するタンパク質をコードする外因性核酸分子の導入を含む、遺伝的に修飾された単子葉植物の細胞および植物体に関する。本発明はさらに、これらを作製する手段および方法に関する。この種の植物細胞および植物体は、対応する遺伝的に修飾されていない単子葉植物に由来するデンプンと比較して、リン酸含量が高いこと、および／またはリン酸化パターンが修飾されていること、および／またはＲＶＡプロファイルにおける最終粘性が高いこと、および／またはＤＳＣ解析におけるピーク温度が低いこと、および／またはテクスチャー解析におけるゲル強度が高いことを特徴とする修飾型デンプンを合成する。したがって本発明は、本発明の植物細胞および植物体から合成されるデンプンに関し、またこのようなデンプンを作製する方法にも関する。本発明はさらに、このような修飾型デンプンを含む小麦粉に関し、またこのような小麦粉および／またはデンプンを含む食品およびパン製品に関する。
【０００２】
原材料の再生可能な供給源として植物内容物に最近付け加えられた高い重要性に関して、生物工学的研究の課題の一つは、このような植物原材料の、これらを加工する業界の求める要件への適合を試みることである。できるだけ多くの使用分野において、再生可能な原材料の使用を促進するためには、極めて多様な物質を提供することも必要である。
【０００３】
油分、脂肪、およびタンパク質に加えて、多糖類は、植物に由来する重要な再生可能原材料を構成する。セルロースに加えて、高等植物の極めて重要な貯蔵材料の１種であるデンプンは、多糖類の中心的存在と考えられている。
【０００４】
多糖であるデンプンは、化学的に均一な基本成分、すなわちグルコース分子の重合体である。しかしデンプンは、重合およびグルコース鎖における分枝領域の出現の程度が異なる、さまざまな形状の分子の極めて複雑な混合物である。したがってデンプンは均一な原材料を含まない。区別は、デンプンの２種の化学的に異なる成分（アミロースおよびアミロペクチン）間においてなされる。トウモロコシ、コムギ、ジャガイモなどの、デンプン生産に使用される典型的な植物体では、合成されたデンプンは、最大約２０～３０％のアミロースデンプン、および最大約７０～８０％のアミロペクチンデンプンを含む。
【０００５】
アミロースは、α－１，４－グリコシド結合で結合されたα－Ｄ－グルコース単量体を含む直鎖状の重合体であると長く考えられてきた。しかし最近の研究で、α－１，６－グリコシド結合分岐部位の存在率が約０．１％であることが明らかにされた（Ｈｉｚｕｋｕｒ

ｉおよびＴａｋａｇｉ、Ｃａｒｂｏｈｙｄｒ．Ｒｅｓ．１３４．（１９８４）、１～１０；Ｔａｋｅｄａら、Ｃａｒｂｏｈｙｄｒ．Ｒｅｓ．１３２、（１９８４）、８３～９２）。しかし基本的には、アミロースとアミロペクチンを完全に分離することは極めて困難であり、このためアミロースの質は、選択される分離法の種類によって大きく変わる。アミロースとは対照的に、アミロペクチンは、より強く枝分かれしており、付加的なα－１，６－グリコシド結合によって形成される分岐部位の約４％を含む。アミロペクチンは、さまざまな分岐状のグルコース鎖の複合混合物を含む。

【０００６】

以上２種の分子間におけるさらに重要な差が分子量である。デンプンの起源に依存するアミロースの分子量が5×10^{5}～10^{6} Ｄａであるのに対し、アミロペクチンの分子量は10^{7}～10^{8} Ｄａである。これら２種の巨大分子は、その分子量により、また異なるヨウ素結合特性の差によって最も容易に明らかとなる物理化学的性質の差により区別することができる。

【０００７】

アミロースとアミロペクチンのさらに重要な差は、これらの巨大分子と結合して存在する場合がある微量物質の相対量である。アミロースは、疎水性分子に対して親和性が高い。特に穀物類では、アミロースは、比較的多量の脂質と複合体を形成する場合がある（Ｍｏｒｒｉｓｏｎ、Ｃｅｒｅａｌ Ｆｏｏｄｓ Ｗｏｒｌｄ ４０、（１９９５）、４３７～４４６）。一方、アミロペクチンは、共有結合で結合された無機リン酸を、デンプンのリン酸モノエステルの状態で含む場合がある（この知見は、これまでアミロースについては報告されていない）。高量のリン酸モノエステルは、塊茎から得られるデンプン中に特に見出される。市販のデンプンのなかでは、ジャガイモのデンプンのリン酸含量が最も高く、その範囲はデンプン１ｍｇあたり１０～３０ ｎｍｏｌである。ある種のウコン（Ｃｕｒｃｕｍａ）では、リン酸含量は２～４倍高くなることがある（Ｂａｙ－Ｓｍｉｄｔら、第５回ＩＳＰＭＰ会議予稿集、（１９９７）、７４１）一方で、穀物類では約１００倍少ない（ＫａｓｅｍｓｕｗａｎおよびＪａｎｅ、Ｃｅｒｅａｌ Ｃｈｅｍ．７３、（１９９６）、７０２～７０７）。塊茎、根およびマメ科植物に由来するデンプン対して、穀物類（単子葉植物）のデンプンに含まれる検出可能なリン酸は、デンプンモノエステル誘導体の状態ではほとんどみられず、主にリン脂質の状態で存在する（Ｊａｎｅら、Ｃｅｒｅａｌ Ｆｏｏｄｓ Ｗｏｒｌｄ ４１、（１９９６）、８２７～８３２）。

【０００８】

アミロース／アミロペクチン比、およびリン酸含量とは異なり、デンプンの機能的特性は、分子量、側鎖分布のパターン、イオン含量、脂質およびタンパク質の含量などの影響を受ける。本明細書で言及すべき重要な機能的特性は、可溶性、老化挙動、吸水能、造膜特性、粘性、膠着特性、凍結融解安定性、酸に対する安定性、ゲル強度などである。デンプン粒の大きさも、さまざまな応用上、重要な場合がある。

【０００９】

原理的には、リン酸含量は、遺伝子工学的なアプローチにより、または天然デンプンの化学的なリン酸化により修飾することができる（例えば、Ｓｔａｒｃｈ Ｃｈｅｍｉｓｔｒｙ ａｎｄ Ｔｅｃｈｎｏｌｏｇｙ、Ｒ．Ｌ．Ｗｈｉｓｔｌｅｒ、Ｊ．Ｎ．ＢｅＭｉｌｌｅｒ、およびＥ．Ｆ．Ｐａｓｃｈａｌｌ編、Ａｃａｄｅｍｉｃ Ｐｒｅｓｓ、Ｎｅｗ Ｙｏｒｋ、１９８８、３４９～３６４を参照）。しかし、化学的な修飾は一般に費用および時間がかかり、物理化学的特性がインビボで修飾されたデンプンの特性とは異なるデンプンが生じる。

【００１０】

野生型の単子葉植物に由来するデンプン、特に穀物類の植物（コムギ、イネ、トウモロコシ、オーツムギ、ミレット、ライムギ）のデンプンは、デンプンのリン酸モノエステルの状態で極めて少量のリン酸しか含まないので（Ｌｉｍら、Ｃｅｒｅａｌ Ｃｈｅｍ．７１、（１９９４）、４８８）、本発明の一つの目的は、対応する野生型の植物細胞および植物体と比較して、高いリン酸含量（デンプンのリン酸モノエステルの量）、および修飾さ

れた物理化学的特性を有するデンプンを合成する単子葉植物を提供することにある。
【００１１】
したがって本発明の基本的な目的は、対応する遺伝的に修飾されていない野生型の植物細胞および植物体と比較して、修飾された構造および／または機能的特性を有するデンプンを合成する遺伝的に修飾された単子葉植物の細胞および植物体を提供すること、および構造および／または機能の特性が、対応する遺伝的に修飾されていない野生型の植物細胞および植物体のデンプンの特性、および化学的に修飾されたデンプンの特性とは異なることから一般的および／または特別の工業的な使用目的により適しているデンプンを提供することである。
【００１２】
以上の目的は、特許請求の範囲に記載された態様を提供することで達成される。というのは驚くべきことに、外因性核酸分子の単子葉植物の細胞および植物体のゲノムへの導入が、このような単子葉植物の細胞および植物体で合成されるデンプンの構造および／または機能の特性に修飾を生じることがわかっているからである。
【００１３】
外因性核酸分子の発現は主に、単子葉植物、特にコムギ植物体のデンプン貯蔵器官に有利であり、対応する遺伝的に修飾されていない野生型の植物体、特にコムギ植物体のデンプン貯蔵器官から単離可能なデンプンと比較して、デンプン貯蔵器官から単離可能なデンプンのリン酸含量および粘性特性の修飾を高める。また本発明のデンプンは、修飾されたリン酸化パターンおよび修飾された粘性特性によって、化学的にリン酸化されたデンプンと区別され、またデンプンの膨着反応およびゲル形成の後では、修飾されたゲル強度によっても区別される。
【００１４】
したがって本発明は、遺伝的修飾が、少なくとも１種の外因性核酸分子の導入を含み、このような外因性核酸分子が以下からなる群より選択される、遺伝的に修飾された単子葉植物の細胞に関する：
ａ）配列番号：１に記載されたヌクレオチド配列のコード領域を含む核酸分子；
ｂ）本明細書２に記載されたアミノ酸配列を有するジャガイモ（Ｓｏｌａｎｕｍ ｔｕｂｅｒｏｓｕｍ）のＲ１タンパク質をコードする核酸分子；
ｃ）配列番号：１に記載されたヌクレオチド配列の誘導体を含む核酸分子；および
ｄ）（ａ）、（ｂ）、または（ｃ）記載の核酸分子の断片を含む核酸分子。
【００１５】
本発明では、「遺伝的に修飾された」という表現は、植物細胞の遺伝情報が、外因性核酸分子の導入によって修飾されること、また外因性核酸分子の存在または発現が表現型の修飾をもたらすことを意味する。「表現型の修飾」という用語は、好ましくは、細胞の一つまたは複数の機能の測定可能な修飾を意味する。例えば、本発明の遺伝的に修飾された植物細胞は、修飾された発現パターンを示す。本発明では、「遺伝的に修飾された」という表現は、本発明の単子葉植物の細胞が、ゲノムに安定に組み込まれた少なくとも１個の外因性核酸分子を含むことを意味する。
【００１６】
本発明では、「外因性核酸分子」という用語は、対応する遺伝的に修飾されていない野生型植物細胞に天然には存在しないＲ１タンパク質、好ましくはジャガイモのＲ１タンパク質の生物学的活性を有するタンパク質をコードする核酸分子を意味すると理解される。外因性核酸分子は好ましくは、植物細胞に天然にみられない異なる配列、組み合わせ、または特異的な空間配置を含む組換え体分子である。本発明の単子葉植物の細胞は少なくとも１種の外因性核酸分子を含む（後者は好ましくは、植物細胞における転写を確実なものとする調節性ＤＮＡ配列（特にプロモーター）に連結される）。
【００１７】
ジャガイモのＲ１タンパク質をコードする外因性核酸分子の例を配列番号：１に示す。ジャガイモのＲ１タンパク質をコードするヌクレオチド配列は、国際公開公報第９７／１１

１８８Ａ１号、およびローバース（Ｌｏｒｂｅｒｔｈ）らの論文（Ｎａｔｕｒｅ Ｂｉｏｔｅｃｈ．１６．（１９９８）、４７３～４７７）にも記載されている。

【００１８】

ジャガイモのＲ１タンパク質の重要な特徴は、（ｉ）アミノ酸配列（例えば配列番号：２を参照）；（ｉｉ）植物細胞の色素体のストロマにおける位置（デンプン粒に結合した状態、また可溶性の状態の両方で存在する場合がある）；ならびに（ｉｉｉ）植物体に含まれるデンプンのリン酸化の程度に影響を及ぼす能力。例えば、トランスジェニックジャガイモ植物体のジャガイモのＲ１タンパク質をコードするＲ１遺伝子が抑制されると、ジャガイモの塊茎から単離可能なデンプンのリン酸含量が低下する。またローバースらは、ジャガイモのＲ１タンパク質が、対応するＲ１ ｃＤＮＡを大腸菌で発現させた場合に細菌由来のグリコーゲンをリン酸化する能力をもつことを報告している（Ｌｏｒｂｅｒｔｈら、Ｎａｔｕｒｅ Ｂｉｏｔｅｃｈ．１６、（１９９８）、４７３～４７７）。

【００１９】

リッテ（Ｒｉｔｔｅ）ら（Ｐｌａｎｔ Ｊ．２１、（２０００）、３８７～３９１）は、ジャガイモのＲ１タンパク質が、ジャガイモの植物体に含まれるデンプン粒に可逆的に結合することを報告している（デンプン粒に対する結合強度は植物体の代謝状態によって変わる）。デンプン粒に結合した状態では、ジャガイモ植物体に含まれるタンパク質は、暗条件で維持される葉に主に存在する。しかし、葉に光を照射すると、このタンパク質は、デンプン粒に結合していない可溶性状態で主に存在する。また、トランスジェニックジャガイモ植物体のジャガイモのＲ１遺伝子の発現を抑制すると、「デンプンが過剰な」表現型となる。すなわち、対応する植物体の葉に含まれるデンプン量が多くなる（Ｌｏｒｂｅｒｔｈら、Ｎａｔｕｒｅ Ｂｉｏｔｅｃｈ．１６、（１９９８）、４７３～４７７）。また、このようなジャガイモ植物体の塊茎は、冷所保存後に「冷却による甘味発生」の低下を示す点で区別される（Ｌｏｒｂｅｒｔｈら、Ｎａｔｕｒｅ Ｂｉｏｔｅｃｈ．１６、（１９９８）、４７３～４７７）。

【００２０】

原理的には、Ｒ１タンパク質をコードする外因性核酸分子は、任意の種類のジャガイモまたはジャガイモ植物体、好ましくは、トメンサ（Ｔｏｍｅｎｓａ）、デジレ（Ｄｅｓｉｒｅｅ）、テンポラ（Ｔｅｍｐｏｒａ）、およびソマナ（Ｔｈｏｍａｎａ）の各品種のジャガイモに起源を有する場合がある。

【００２１】

一つの好ましい態様では、このような外因性核酸分子は、配列番号：１に記載されたヌクレオチド配列を有する。

【００２２】

さらに好ましい態様では、外因性核酸分子は、配列番号：１に記載されたヌクレオチド配列を有する

【００２３】

本発明のさらに好ましい態様では、外因性核酸分子は、配列番号：２に記載されたアミノ酸配列を有する、ジャガイモ（Ｓｏｌａｎｕｍ ｔｕｂｅｒｓｕｍ）に由来するＲ１タンパク質をコードする。

【００２４】

本発明のさらに別の態様では、外因性核酸分子は、成熟型（色素体シグナルペプチドを含まない）タンパク質のコード領域（配列番号：１に記載されたヌクレオチド配列のｂｐ４４７～ｂｐ ４６０７）を含む。ジャガイモのＲ１タンパク質の色素体のＮ末端シグナルペプチド（配列番号：１に記載されたヌクレオチド配列のヌクレオチド２１６～４４６にコードされたアミノ酸１～７７）の代わりに、本発明のこの態様では、外因性核酸分子は、異種の色素体シグナル配列、すなわち、成熟型Ｒ１タンパク質と関連する、天然には存在しないシグナル配列を含む。色素体のシグナル配列は当業者に周知である。

【００２５】

例えばホウレンソウのフェレドキシン：ＮＡＤＰ$^{+}$酸化還元酵素（ＦＮＲ）のようなシグ

ナル配列は、色素体シグナル配列として使用されることがある。この配列は、ホウレンソウの色素体タンパク質フェレドキシン：ＮＡＤＰ$^{+}$酸化還元酵素のｃＤＮＡの５’側の非翻訳領域、ならびに隣接する輸送ペプチド配列を含む（ヌクレオチド －１７１～＋１６５；Ｊａｎｓｅｎら、Ｃｕｒｒｅｎｔ Ｇｅｎｅｔｉｃｓ １３、（１９８８）、５１７～５２２）。

【００２６】

また例えば、トウモロコシのｗａｘｙタンパク質の輸送ペプチド＋成熟型ｗａｘｙタンパク質の先頭の３４アミノ酸がシグナル配列として使用されることがある（Ｋｌｏｓｇｅｎら、Ｍｏｌ．Ｇｅｎ．Ｇｅｎｅｔ． ２１７、（１９８９）、１５５～１６１）。また、トウモロコシのｗａｘｙタンパク質の輸送ペプチドは、成熟型ｗａｘｙタンパク質の先頭の３４アミノ酸を必要とすることなく使用される場合がある。

【００２７】

本発明のさらに好ましい態様では、外因性核酸分子は、配列番号：１に記載されたヌクレオチド配列の誘導体を含む。

【００２８】

本発明では、「誘導体」という用語は、配列番号：１に記載されたヌクレオチド配列とは１個または複数の位置が異なり、配列番号：１に記載されたヌクレオチド配列のコード領域と高度の相同性を示す分子の配列を意味する。また誘導体は、それらがＲ１タンパク質、好ましくはジャガイモのＲ１タンパク質の生物学的特性を有するタンパク質をコードする点において区別される。本発明では、「相同性」とは、ヌクレオチド配列の、少なくとも６５％、特に少なくとも８５％、より詳細には少なくとも９０％の同一性、好ましくは９５％以上、また最も好ましくは９８％以上の配列の同一性を意味する。上述の核酸分子との差は、欠失、付加、置換、挿入、または組換えによって生じる場合がある。

【００２９】

相同性の程度は好ましくは、個々のヌクレオチド配列を、配列番号：１のコード領域、特に配列番号：１に記載されたヌクレオチド配列の成熟型タンパク質をコードする配列番号：１の領域（ｂｐ４４７～ｂｐ４６０７）と比較することで決定される。比較対象の配列が異なる長さをもつ場合は、相同性の程度は、好ましくは、より長い配列のヌクレオチドと同一な短いヌクレオチド配列中においてこのようなヌクレオチドが存在するパーセンテージで表される（すなわち配列の同一性は、個々のヌクレオチド配列が重複する領域に関して決定される）。配列の比較は、トンプソン（Ｊｕｌｉｅ Ｔｈｏｍｐｓｏｎ）（Ｔｈｏｍｆｉｓｏｎ@ＥＭＢＬ－Ｈｅｉｄｅｌｂｅｒｇ．ＤＥ）、およびギブソン（Ｔｏｂｙ Ｇｉｂｓｏｎ）（Ｇｉｂｓｏｎ@ＥＭＢＬ－Ｈｅｉｄｅｌｂｅｒｇ．ＤＥ；Ｅｕｒｏｐｅａｎ Ｍｏｌｅｃｕｌａｒ Ｂｉｏｌｏｇｙ Ｌａｂｏｒａｔｏｒｙ、Ｍｅｙｅｒｈｏｆｓｔｒａｓｓｅ １、Ｄ６９１１７ Ｈｅｉｄｅｌｂｅｒｇ、Ｇｅｒｍａｎｙ）から配布されているＣｌｕｓｔａｌ Ｗプログラム（Ｔｈｏｍｐｓｏｎら、Ｎｕｃｌｅｉｃ Ａｃｉｄ Ｒｅｓｅａｒｃｈ ２２、（１９９４）、４６７３～４６８０）などの既知のコンピュータプログラムを用いて行うことができる。Ｃｌｕｓｔａｌ Ｗは、さまざまなインターネットサイト（例えばＩＧＢＭＣ（Ｉｎｓｔｉｔｕｔ ｄｅ Ｇｅｎｅｔｉｑｕｅ ｅｔ ｄｅ Ｂｉｏｌｏｇｉｅ Ｍｏｌｅｃｕｌａｉｒｅ ｅｔ Ｃｅｌｌｕｌａｉｒｅ、Ｂ．Ｐ．１６３、６７４０４ Ｉｌｌｋｉｒｃｈ Ｃｅｄｅｘ、Ｆｒａｎｃｅ；ｆｔｐ：／／ｆｔｐ－ｉｇｂｍｃ．ｕ－ｓｔｒａｓｂｇ．ｆｒ／ｐｕｂ／）のサイト、およびＥＢＩ（Ｅｕｒｏｐｅａｎ Ｂｉｏｉｎｆｏｒｍａｔｉｃｓ Ｉｎｓｔｉｔｕｔｅ）のサイト（ｆｔｐ：／／ｆｔｐ．ａｃ．ｕｋ／ｐｕｂ／ｓｏｆｔｗａｒｅ／）、ならびにＥＢＩ（Ｅｕｒｏｐｅａｎ Ｂｉｏｉｎｆｏｒｍａｔｉｃｓ Ｉｎｓｔｉｔｕｔｅ、Ｗｅｌｌｃｏｍｅ Ｔｒｕｓｔ Ｇｅｎｏｍｅ Ｃａｍｐｕｓ、Ｈｉｎｘｔｏｎ、Ｃａｍｂｒｉｄｇｅ ＣＢ１０ １ＳＤ、ＵＫ）にリンクされているインターネットサイト）からダウンロードすることもできる。

【００３０】

Ｃｌｕｓｔａｌ Ｗプログラム（バージョン１．８）を使用する際には、さまざまなパラメータのデフォルト値を使用する。ＤＮＡ配列を比較する場合は、このようなパラメータ

は以下の値をとる：ＫＴＵＰＬＥ＝２、ＴＯＰＤＩＡＧＳ＝４、ＰＡＩＲＧＡＰ＝５、ＤＮＡＭＡＴＲＩＸ：ＩＵＢ、ＧＡＰＯＰＥＮ＝１０、ＧＡＰＥＸＴ＝５、ＭＡＸＤＩＶ＝４０、ＴＲＡＮＳＩＴＩＯＮＳ：非荷重。

【００３１】

タンパク質配列を比較する場合は、Ｃｌｕｓｔａｌ Ｗプログラムのパラメータのデフォルト値を同様に使用する。このようなパラメータは以下の値をとる：ＫＴＵＰＬＥ＝１、ＴＯＰＤＩＡＧ＝５、ＷＩＮＤＯＷ＝５、ＰＡＩＲＧＡＰ＝３、ＧＡＰＯＰＥＮ＝１０、ＧＡＰＥＸＴＥＮＤ＝０．０５、ＧＡＰＤＩＳＴ＝８、ＭＡＸＤＩＶ＝４０、ＭＡＴＲＩＸ＝ＧＯＮＮＥＴ、ＥＮＤＧＡＰＳ（ＯＦＦ）、ＮＯＰＧＡＰ、ＮＯＨＧＡＰ。

【００３２】

相同性の程度は、例えばＭｖｉｅｗ（ｈｔｔｐ：／／ｗｗｗ．ｓａｃｅｅｕｃｓｆ．ｅｄｕ／ｄｏｃｕｍｅｎｔａｔｉｏｎ／ｓｅｑｓｏｆｔｗａｒｅ／ｍｖｉｅｗ；Ｂｒｏｗｎ、Ｎ．Ｐ．、Ｌｅｒｏｙ Ｃ．、Ｓａｎｄｅｒ Ｃ．（１９９８）、ＭＶｉｅｗ：ａ ｗｅｂ－ｃｏｍｐａｔｉｂｌｅ ｄａｔａｂａｓｅ ｓｅａｒｃｈ ｏｒ ｍｕｌｔｉｐｌｅ ａｌｉｇｎｍｅｎｔ ｖｉｅｗｅｒ、Ｂｉｏｉｎｆｏｒｍａｔｉｃｓ、１４（４）：３８０～３８１）などの既知のコンピュータプログラムを用いて決定することができる。

【００３３】

本発明では、「相同性」は、機能および／または構造が個々の核酸分子間、または核酸分子がコードするタンパク質間で等価であることも意味する。配列番号：１に記載された核酸分子に相同な核酸分子、およびこれらの分子の誘導体を含む核酸分子は、同じ生物学的機能を果たす修飾、およびインビボにおいて単子葉植物に含まれるデンプンのリン酸含量を上昇させる能力を含む、このような分子の変形例である。これらは、天然の変形例、例えば他の種類のジャガイモに由来する配列、または変異である場合がある（このような変異は天然に形成される場合があるほか、標的変異誘発で導入される場合がある）。

【００３４】

本発明のさらに別のの態様では、配列番号：１の誘導体は、配列番号：１に記載された核酸分子の対立遺伝子変異体を含む。

【００３５】

このような対立遺伝子変異体は、デンプンのリン酸含量を上昇可能な天然の変異体である。

【００３６】

本発明の他の態様では、外因性核酸分子は、本発明で定義される外因性核酸分子の断片を含む。

【００３７】

本発明では、「断片」という用語は、Ｒ１タンパク質の生物学的に活性のある部分、好ましくは酵素活性をもつ部分をコードする外因性核酸分子の一部を意味する。

【００３８】

Ｒ１タンパク質の生物学的に活性のある部分は、植物では、外因性核酸分子の過剰発現によってリン酸含量の上昇を招くことが可能な点によって、および／または大腸菌における発現後にグリコーゲンのリン酸化を促進する点によって区別される（国際公開公報第９７／１１１８８ Ａ１号、実施例９を参照）。

【００３９】

外因性核酸分子のさまざまな異型（断片、誘導体、対立遺伝子変異体）にコードされているタンパク質は、特定の一般的な特徴を示す。後者の例には、タンパク質の相同性を比較する上述の方法で調査可能な生物学的活性、酵素活性、または類似の一次構造が含まれる。このようなタンパク質のアミノ酸配列は相互に少なくとも６０％、好ましくは少なくとも８５％、特に少なくとも９５％、また最も好ましくは少なくとも９７％の相同性を示す。

【００４０】

上述の特徴に加えて、外因性核酸分子は、以下の特徴的なペプチドモチーフの少なくとも

１個、好ましくは少なくとも５個、特に少なくとも１０個、また最も好ましくはすべてを含むＲ１タンパク質の生物学的活性を有するタンパク質をコードすることを特徴とする場合がある：

DKAAET (配列番号 . 3), IADME (配列番号 . 4), VWMRFM (配列番号 . 5),
MQEWHQ (配列番号 . 6), LGHYM (配列番号 . 7), ERGYEE (配列番号 .8),
KAVLDR (配列番号 . 9), LSSLL (配列番号 . 10), IPDGAV (配列番号 .11),
KVCFAT (配列番号 . 12), ISADEF (配列番号 . 13), PFGVFE (配列番号 . 14),
SSGDD (配列番号 . 15), SFICKK (配列番号 . 16).

【００４１】
他の差には、本発明の植物細胞は、天然には同細胞に存在しない外因性核酸分子を含む点により、またはこのような分子が、天然には存在しない、細胞のゲノム中のある位置（すなわち異なるゲノム環境）に組み込まれる点により、天然の植物細胞と区別できるものもある。また、本発明のこの種のトランスジェニック植物細胞は、ゲノムに安定に組み込まれる少なくとも１コピーの外因性核酸分子を含む点で、また任意選択で、細胞内に天然に存在するこのような分子のコピーに加えて含む点で、天然の植物細胞と区別することができる。細胞に導入される核酸分子が、既に細胞内に天然に存在する分子の付加的なコピーである場合、本発明の植物細胞は、特に天然の植物細胞と、付加的なコピーが、天然には存在しないゲノム上のある部位に位置する点で区別することができる。これは後に、例えばサザンブロッティング解析により検出することができる。
【００４２】
また、本発明に係る植物細胞は好ましくは、以下の少なくとも一つの特徴により、天然の植物細胞と区別される：導入される核酸分子が植物体に関して異種である場合、本発明のトランスジェニック植物の細胞が、導入される核酸分子の転写物を含むこと、実際には野生型の植物体で転写物が検出されない器官であっても含むこと。本発明の植物細胞は好ましくは、外因性核酸分子の転写物を含む。これは例えばノーザンブロッティング解析で検出することができる。本発明の植物細胞は、好ましくは、導入された外因性核酸分子にコードされるタンパク質を含む。このような付加的なタンパク質は、免疫学的方法、例えば特にウェスタンブロッティング解析で検出することができる。
【００４３】
一つの好ましい態様では、本発明の植物細胞および植物体は、対応する遺伝的に修飾されていない野生型植物細胞および野生型植物体と比較して、Ｒ１タンパク質の高い生物学的活性を示す。本発明に関連して、「Ｒ１タンパク質の高い生物学的活性」は例えば、本発明の植物細胞および植物体で合成されるデンプンのリン酸含量を測定することで決定することができる。対応する遺伝学的に修飾されていない野生型の植物細胞のデンプンと比較して、Ｒ１タンパク質の高い生物学的活性を示す、本発明の植物細胞のデンプンは、これが、リン酸含量が高いデンプン、好ましくはグルコース単量体のＣ６位におけるリン酸含量が高いデンプンを合成する点で区別される。
【００４４】
また、Ｒ１タンパク質の生物学的活性の上昇は、Ｒ１転写物の量を例えばノーザンブロッティング解析で測定して、対応する遺伝的に修飾されていない野生型植物体のＲ１転写物の量と比較することで決定することができる。
【００４５】
また、Ｒ１タンパク質の高い生物学的活性は、Ｒ１タンパク質の量を例えばウェスタンブロッティング解析で測定して、対応する遺伝的に修飾されていない野生型植物体のＲ１タンパク質の量と比較することで決定することができる。
【００４６】
本発明に係る単子葉植物の細胞が、対応する遺伝的に修飾されていない野生型植物体の植

物細胞のデンプンと比較して、グルコース単量体のＣ６位におけるリン酸含量の上昇、および／または修飾されたリン酸化パターン、および／または修飾された粘性特性を有するデンプンを合成することがわかっている。

【００４７】

したがって本発明は好ましくは、対応する遺伝的に修飾されていない野生型植物体の植物細胞のデンプンと比較して、グルコース単量体のＣ６位におけるリン酸含量が高く、および／または修飾されたリン酸化パターンを有し、および／または修飾された粘性特性を有するデンプンを合成する、本発明の植物細胞に関する。

【００４８】

本発明では、「リン酸含量」という用語は、デンプンのリン酸モノエスエルの状態で共有結合で結合されたリン酸の量を意味する。

【００４９】

本発明では、「Ｃ６位」という用語は、炭素原子の「６」位において、デンプンのグルコース単量体に結合されるリン酸基を意味すると理解される。

【００５０】

本発明に関連して、「Ｃ６位におけるリン酸含量の上昇」という用語は、グルコース単量体のリン酸基が、光学的－酵素学的試験により、Ｃ６位で検出可能であることを意味すると理解される（Ｎｉｅｌｓｅｎら、Ｐｌａｎｔ Ｐｈｙｓｉｏｌ．１０５、（１９９４）、１１１～１１７、方法を参照）。「Ｃ６位におけるリン酸含量の上昇」という用語は、好ましくは、対応する遺伝的に修飾されていない野生型植物体のデンプンのリン酸含量と比較して、グルコース単量体のＣ６位におけるデンプンのリン酸含量の少なくとも２０％、好ましくは少なくとも５０％、また最も好ましくは少なくとも１００％の上昇を意味すると理解される。

【００５１】

原理的には、グルコース単位のＣ２位、Ｃ３位、およびＣ６位が、インビボにおいてデンプン中でリン酸化される場合がある。本発明に関連して、Ｃ６位におけるリン酸含量（＝Ｃ６ Ｐ含量）は、光学的－酵素学的試験を用いるグルコース－６－リン酸の決定により決定することができる（Ｎｉｅｌｓｅｎら、Ｐｌａｎｔ Ｐｈｙｓｉｏｌ．１０５、（１９９４）、１１１～１１７）（方法参照）。

【００５２】

本発明では、「修飾されたリン酸化パターン」という用語は、対応する遺伝的に修飾されていない植物体のデンプンから作製される化学的にリン酸化されたデンプンと比較して、デンプン粒のさまざまな層内においてデンプンに共有結合で結合されるリン酸基の総リン酸含量が修飾されていることを意味する。化学的にリン酸化されたデンプンは、デンプン粒内のリン酸勾配により区別される（外側の層は内側の層より一般に強くリン酸化されている）。これに対して本発明のデンプンは、リン酸基が、デンプン粒のさまざまな層に異なって分布する点で区別される。すなわち本発明のデンプンは、化学的にリン酸化されたデンプンに典型的にみられる外側から内側への勾配を含まないことを特徴とするといえる。

【００５３】

デンプンのリン酸化パターンを調べる方法は当業者に周知である（例えば、ＪａｎｅおよびＳｈｅｎ、Ｃａｒｂｏｈｙｄｒａｔｅ Ｒｅｓｅａｒｃｈ ２４７、（１９９３）、２７９～２９０；ＧｏｕｇｈおよびＰｙｂｕｓ、Ｓｔａｅｒｋｅ ２５、（１９７３）、１２３～１３０を参照）。これらの方法は、内部でデンプン粒の膠着層が機械的に段階的に除かれる、デンプン粒のさまざまな層の段階的な化学的膠着を基礎としている。このような脱殻的手法（ｓｈｅｌｌｉｎｇ ｐｒｏｃｅｄｕｒｅ）に続いて、さまざまなデンプン粒の層の総リン酸含量が標準的な方法で決定される。

【００５４】

本発明では、「化学的にリン酸化されたデンプン」という用語は、対応する遺伝的に修飾されていない植物細胞および／または植物体の天然のデンプンの化学的なリン酸化で作ら

れるデンプンを意味すると理解される。本発明に関連して、化学的にリン酸化されたデンプンは、好ましくは、本発明のデンプンのアミロース含量と同等のアミロース含量と区別される。天然のデンプンの化学的リン酸化の間、Ｃ６位におけるリン酸含量は、適切な試験条件を選択して、化学的にリン酸化されたデンプンのＣ６位におけるリン酸含量が、本発明のデンプンのグルコース単量体のＣ６位におけるリン酸含量と同一および／または同等になるように調節される。

【００５５】

本発明に関連して、「修飾された粘性特性」という用語は、ＲＶＡプロファイルにおける最終粘性の上昇、および／またはＤＳＣ解析におけるピーク温度の低下を特に意味すると理解される。

【００５６】

他の好ましい態様では、本発明の植物細胞は、対応する遺伝的に修飾されていない野生型植物体のデンプンと比較して、最終粘性が高いデンプンを合成する。

【００５７】

本発明に関連して、「最終粘性」という用語は、粘性プロファイル（図１参照）を元に決定可能で、また図１で「最終粘性＝Ｆｉｎ」と示されている粘性を意味すると理解される。粘性プロファイルは、ラピッドビスコアナライザー（Ｒａｐｉｄ Ｖｉｓｃｏ Ａｎａｌｙｚｅｒ；ＲＶＡ）（Ｎｅｗｐｏｒｔ Ｓｃｉｅｎｔｉｆｉｃ Ｐｔｙ Ｌｔｄ、Ｉｎｖｅｓｔｍｅｎｔ Ｓｕｐｐｏｒｔ Ｇｒｏｕｐ、Ｗａｒｒｉｅｗｏｏｄ、ＮＳＷ２１０２、Ａｕｓｔｒａｌｉａ）を使用して得られる。コムギデンプンの解析では、粘性プロファイルは以下の手順で決定される：２．５ ｇのデンプン（乾燥物質）を２５ ｍｌのH_2Oに溶解し、ラピッドビスコアナライザー（Ｎｅｗｐｏｒｔ Ｓｃｉｅｎｔｉｆｉｃ Ｐｔｙ Ｌｔｄ．、Ｉｎｖｅｓｔｍｅｎｔ Ｓｕｐｐｏｒｔ Ｇｒｏｕｐ、Ｗａｒｒｉｅｗｏｏｄ ＮＳＷ ２１０２、Ａｕｓｔｒａｌｉａ）による解析に用いる。同装置は、製造業者の指示書通りに操作する。完全な温度プログラムを図１に示す。ＲＶＡ解析の実施の詳細は後述する（方法参照）。

【００５８】

本発明では、「最終粘性の上昇」という用語は、最終粘性が、対応する遺伝的に修飾されていない野生型植物の細胞に由来するデンプンと比較して、少なくとも１０％、好ましくは少なくとも３０％、特に少なくとも５０％、また最も好ましくは少なくとも８０％上昇することを意味する（最終粘性は、対応する遺伝的に修飾されていない野生型植物の細胞のデンプンと比較して、最大１０００％、好ましくは最大５００％、特に最大２５０％上昇する場合がある）。

【００５９】

他の好ましい態様では、本発明の植物細胞は、対応する遺伝的に修飾されていない野生型植物のデンプンと比較して、および／または化学的にリン酸化されたデンプンと比較して、グルコース単量体のＣ６位におけるリン酸含量が同一または同等のデンプン、および／またはピーク温度が低いデンプンを合成する。

【００６０】

本発明では、「ピーク温度」という用語は、当業者に周知の差分走査熱量測定（ＤＳＣ）で決定可能な温度Ｔｐを意味すると理解されるべきである。ピーク温度は、ＤＳＣ曲線の最初のピーク極大を一般に指す温度である。これは例えば、大容量カプセルを用いて、Ｐｅｒｋｉｎ Ｅｌｍｅｒから入手される装置（装置番号：ＤＳＣ－７）を用いて決定することができる（調査対象の試料は、デンプン：総水量の比が約１：４であり、測定は１０℃／分の加熱速度で温度範囲１０～１６０℃で実施される）（実施例４参照）。

【００６１】

「ピーク温度Ｔｐの低下」という用語は、対応する遺伝的に修飾されていない野生型植物細胞のデンプンと比較して、ピーク温度Ｔｐが少なくとも１．５℃、好ましくは少なくとも２．５℃、特に少なくとも４℃、また最も好ましくは少なくとも６℃低下すること、および最大５℃、１２℃、または９℃低下することを意味する。

【００６２】
他の好ましい態様では、本発明は、膠着後にゲルを形成し、対応する遺伝的に修飾されていない野生型植物の細胞のデンプンから形成されるゲルと比較して、高いゲル強度を有するデンプンを合成する本発明の植物細胞に関する。
【００６３】
本発明では、「ゲル強度の上昇」という用語は、対応する遺伝的に修飾されていない野生型植物の細胞のデンプンのゲル強度と比較して、ゲル強度の上昇が少なくとも２０％、特に少なくとも５０％、好ましくは少なくとも８０％、また最も好ましくは少なくとも１００％であり、最大低下率が５００％、または最大２５０％であることを意味すると理解される。
【００６４】
本発明に関連して、ゲル強度は、後述する条件下におけるテクスチャー解析法で決定することができる（方法参照）。
【００６５】
本発明の別の態様では、デンプンのリン酸含量の上昇は、デンプンのアミロペクチン成分に関する場合がある。
【００６６】
したがって本発明は、デンプンを合成する本発明の植物細胞にも関し、このアミロペクチン成分はリン酸化されており、またこのアミロース成分の総リン酸含量は、グルコース単量体のＣ６位におけるデンプンのリン酸含量が同じである、対応する化学的にリン酸化されたデンプンのアミロース成分と比較して低い。
【００６７】
したがって、グルコース単量体のＣ６位におけるリン酸含量が同じである化学的にリン酸化されたデンプンに対し、本発明の植物細胞のデンプンは、デンプンから作られ、好ましくは対応する遺伝的に修飾されていない植物体と同等のアミロース量を有するグルコース単量体のＣ６位におけるリン酸含量が同じである、化学的に合成されたデンプンのアミロース成分と比較して、本発明の植物細胞のデンプンのアミロース成分の総リン酸含量が低い点、および／またはグルコース単量体のＣ６位におけるリン酸含量が低い点において区別される。
【００６８】
本発明に関連して、「総リン酸含量」という用語は、グルコース単位のＣ２位、Ｃ３位、およびＣ６位におけるデンプンのリン酸モノエステルの状態で共有結合で結合されたリン酸の量を意味すると理解される。本発明では、リン酸化された非グルカン（リン脂質など）の量は、「総リン酸含量」という用語には含まれない。したがって、リン酸化された非グルカンは、総リン酸含量を決定する前に量的に分離されなければならばい。リン酸化された非グルカン（例えばリン脂質）をデンプンから分離する方法は当業者に周知である。
【００６９】
本発明では、「総リン酸含量の低下」という用語は、対応する遺伝的に修飾されていない植物体に由来するデンプンから作られるＣ６位のリン酸含量が同じ化学的にリン酸化されたデンプンのアミロース成分の総リン酸含量と比較して、少なくとも５％、特に少なくとも２０％、好ましくは少なくとも５０％、また最も好ましくは少なくとも８０％の総リン酸含量の低下を意味すると理解される。
【００７０】
総リン酸含量を決定する方法は当業者に周知であり、後述する（方法参照）。
【００７１】
本発明では、「グルコース単量体のＣ６位におけるリン酸含量の低下」という表現は、対応する遺伝的に修飾されていない植物体に由来するデンプンから作られるＣ６位におけるリン酸含量が同じ化学的にリン酸化されたデンプンのアミロース成分のグルコース単量体のＣ６位におけるリン酸含量と比較して、アミロース成分のグルコース単量体のＣ６位におけるリン酸含量が少なくとも１０％、特に少なくとも２０％、好ましくは少なくとも２

０％、好ましくは少なくとも５０％、また最も好ましくは少なくとも８０％低下することを意味すると理解されるべきである。グルコース単量体のＣ６位におけるリン酸含量を決定する方法は当業者に周知であり、後述する（方法参照）。

【００７２】

本発明では、「アミロペクチン成分」という用語は、デンプンのアミロペクチンを意味すると理解される。

【００７３】

本発明では、「アミロース成分」という用語は、デンプンのアミロースを意味すると理解される。

【００７４】

アミロースとアミロペクチンを分離する方法は当業者に周知である。例えばアミロース成分は、例えばロジャー（Ｒｏｇｅｒ）およびコロナ（Ｃｏｌｏｎｎａ）（Ｉｎｔｅｒｎａｔｉｏｎａｌ Ｊｏｕｒｎａｌ ｏｆ Ｂｉｏｌｏｇｉｃａｌ Ｍａｃｒｏｍｏｌｅｃｕｌｅｓ １９、（１９９６）、５１～６１）に記載されている手順で、デンプンの水性リーチング（ａｑｕｅｏｕｓ ｌｅａｃｈｉｎｇ）で得られる。

【００７５】

本発明の他の好ましい態様では、本発明のデンプンは、デンプンのＣ６位におけるグルコース単量体のＣ６位のリン酸含量に対するアミロース成分のＣ６位におけるグルコース単量体のＣ６リン酸含量の比が０．７５未満、特に０．５未満、好ましくは０．２５未満、また最も好ましくは０．２０未満であることをさらに特徴とする。化学的にリン酸化されたデンプンに対し、本発明のデンプンは、グルコース単量体のＣ６位におけるリン酸の主要な部分が、デンプンのアミロペクチン成分に検出され、アミロース成分に検出されない点で区別される。

【００７６】

本発明の植物細胞は、単子葉植物に起源を有する。これらは好ましくは、農業分野で使用される植物、すなわち栄養分を得る目的、または技術上の目的（特に工業的な目的）で、人間によって栽培される植物に由来する植物細胞である。したがって本発明は、好ましくは、デンプンを合成する植物、またはデンプンを貯蔵する植物（例えば、ライムギ、オオムギ、オーツムギ、コムギ、ミレット、イネ、またはトウモロコシ）に由来する植物細胞に関する。

【００７７】

本発明の一つの好ましい態様では、本発明の植物細胞は、コムギ、イネ、オオムギ、オーツムギ、ライムギ、およびトウモロコシからなる群より選択される植物体に起源を有する。コムギ、イネ、およびトウモロコシの植物体の植物細胞が好ましく；コムギ植物体の植物細胞が特に好ましい。

【００７８】

特に好ましい一つの態様では、本発明の植物細胞は、グルコース単量体のＣ６位におけるリン酸含量が少なくとも０．１ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$ デンプン、特に少なくとも０．５ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$、好ましくは少なくとも１ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$ デンプン、より好ましくは少なくとも２ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$ デンプン、最も好ましくは少なくとも５ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$ デンプン、最も詳細には好ましくは少なくとも１０ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$ デンプンであるデンプンを合成する（本発明の植物細胞は、グルコース単量体のＣ６位におけるリン酸含量が最大１００ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$ デンプン、詳細には最大５０ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$ デンプン、また最も詳細には最大２５ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$ デンプンであるデンプンを合成する）。

【００７９】

本発明の他の態様では、本発明の植物細胞は、グルコース単量体のＣ６位におけるリン酸含量が少なくとも１５ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$ デンプンであるデンプンを合成する場合がある（本発明の植物細胞は、グルコース単量体のＣ６位におけるリン酸含量が最大１００ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$ デンプン、詳細には最大５０ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-}$

^{1} デンプン、また最も詳細には最大２５ｎｍｏｌ Ｃ６ Ｐ mg^{-1} デンプンであるデンプンを合成する）。

【００８０】

他の態様では、本発明は、本発明の植物細胞を作製する方法に関し、単子葉植物の細胞が遺伝的に修飾され、またこの遺伝的修飾は少なくとも１個の外因性核酸分子の導入を含む。

【００８１】

植物の宿主細胞へＤＮＡを輸送する際に利用可能な多様な手法がある。このような手法には、形質転換の媒介物としてアグロバクテリウム・ツメファシエンス（Ａｇｒｏｂａｃｔｅｒｉｕｍ ｔｕｍｅｆａｃｉｅｎｓ）またはアグロバクテリウム・リゾジェネス（Ａｇｒｏｂａｃｔｅｒｉｕｍ ｒｈｉｚｏｇｅｎｅｓ）を用いるＴ－ＤＮＡによる植物細胞の形質転換、プロトプラストの融合、注入、ＤＮＡのエレクトロポレーション、微粒子銃を用いた手段によるＤＮＡの導入、および他の可能な手法が含まれる。

【００８２】

アグロバクテリウムにより促進される植物細胞の形質転換の使用は詳しく検討されており、また欧州特許１２０５１６号；フーケマ（Ｈｏｅｋｅｍａ）、「Ｔｈｅ Ｂｉｎａｒｙ Ｐｌａｎｔ Ｖｅｃｔｏｒ Ｓｙｓｔｅｍ Ｏｆｆｓｅｔｄｒｕｋｋｅｒｉｊ Ｋａｎｔｅｒｓ Ｂ．Ｖ．、Ａｌｂｌａｓｓｅｒｄａｍ（１９８５）、第Ｖ章；フレイリー（Ｆｒａｌｅｙ）ら、Ｃｒｉｔ．Ｒｅｖ．Ｐｌａｎｔ Ｓｃｉ．４、１～４６、およびアン（Ａｎ）ら、ＥＭＢＯ Ｊ．４、（１９８５）、２７７～２８７に詳述されている。ジャガイモの形質転換については、例えばロシュソサ（Ｒｏｃｈａ－Ｓｏｓａ）ら、ＥＭＢＯ Ｊ．８、（１９８９）、２９～３３を参照されたい。

【００８３】

一方、単子葉植物の形質転換は、微粒子銃を用いた手段で、またアグロバクテリウムを用いた手段で日常的に達成されている（Ｋｏｍａｒｉら、（１９９８）、Ａｄｖａｎｃｅｓ ｉｎ ｃｅｒｅａｌ ｇｅｎｅ ｔｒａｎｓｆｅｒ；Ｃｕｒｒｅｎｔ Ｏｐｉｎｉｏｎ ｉｎ Ｐｌａｎｔ Ｂｉｏｔｅｃｈｎｏｌｏｇｙ １、１６１、以下参照；Ｂｉｌａｎｇら（１９９９）、Ｔｒａｎｓｆｏｒｍａｔｉｏｎ ｏｆ Ｃｅｒｅａｌｓ、Ｇｅｎｅｔｉｃ Ｅｎｇｉｎｅｅｒｉｎｇ、１２、１１３～１４８、ＪＫ Ｓｅｔｌｏｗ編、Ｋｌｕｗｅｒ Ａｃａｄｅｍｉｃ／Ｐｌｅｎｕｍ Ｐｕｂｌｉｓｈｅｒ、Ｎｅｗ Ｙｏｒｋ）。アグロバクテリウムに基づくベクターについては諸文献に記載されている（Ｃｈａｎら、Ｐｌａｎｔ Ｍｏｌ．Ｂｉｏｌ．２２、（１９９３）、４９１～５０６；Ｈｉｅｉら、Ｐｌａｎｔ Ｊ．６、（１９９４）２７１～２８２；Ｄｅｎｇら、Ｓｃｉｅｎｃｅ ｉｎ Ｃｈｉｎａ ３３、（１９９０）、２８～３４、Ｗｉｌｍｉｎｋら、Ｐｌａｎｔ Ｃｅｌｌ Ｒｅｐｏｒｔｓ １１、（１９９２）、７６～８０；Ｍａｙら、Ｂｉｏ／Ｔｅｃｈｎｏｌｏｇｙ １３、（１９９５）、４８６～４９２；ＣｏｎｎｅｒおよびＤｏｍｉｓｓｅ、Ｉｎｔ．Ｊ．Ｐｌａｎｔ Ｓｃｉ．１５３（１９９２）、５５０～５５５；Ｒｉｔｃｈｉｅら、Ｔｒａｎｓｇｅｎｉｃ Ｒｅｓ．２、（１９９３）、２５２～２６５）。単子葉植物の形質転換の別の系には、微粒子銃を用いた手段による形質転換（ＷａｎおよびＬｅｍａｕｘ、Ｐｌａｎｔ Ｐｈｙｓｉｏｌ．１０４、（１９８４）、３７～４８；Ｖａｓｉｌら、Ｂｉｏ／Ｔｅｃｈｎｏｌｏｇｙ １１（１９９３）、１５５３～１５５８；Ｒｉｔａｌａら、Ｐｌａｎｔ Ｍｏｌ．Ｂｉｏｌ．２４、（１９９４）、３１７～３２５；Ｓｐｅｎｃｅｒら、Ｔｈｅｏｒ．Ａｐｐｌ．Ｇｅｎｅｔ．７９、（１９９０）、６２５～６３１）、プロトプラストによる形質転換、部分透過性をもつ細胞を対象としたエレクトロポレーション、およびガラス繊維を用いたＤＮＡの導入などがある。特にトウモロコシの形質転換については諸文献に数回にわたって記載されている（例えば、国際公開公報第９５／０６１２８号、欧州特許第０５１３８４９号、ＥＯ ０４６５８７５、欧州特許第２９２４３５号；Ｆｒｏｍｍら、Ｂｉｏｔｅｃｈｎｏｌｏｇｙ ８、（１９９０）、８３３～８４４；Ｇｏｒｄｏｎ－Ｋａｍｍら、Ｐｌａｎｔ Ｃｅｌｌ ２、（１９９０）、６０３～６１８；Ｋｏｚｉｅｌら、Ｂｉｏｔｅｃｈｎｏｌｏｇｙ １１（１９９３）、１９４～２００；Ｍｏ

ｒｏｃら、Ｔｈｅｏｒ．Ａｐｐｌ．Ｇｅｎｅｔ．８０、（１９９０）、７２１～７２６を参照）。

【００８４】

他の種の穀物類の形質転換がうまくゆくことについても、例えばオオムギ（ＷａｎおよびＬｅｍａｕｘ、上述；Ｒｉｔａｌａら、上述；Ｋｒｅｎｓら、Ｎａｔｕｒｅ ２９６、（１９８２）、７２～７４）、およびコムギ（Ｂｅｃｋｅｒら、Ｐｌａｎｔ Ｊ．５（２）、（１９９４）、２２９～３０７；Ｎｅｈｒａら、Ｐｌａｎｔ Ｊ．５、（１９９４）、２８５～２９７）について報告されている

【００８５】

イネについては、さまざまな形質転換法、例えばアグロバクテリウムによって促進される形質転換（Ｈｉｅｉら、Ｐｌａｎｔ Ｊ．６（１９９４）、２７１～２８２；Ｈｉｅｉら、Ｐｌａｎｔ Ｍｏｌ．Ｂｉｏｌ．３５（１９９７）、２０５～２１８；Ｐａｒｋら、Ｊ．Ｐｌａｎｔ Ｂｉｏｌ．３８（１９９５）、３６５～３７１）、プロトプラストを用いた形質転換（Ｄａｔｔａ、「Ｇｅｎｅ ｔｒａｎｓｆｅｒ ｔｏ ｐｌａｎｔｓ」、Ｐｏｔｒｙｋｕｓ、Ｓｐａｎｇｅｎｂｅｒｇ（編）、Ｓｐｒｉｎｇｅｒ－Ｖｅｒｌａｇ、Ｂｅｒｌｉｎ、Ｈｅｉｄｅｌｂｅｒｇ、１９９５、６６～７５；Ｄａｔｔａら、Ｐｌａｎｔ Ｍｏｌ．Ｂｉｏｌ．２０（１９９２）、６１９～６２９；Ｓａｄａｓｉｖａｍら、Ｐｌａｎｔ Ｃｅｌｌ Ｒｅｐ．１３（１９９４）、３９４～３９６）、微粒子銃を用いる植物形質転換法（Ｌｉら、Ｐｌａｎｔ Ｃｅｌｌ Ｒｅｐ．１２（１９９３）、２５０～２５５；Ｃａｏら、Ｐｌａｎｔ Ｃｅｌｌ Ｒｅｐ．１１（１９９２）、５８６～５９１；Ｃｈｒｉｓｔｏｕ、Ｐｌａｎｔ Ｍｏｌ．Ｂｉｏｌ．（１９９７）、１９７～２０３）、およびエレクトロポレーション（Ｘｕら、「Ｇｅｎｅ ｔｒａｎｓｆｅｒ ｔｏ ｐｌａｎｔｓ」、Ｐｏｔｒｙｋｕｓ、Ｓｐａｎｇｅｎｂｅｒｇ（編）、Ｓｐｒｉｎｇｅｒ－Ｖｅｒｌａｇ、Ｂｅｒｌｉｎ、Ｈｅｉｄｅｌｂｅｒｇ、１９９５、２０１～２０８）が報告されている。

【００８６】

本発明はさらに、本発明の植物細胞を含む植物体、および／または本発明の植物細胞から再生させることで作製可能な植物体に関する。このような植物体は好ましくは有用な単子葉植物、例えばライムギ、オオムギ、オーツムギ、コムギ、ミレット、イネ、およびトウモロコシである。コムギ、イネ、およびトウモロコシが好ましく；コムギが特に好ましい。

【００８７】

本発明の植物体は、修飾型のデンプンを合成する。このようなデンプンのリン酸含量、および／またはリン酸化パターン、および／または粘性特性は、本発明の植物細胞に関連して記載されているように、対応する遺伝的に修飾されていない野生型植物体のデンプンとは異なる。

【００８８】

したがって本発明は、対応する遺伝的に修飾されていない植物体のデンプンと比較して、グルコース単量体のＣ６位におけるリン酸含量が高いデンプン、および／または修飾されたリン酸化パターンを示すデンプン、および／または修飾された粘性特性を示すデンプンを合成する本発明の植物体にも関する。

【００８９】

本発明の植物細胞に関連してなされた記述は、デンプンのグルコース単量体のＣ６位におけるリン酸含量の上昇、デンプンのリン酸化パターンの修飾、およびデンプンの粘性特性の修飾についても言える。

【００９０】

本発明は好ましくは、対応する遺伝的に修飾されていない野生型植物体のデンプンから形成されるゲルと比較して、膨着後にゲル強度が高いゲルを形成するデンプンを合成する本発明の植物体に関する。

【００９１】

「高いゲル強度」という用語は、本発明の植物細胞に関連して定義されている。

【００９２】

本発明の他の好ましい態様では、本発明の植物体、好ましくはコムギ植物体は、グルコース単量体のＣ６位におけるリン酸含量が少なくとも０．１ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$ デンプン、特に少なくとも０．５ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$、好ましくは少なくとも１ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$ デンプン、最も好ましくは少なくとも２ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$ デンプン、特に少なくとも５ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$ デンプン、極めて最も好ましくは少なくとも１０ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$ デンプンであるデンプン、好ましくはコムギデンプンを合成する（本発明の植物体は、グルコース単量体のＣ６位におけるリン酸含量が、最大１００ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$ デンプン、特に最大５０ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$ デンプン、または最大２５ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$ デンプンであるデンプンを合成する）。

【００９３】

本発明の他の態様では、本発明の植物体は、グルコース単量体のＣ６位におけるリン酸含量が少なくとも１５ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$ デンプンであるデンプンを合成することができる（本発明の植物体は、グルコース単量体のＣ６位におけるリン酸含量が最大１００ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$ デンプン、特に最大５０ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$ デンプン、または最大２５ｎｍｏｌ Ｃ６ Ｐ ｍｇ$^{-1}$ デンプンであるデンプンを合成する）。

【００９４】

本発明の他の好ましい態様では、本発明の植物体は、本発明の植物体のデンプン貯蔵器官内に本発明のデンプンを合成する。

【００９５】

最も好ましい態様では、本発明の植物体は、そのデンプン貯蔵器官内で、対応する遺伝的に修飾されていない野生型植物体のデンプン貯蔵器官に存在するデンプンと比較して、グルコース単量体のＣ６位におけるリン酸含量が上昇したデンプン、および／または修飾型のリン酸化パターンを有するデンプン、および／または修飾型の粘性特性を有するデンプンを合成する。

【００９６】

これと関連して、「デンプン貯蔵器官」という用語は、デンプンを一過的にのみ合成する葉などの器官に対して、貯蔵型デンプンを取り込む器官、例えばトウモロコシ、イネ、コムギの植物体の種子を意味すると理解される。

【００９７】

外因性核酸分子の発現は、単子葉植物、特にコムギ植物体のデンプン貯蔵器官内で特に有利であり、対応する遺伝的に修飾されていない野生型植物体（特にコムギ植物体）のデンプン貯蔵器官から単離可能なデンプンと比較して、デンプン貯蔵器官から単離可能なデンプンのリン酸含量の上昇を招く。

【００９８】

単子葉植物のデンプン貯蔵器官内における外因性核酸分子の発現は、構成的プロモーター、例えばカリフラワーモザイクウイルスの３５Ｓ ＲＮＡのプロモーター（例えば米国特許Ａ－５，３５２，６０５号を参照）、およびトウモロコシのユビキチンプロモーター（例えば米国特許Ａ－５，６１４，３９９号を参照）を使用することで初めて達成される。

【００９９】

プロモーターは好ましくは、デンプン貯蔵器官に特異的なもの、例えばｇｌｕｔｅｌｉｎプロモーター（Ｌｅｉｓｙら、Ｐｌａｎｔ Ｍｏｌ．Ｂｉｏｌ．１４、（１９９０）、４１～５０；Ｚｈｅｎｇら、Ｐｌａｎｔ Ｊ．４、（１９９３）、３５７～３６６；Ｙｏｓｈｉｈａｒａら、ＦＥＢＳ Ｌｅｔｔ．３８３、（１９９６）、２１３～２１８）、ｓｈｒｕｎｋｅｎ－１プロモーター（Ｗｅｒｒら、ＥＭＢＯ Ｊ．４、（１９８５）、１３７３～１３８０）、コムギのＨＭＷプロモーター（Ａｎｄｅｒｓｏｎ、Ｔｈｅｏｒｅｔｉｃａｌ ａｎｄ Ａｐｐｌｉｅｄ Ｇｅｎｅｔｉｃｓ ９６、（１９９８）、５６８～５７６、

Ｔｈｏｍａｓ、Ｐｌａｎｔ Ｃｅｌｌ ２（１２）、（１９９０）、１１７１～１１８０）、ＵＳＰプロモーター、ファゼオリンプロモーター（Ｓｅｎｇｕｐｔａ－Ｇｏｐａｌａｎ、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ８２（１９８５） ３３２０～３３２４、Ｂｕｓｔｏｓ、Ｐｌａｎｔ Ｃｅｌｌ １（９）（１９８９）、８３９～８５３）などの胚乳特異的なプロモーター、またはトウモロコシのｚｅｉｎ遺伝子のプロモーター（Ｐｅｄｅｒｓｅｎら、Ｃｅｌｌ ２９、（１９８２）、１０１５～１０２６；Ｑｕａｔｒｏｃｃｉｏら、Ｐｌａｎｔ Ｍｏｌ．Ｂｉｏｌ． １５（１９９０）、８１～９３）、またはＧＢＳＳＩ（顆粒性デンプン合成酵素Ｉ）（ＤＥ１００４１８６１．９）およびコムギのＳＳＩＩ（可溶性デンプン合成酵素ＩＩ）（ＤＥ１００３２３７９．０）の穎花特異的プロモーターが使用される。

【０１００】
一つの特に好ましい態様では、本発明の植物体は、植物体のデンプン貯蔵器官内でＲ１遺伝子の発現を示す。

【０１０１】
Ｒ１遺伝子の発現は、例えばＲ１転写物の量を例えばノーザンブロッティング解析で測定し、野生型植物体のＲ１転写物の量と比較することで決定することができる。

【０１０２】
また本発明に関連して、Ｒ１遺伝子の発現は、Ｒ１タンパク質の量を例えばウェスタンブロッティング解析で測定することで決定することができる。Ｒ１タンパク質の量は好ましくは、胚乳の植物細胞から単離されるタンパク質抽出物を対象に決定される。

【０１０３】
本発明の他の態様では、本発明の植物体は、植物体のデンプン貯蔵器官内でＲ１遺伝子の発現を示し、この発現は、対応する遺伝的に修飾されていない野生型植物体のデンプン貯蔵器官内におけるＲ１遺伝子の発現と比較して、好ましくは少なくとも５０％、最も好ましくは少なくとも１００％、特に少なくとも２５０％、また最も詳細には少なくとも５００％上昇している。

【０１０４】
一つの最も好ましい態様では、本発明の植物体は、本発明の植物体のデンプン貯蔵器官内における外因性核酸分子の器官特異的な発現を示す。

【０１０５】
本発明では、「器官特異的」という用語は、選択されたプロモーターが、成熟した葉と比較して、デンプン貯蔵器官内における外因性核酸分子の発現に望ましく、また少なくとも２～５倍、好ましくは５～１０倍、最も好ましくは１０～１００倍上昇する発現レベルといった、発現の程度の有意な上昇をもたらすことを意味すると理解される。

【０１０６】
本発明のこの態様では、本発明の植物体は、それが示す外因性核酸分子の器官特異的な発現が、対応する遺伝的に修飾されていない野生型植物体のデンプン貯蔵器官内における内因性Ｒ１遺伝子の遺伝子発現と比較して、デンプン貯蔵器官内におけるＲ１遺伝子の発現を上昇させるという点で区別される。本発明のこの態様では、本発明の植物体の葉におけるＲ１遺伝子発現の上昇は、デンプン貯蔵器官内における発現と同程度に検出されない。Ｒ１遺伝子発現の上昇は好ましくは、デンプン貯蔵器官のみと関連する。

【０１０７】
本発明の好ましい態様では、本発明の植物体は、コムギ、イネ、オオムギ、ミレット、オーツムギ、ライムギ、およびトウモロコシからなる群より選択される植物体である。コムギ、イネ、およびトウモロコシの植物体が好ましく；コムギ植物体が特に好ましい。

【０１０８】
本発明は、以下のように、本発明で定義される植物体を作製する方法にも関する：
（ａ）単子葉植物の細胞が遺伝的に修飾され、遺伝的修飾が少なくとも１種の外因性核酸分子の導入を含み；
（ｂ）段階（ａ）の細胞から植物体が再生され；また任意選択で

（ｃ）段階（ｂ）で作製された植物体から他の植物体を作製する。

【０１０９】

本発明はさらに、植物のデンプン貯蔵器官で、以下のように野生型植物体のデンプン貯蔵器官のデンプンと比較して、リン酸含量が高いデンプン、および／またはリン酸化パターンが修飾されたデンプン、および／または最終粘性が高いデンプン、および／またはピーク温度が低いデンプンが合成される、本発明で定義される植物体を作製する方法に関する：

（ａ）単子葉植物の細胞が遺伝的に修飾され、遺伝的修飾が本発明で定義される少なくとも１種の外因性核酸分子の導入を含み；

（ｂ）段階（ａ）の細胞から植物体が再生され；また任意選択で

（ｃ）段階（ｂ）で作製された植物体から他の植物体を作製する。

【０１１０】

同じことが、本発明の植物細胞および植物体に関連して既に説明したように、段階（ａ）で導入される遺伝的修飾について適用できる。

【０１１１】

段階（ｂ）の植物体の再生は、当業者に周知の方法で達成することができる。

【０１１２】

本発明の方法の段階（ｃ）の他の植物体の作製は例えば、栄養繁殖（例えば、挿し木、塊茎またはカルスの栽培、および植物体全体の再生）により、または種子繁殖により達成することができる。種子繁殖は好ましくは、制御された状態で行う。すなわち性質が明らかな選択された植物体を相互に交配させて繁殖させる。

【０１１３】

本発明の一つの好ましい態様では、本発明の方法は、段階（ａ）の植物細胞に導入される外因性核酸分子が以下からなる群より選択される点で区別される：

（ｉ）配列番号：１に記載されたヌクレオチド配列のコード領域を含む核酸分子；

（ｉｉ）配列番号：２に記載されたアミノ酸配列を有するジャガイモ（Ｓｏｌａｎｕｍ ｔｕｂｅｒｏｓｕｍ）のＲ１タンパク質をコードする核酸分子；

（ｉｉｉ）配列番号：１に記載されたヌクレオチド配列の誘導体を含む核酸分子；および

（ｉｖ）（ｉ）、（ｉｉ）、または（ｉｉｉ）に記載された核酸分子の断片を含む核酸分子。

【０１１４】

一つの特に好ましい態様では、本発明の方法は、外因性核酸分子が、プロモーター、好ましくは、デンプン貯蔵組織においてＲ１遺伝子の発現を器官特異的に促進する、胚乳特異的なプロモーター、および／または穎花特異的なプロモーターの制御下にあるという点で区別される。

【０１１５】

胚乳特異的なプロモーター、また穎花特異的なプロモーターなどの、この種のプロモーターは、本発明の植物体に関連して既に本明細書で例示されている。

【０１１６】

他の態様では、本発明の方法は、有用な単子葉植物、例えば、ライムギ、オオムギ、オーツムギ、コムギ、ミレット、イネ、およびトウモロコシに関する。コムギ、イネ、およびトウモロコシが好ましく；コムギが特に好ましい。

【０１１７】

本発明は、本発明の方法で得られる植物体にも関する。

【０１１８】

本発明は、本発明の植物細胞を含む植物体の増殖材料にも関し、また本発明の方法で作製される植物体に関する。本発明では、「増殖材料」という用語は、栄養的経路または生殖的経路による作製または増殖に適した植物体の任意の構成成分を含む。例えば、挿し木、カルス培養、根茎、または塊茎は栄養繁殖に適している。他の増殖材料には例えば、果実、種子、シュート、プロトプラスト、細胞培養などがある。種子は好ましい増殖材料であ

る。
【０１１９】
一つの好ましい態様では、本発明はトウモロコシの種子に関する。
【０１２０】
最も好ましい態様では、本発明はコムギの種子に関する。
【０１２１】
本発明のトウモロコシおよびコムギの種子は、飼料および食品の生産に特に適している。
【０１２２】
本発明はさらに、本発明の植物体、好ましくはコムギ、トウモロコシ、およびイネの植物体、最も好ましくはコムギ植物体の作製に関連して、または本発明の単子葉植物細胞の作製に関連して本発明で定義される外因性核酸分子の使用に関する。
【０１２３】
本発明の植物細胞および植物体は、特にデンプン貯蔵器官において、物理化学的性質、特にリン酸含量、および／または粘性挙動、および／またはリン酸化パターンが、野生型植物体で合成されたデンプンと比較して、また化学的にリン酸化されたデンプンと比較して修飾されているデンプンを合成する。
【０１２４】
したがって本発明は、本発明の植物細胞、植物体、および／または増殖材料から得られるデンプンにも関する。
【０１２５】
本発明の一つの好ましい態様では、本発明のデンプンは、対応する遺伝的に修飾されていない野生型植物体のデンプンと比較して、グルコース単量体のＣ６位におけるリン酸含量が高いこと、および／またはリン酸化パターンが修飾されていること、および／または最終粘性が高いこと、および／またはピーク温度が低いことを特徴とする。
【０１２６】
本発明はまた、対応する遺伝的に修飾されていない野生型植物体のデンプンと比較して、グルコース単量体のＣ６位におけるリン酸含量が高いこと、および／またはリン酸化パターンが修飾されていること、および／または最終粘性が高いこと、および／またはピーク温度が低いことを特徴とするデンプンにも関する。
【０１２７】
本発明の植物細胞に関連してなされる記述は、Ｃ６位におけるリン酸含量の上昇、リン酸化パターンの修飾、および粘性特性（最終粘性、ピーク温度）の修飾にあついても言える。
【０１２８】
他の態様では、本発明は、グルコース単量体のＣ６位におけるリン酸含量が少なくとも０．１ｎｍｏｌ Ｃ６ Ｐ mg^{-1} デンプン、特に少なくとも０．５ｎｍｏｌ Ｃ６ Ｐ mg^{-1} デンプン、好ましくは少なくとも１ｎｍｏｌ Ｃ６ Ｐ mg^{-1} デンプン、最も好ましくは少なくとも２ｎｍｏｌ Ｃ６ Ｐ mg^{-1} デンプン、最も詳細には少なくとも５ｎｍｏｌ Ｃ６ Ｐ mg^{-1} デンプン、かなり最も好ましくは少なくとも１０ｎｍｏｌ Ｃ６ Ｐ mg^{-1} デンプンであること、および／または最終粘性が高いこと、および／またはピーク温度が低いことを特徴とするデンプン、好ましくはコムギデンプンに関する（グルコース単量体のＣ６位において、本発明のデンプンのリン酸含量は最大１００ｎｍｏｌ Ｃ６ Ｐ mg^{-1} デンプン、特に最大５０ｎｍｏｌ Ｃ６ Ｐ mg^{-1} デンプン、または最大２５ｎｍｏｌ Ｃ６ Ｐ mg^{-1} デンプンである）。
【０１２９】
本発明の他の態様では、本発明のデンプンは、グルコース単量体のＣ６位におけるリン酸含量が少なくとも１５ｎｍｏｌ Ｃ６ Ｐ mg^{-1} デンプンであることを特徴とする場合がある（グルコース単量体のＣ６位において、本発明のデンプンのリン酸含量は最大１００ｎｍｏｌ Ｃ６ Ｐ mg^{-1} デンプン、特に最大５０ｎｍｏｌ Ｃ６ Ｐ mg^{-1} デンプン、または最大２５ｎｍｏｌ Ｃ６ Ｐ mg^{-1} デンプンである）。

【０１３０】
他の好ましい態様では、本発明のデンプン、好ましくはコムギのデンプンは、膨着後に本発明のデンプンが、同じリン酸含量の対応する化学的にリン酸化されたデンプンのゲルと比較して、および／または対応する遺伝的に修飾されていない植物体のデンプンのゲルと比較して、高いゲル強度を示すゲルを形成することを特徴とする。
【０１３１】
本発明の他の好ましい態様では、本発明のデンプンは、リン酸基の大部分がデンプンのアミロペクチン成分に結合されており、アミロースは、共有結合で結合されたデンプンのモノリン酸エステルを極めて少量しか有さないことを特徴とする場合がある。Ｃ６リン酸含量が同等の化学的にリン酸化されたデンプンと比較して、本発明の植物細胞の本発明のデンプンは、本発明のデンプンのアミロースがアミロペクチン成分ほど強くリン酸化されていない点において区別される。
【０１３２】
本発明の他の態様では、本発明のデンプンは、化学的にリン酸化されたデンプンのアミロース成分と比較して、アミロース成分に含まれる総リン酸含量が低いこと、好ましくは、グルコース単量体のＣ６位におけるリン酸含量が同じであることを特徴とする場合がある。
【０１３３】
総リン酸含量を決定する方法は当業者に周知であり、後述する（方法参照）。
【０１３４】
本発明の他の態様では、本発明のデンプンは、デンプンのアミロース成分の３１Ｐ ＮＭＲ（ＫａｓｅｍｓｕｗａｎおよびＪａｎｅ（Ｃｅｒｅａｌ Ｃｈｅｍｉｓｔｒｙ ７３（６）、（１９９６）、７０２～７０７）で検出可能な総リン酸含量が、対応する遺伝的に修飾されていない植物体のデンプンから作られる、グルコース単量体のＣ６位におけるリン酸含量が同じ化学的にリン酸化されたデンプンのアミロース成分と比較して、少なくとも５％、好ましくは少なくとも２０％、特に少なくとも５０％、まと最も好ましくは少なくとも８０％低いことを特徴とする場合がある。
【０１３５】
本発明の一つの好ましい態様では、本発明のデンプンは、化学的にリン酸化されたデンプンと比較して、修飾されたリン酸化パターンを示すことを特徴とする場合がある。
【０１３６】
「リン酸化パターンの修飾」という用語は、本発明の植物細胞に関連して既に定義した。
【０１３７】
本発明の一つの好ましい態様では、デンプンは、ライムギ、オオムギ、オーツムギ、コムギ、ミレット、イネ、トウモロコシのデンプンなどの有用な単子葉植物のデンプンである。コムギ、イネ、およびトウモロコシのデンプンが好ましく；コムギデンプンが特に好ましい。
【０１３８】
本発明はさらに、上述の植物体および植物細胞から、および／または、このような植物体のデンプン貯蔵部分から、および／または本発明の増殖材料からデンプンを抽出する段階を含む、本発明のデンプンを作製する方法に関する。このような方法は、好ましくは、栽培された植物体、および／または同植物体のデンプン貯蔵部分を回収してからデンプンを抽出する段階も含み、また最も好ましくは、本発明の植物体、および／または本発明の増殖材料を栽培してから収穫する段階をさらに含む。植物体、または植物体のデンプン貯蔵部分からデンプンを抽出する段階は当業者に周知である。さまざまなデンプン貯蔵性植物からデンプンを抽出する方法は例えば、「Ｓｔａｒｃｈ：Ｃｈｅｍｉｓｔｒｙ ａｎｄ Ｔｅｃｈｎｏｌｏｇｙ（Ｗｈｉｓｔｌｅｒ、ＢｅＭｉｌｌｅｒおよびＰａｓｃｈａｌｌ編、（１９９４）、第２版、Ａｃａｄｅｍｉｃ Ｐｒｅｓｓ Ｉｎｃ．Ｌｏｎｄｏｎ Ｌｔｄ；ＩＳＢＮ ０－１２－７４６２７０－８；第ＸＩＩ章の４１２～４６８参照：トウモロコシおよびソルガムのデンプン：生産；Ｗａｔｓｏｎ；第ＸＩＩＩ章の４６９～４７９：

タピオカ、クズウコン、およびサゴのデンプン：生産；ＣｏｒｂｉｓｈｌｅｙおよびＭｉｌｌｅｒ；第ＸＩＶ章の４７９～４９０：ジャガイモのデンプン：生産および使用；Ｍｉｌｃｈ；第ＸＶ章の４９１～５０６：コムギのデンプン：生産、修飾、および使用；ＫｎｉｇｈｔおよびＯｓｏｎ；ならびに第ＸＶＩ章の５０７～５２８：イネのデンプン：生産および使用；ＲｏｈｍｅｒおよびＫｌｅｍ；ならびにＭａｉｚｅ ｓｔａｒｃｈｅｓ：Ｅｃｋｈｏｆｆら、Ｃｅｒｅａｌ Ｃｈｅｍ．７３（１９９６）５４～５７参照）に記載されている。トウモロコシデンプンの工業スケールにおける抽出は一般に、いわゆる「湿式粉砕」により行われている。植物材料からデンプンを抽出する方法に一般に使用されている装置には、ミル、セパレーター、デカンター、ハイドロサイクロン、スプレー乾燥機、および流動層乾燥機などがある。

【０１３９】

本発明は、上述の本発明の方法で得られる本発明のデンプンにも関する。

【０１４０】

本発明のデンプンは次に、当業者に周知の方法で修飾することが可能であり、未修飾状態および修飾状態のデンプンは、食品産業部門または非食品産業部門におけるさまざまな使用に適している。

【０１４１】

したがって本発明は、後に化学的に修飾、および／または物理的に修飾された、本発明で定義されるデンプンにも関する。

【０１４２】

化学的および／または物理的な修飾に使用可能なさまざまな選択肢については詳しく後述する。

【０１４３】

植物体を対象とした遺伝子工学的操作による修飾型デンプンの作製では第一に、植物体から得られるデンプンの性質を修飾することが可能であり、化学的方法または物理的方法によるさらなる修飾は必要でないように思われる。第二に、遺伝子工学的な方法で修飾されたデンプンを対象に、さらに化学的修飾および／または物理的修飾を行うことが可能であり、結果的に、上述した使用のいくつかの領域における質に関連して付加的な改善が得られる。このような化学的修飾および物理的修飾は基本的に既知である。特にこのような修飾は、以下の方法による修飾を含む：

－加熱処理、

－酸処理、

－デンプンエーテル、デンプンのアルキルエーテル、Ｏ－アリルエーテル、ヒドロキシアルキルエーテル、Ｏ－カルボキシメチルエーテル、Ｎ含有デンプンエーテル、Ｐ含有デンプンエーテル、Ｓ含有デンプンエーテルの生産、

－架橋デンプンの生産、

－デンプンのグラフトポリマーの生産

－酸化、および

－デンプンのリン酸塩、硝酸塩、硫酸塩、キサントゲン酸塩、酢酸塩、およびクエン酸塩の生産目的で使用されるエステル化操作。エステル化には他の有機酸も使用可能である。

【０１４４】

他の態様では、本発明は、工業分野、好ましくは食品材料を生産するための、本発明のデンプンの使用に関する。

【０１４５】

他の態様では、本発明は、本発明のデンプンを含む小麦粉に関する。他の性質のなかでは、本発明の小麦粉は、従来の小麦粉と比較して、修飾されたベーキング特性により区別される。コムギの種子から小麦粉を生産する方法は当業者に周知である。そのような方法により、本発明の小麦粉は、本発明の植物細胞および植物体から、また本発明の増殖材料から単離することができる。

【０１４６】

したがって本発明は、本発明の植物細胞および植物体から、また本発明の増殖材料から得られる小麦粉にも関する。

【０１４７】

対応する遺伝的に修飾されていない野生型植物体に由来する小麦粉と比較して、本発明の小麦粉は、特に高い吸水能をもつ点で区別される。吸水能を決定する方法は当業者に周知であり、詳しく後述する（方法参照）。

【０１４８】

本発明の一つの好ましい態様では、小麦粉は、対応する遺伝的に修飾されていないコムギ植物体に由来する小麦粉と比較して、少なくとも５％、特に少なくとも１５％、好ましくは少なくとも２０％、また最も好ましくは少なくとも２５％高い吸水能を示す。

【０１４９】

吸水能が高いため、本発明の小麦粉は、パン生地の準備段階において水をそれほど多く含まないパン生地を小麦粉に提供しなければならない工業生産上の利点がある。

【０１５０】

他の態様では、本発明は、ベーキングミクスチャーを生産する目的、および／または食品を生産する目的における本発明の小麦粉の使用、および／または本発明のデンプンの使用に関する。

【０１５１】

他の態様では、本発明は、包装材料を被覆する目的における本発明の小麦粉の使用、および／または本発明のデンプンの使用に関する。

【０１５２】

特に好ましい態様では、本発明は、本発明の小麦粉、および／または本発明のデンプンを含むベーキングミクスチャーに関する。これに関連して、「ベーキングミクスチャー」という用語は、パン生地（例えばイースト生地、パスタ生地、タルト生地、サワードウなど）、および／またはパン製品（例えばパン、ケーキ、ロールパン、クロワッサンなど）の生産に使用可能な任意の混合物を意味すると理解される。

【０１５３】

他の特に好ましい態様では、本発明は、本発明の小麦粉、および／または本発明のベーキングミクスチャー、および／または本発明のデンプンを用いて生産される食品、好ましくはパン製品、およびパン生地製品（例えばパスタ）に関する。

【０１５４】

対応する遺伝的に修飾されていない野生型のコムギ植物体に由来する小麦粉から作られるパン製品と比較して、本発明のパン製品および／または食品は、保存時にパン製品が受ける老化過程を遅らせる点で区別される。

【０１５５】

パン製品の老化過程は、デンプンのアミロペクチン成分の老化（再結晶）の程度に密接に関連している。パン製品および／または食品の老化過程は、アミロペクチン成分の老化の程度が高くなると、さらに進行すると推定されている。ＤＳＣで決定することができるアミロペクチンの融合エンタルピーは、アミロペクチンの老化の程度に関する情報となる。したがって、パン製品の老化は、ＤＳＣ（＝差分走査熱量測定）解析（方法参照）による任意の時点におけるアミロペクチンの融合エンタルピーの決定により決定することができる。

【０１５６】

本発明のパン製品、および／または食品のアミロペクチンの融合エンタルピーが、保存の例えば３日後、および／または７日後、および／または１３日後に決定される場合、同じ期間保存され、対応する遺伝的に修飾されていない野生型植物体の小麦粉の元となる、パン製品および／または食品のアミロペクチンの融合エンタルピーと比較して、アミロペクチンの融合エンタルピーが低下していることがわかる。これは、本発明のパン製品および食品が、従来製品と比較して緩やかな老化過程により区別されることを意味する。

【０１５７】

方法：
デンプンのグルコース単量体のＣ６位におけるリン酸含量（＝Ｃ６ Ｐ含量）の決定（Ｎｉｅｌｓｅｎら、Ｐｌａｎｔ Ｐｈｙｓｉｏｌ．１０５、（１９９４）、１１１～１１７）：
デンプンのＣ６ Ｐ 含量を決定するために、５０ ｍｇのデンプンを、５００ μｌの０．７ Ｍ ＨＣｌ中で９５℃で４時間かけて加水分解した。このバッチを次に１５，５００ｇで１０分間遠心して上清を分離した。７ μｌの上清を、１９３ μｌのイミダゾール緩衝液（１００ ｍＭ イミダゾール、ｐＨ ７．４；５ ｍＭ $MgCl_2$、１ ｍＭ ＥＤＴＡ、および０．４ ｍＭ ＮＡＤ）と混合した。測定はフォトメーターを用いて３４０ ｎｍで行った。基礎吸収を決定した後に、２ Ｕのグルコース－６－リン酸デヒドロゲナーゼ（ロイコノストック・メセンテロイデス（Ｌｅｕｃｏｎｏｓｔｏｃ ｍｅｓｅｎｔｅｒｏｉｄｅｓ）、Ｂｏｅｈｒｉｎｇｅｒ Ｍａｎｎｈｅｉｍから入手）を添加して酵素反応を開始させた。吸収の変化は、デンプンのＣ－６ Ｐ含量の濃度に正比例していた。

【０１５８】
デンプンの総リン酸含量の決定：
デンプンの総リン酸含量を決定する前に、デンプンは、リン脂質などのリン酸化された非グルカンと完全に分離する必要があった。総リン酸含量の決定は、以下に述べるエームズ（Ａｍｅｓ）（Ｍｅｔｈｏｄｓ ｉｎ Ｅｎｚｙｍｏｌｏｇｙ ＶＩＩＩ、（１９６６）、１１５～１１８）の方法で行った：約５０ ｍｇのデンプンを、３０ μｌの１０％ 硝酸マグネシウムエタノール溶液で処理し、マッフル炉内で3時間加熱した。残渣を５００ μｌの０．５ Ｍ 塩酸で処理し、６０℃で３０分間インキュベートした。次に、２０μｌのアリコートを０．５Ｍ 塩酸で３００μｌにメスアップし、１００μｌの１０％アスコルビン酸、および６００μｌの０．４２％モリブデン酸アンモニウム（溶媒：１Ｍ 硫酸）の混合物に添加し、４５℃で２０分間インキュベートした。次に、標準としてリン酸較正段階を用いて、吸光光度法により８２０ ｎｍで測定した。

【０１５９】
ゲル強度の決定（テクスチャーアナライザー）：
２．５ｇのデンプン（ＴＳ）を、ＲＶＡ装置内で２５ｍｌのH_2O中で膨着させ（Ｒａｐｉｄ Ｖｉｓｃｏ Ａｎａｌｙｚｅｒ （ＲＶＡ）による粘性特性の決定を参照）、続いて室温で２４時間保存した。この試料を、Ｓｔａｂｌｅ Ｍｉｃｒｏ Ｓｙｓｔｅｍｓ （Ｓｕｒｒｅｙ、英国）から入手したＴＡ－ＸＴ２テクスチャーアナライザーのセンサー（表面が平らな円筒型プランジャー）の下に固定し、ゲル強度を以下の装置設定を用いて決定した：
－試験速度 ０．５ ｍｍ／秒
－浸透深度 ７ ｍｍ
－接触面積 １１３ mm^2
－圧力 ２ｇ

【０１６０】
ラピッドビスコアナライザー（Ｒａｐｉｄ Ｖｉｓｃｏ Ａｎａｌｙｚｅｒ；ＲＶＡ）による粘性特性（例えば最終粘性）の決定：
２．５ｇ（乾燥重量）のデンプンを２５ｍｌのH_2Oに溶解し、ラピッドビスコアナライザー（Ｎｅｗｐｏｒｔ Ｓｃｉｅｎｔｉｆｉｃ Ｐｔｙ Ｌｔｄ．、Ｉｎｖｅｓｔｍｅｎｔ Ｓｕｐｐｏｒｔ Ｇｒｏｕｐ、Ｗａｒｒｉｅｗｏｄ ＮＳＷ ２１０２、Ａｕｓｔｒａｌｉａ）による解析に使用した。同装置の操作は、製造業者の指示書にしたがった。デンプン水溶液の粘性を決定するために、デンプン懸濁液を最初に１分間５０℃に加熱したのちに、５０℃から９５℃に１２℃／分の速度で加熱した。次に同温度を９５℃で２．５分間維持した。その後、同溶液を９５℃から５０℃へ、１２℃／分の速度で冷却した。最後に同温度を５０℃で６分間維持した。粘性は、試験時間全体を対象に決定した。

【０１６１】
水性リーチングによるデンプンのアミロース成分の単離：

水性リーチングにより、アミロースがデンプンから得られることが知られている（ＲｏｇｅｒおよびＣｏｌｏｎｎａ（Ｉｎｔｅｒｎａｔｉｏｎａｌ Ｊｏｕｒｎａｌ ｏｆ Ｂｉｏｌｏｇｉｃａｌ Ｍａｃｒｏｍｏｌｅｃｕｌｅｓ １９、（１９９６）、５１～６１）。

【０１６２】

デンプンのアミロース成分を単離するために、２５ｍｌの水性デンプン懸濁液（１０％ｗ／ｖ）を調製し、以下のプロファイルで撹拌しながらＲＶＡ（Ｎｅｗｐｏｒｔ Ｓｃｉｅｎｔｉｆｉｃ）中で加熱した：

時間［分］	種類	値[°C]または[rpm]
0	温度	30°
0	速度	960 rpm
0.5	速度	300 rpm
10	温度	95°

【０１６３】

懸濁液または溶液の粘性が２００ ｃＰを越えた直後にプログラムを終了し、デンプンの懸濁液または溶液を、冷却した５０ｍｌ反応容器に移し、２５ｍｌの冷水と混合し、１０分間氷浴中でほぼ室温（ＲＴ）になるまで冷却した。次に、溶解しなかったグルカンのペレットを遠心（２０分、２３００ｇ、室温）して形成させ、清澄な上清を１０ｍｇ ＮａＣｌ／ｍｌで処理した。溶解したグルカンを次に氷上で８０％エタノールから沈殿させ、エタノールで再洗浄し、３７℃で３日間かけて乾燥した。次にこのペレットをすりつぶし（１５秒、３０Ｈｚ、Ｒｅｔｓｃｈ、Ｇｅｒｍａｎｙから入手した振動ミル中でタングステンカーバイドボールを使用）、２５ｍｇのペレットを５００μｌの０．７Ｍ ＨＣｌに懸濁して、９５℃で４時間かけて加水分解を行った。不溶性粒子を次に遠心して除き、グルコース－６Ｐ含量を、酵素を用いて決定した（「グルコース単量体のＣ６位におけるリン酸含量の決定」を参照）。

【０１６４】

コムギを対象とした小規模吸水試験の手順：

材料

スクリューキャップ付きのエッペンドルフカップ（２ｍｌ；Ｓａｒｓｔｅｄ）
エッペンドルフ遠心機（Ｓｉｇｍａ ２０２）
ボルテックス（Ｗｉｌｔｅｎ）
化学天秤（Ｍｅｔｔｌｅｒ）

方法

エッペンドルフカップの重量を化学天秤で小数点以下４桁まで測定する。
カップに１００～１１０ｍｇのコムギを満たし、正確な重量を記載する。
コムギを詰めたカップをボルテックスミキサーで撹拌し、撹拌しながら１．０ｍｌの水を加える。
１０秒間撹拌を続ける。
０．９ｍｌの水を加え、カップをスクリューキャップで閉じる。
１０分間静置する。
カップを遠心機内の６＊１０ローター（スイング型）に据える。
１０，０００ ｒｐｍで３０分間遠心を行う（約８，６００＊ｇ）。
カップから水を除く。
カップの内側の水分を濾紙を用いて除く（水分を含んだ残渣がはがれないように注意する）。
水分を含んだコムギを含むエッペンドルフカップの重量を化学天秤で小数点以下４桁まで

測定する。
吸収された水の重量を計算する。
個々のコムギの吸水量を１０回決定して平均値を得る。
【０１６５】
差分走査熱量測定：｛ｔｃ「差分走査熱量測定」＼１２｝
差分走査熱量測定（ＤＳＣ）は、ブレッドクラムに関して熱の熱転移を調べる手法である。
【０１６６】
この手法を用いることで、試料への熱流が連続的な（比較的低い）加熱速度で決定される。ピーク（ベースラインとの差（吸熱））またはベースラインにおける段階（ガラス転移）のいずれかにより転移が決定される。
【０１６７】
ベースラインにピーク面積を組み入れることで転移のエンタルピーが得られる。ブレッドクラムについては、アミロペクチンの融解熱として４０～７０℃（ピークは６０℃）、またアミロース－脂質の複合体の融解（ｄｅｃｏｍｐｌｅｘａｔｉｏｎ）として約１２０℃が一般に観察されている。
【０１６８】
さまざまなコムギ試料間にみられるアミロペクチンの融解熱に差があることは、老化挙動に差があることを意味し、ひいてはパンが傷むことを意味する。アミロペクチン結晶の融合のエンタルピーは、パンを焼いた後の保存期間が長くなるほど大きくなる。保存中における融合のエンタルピーの上昇は、時間の経過とともに再結晶したアミロペクチン量の増加の尺度の一つである。含水量の差を補正するために、同エンタルピー（ｊ／ｇ）は、乾燥重量ベースで計算した。
【０１６９】
ＤＳＣ測定は、熱量計（ＴＡ Ｉｎｓｔｒｕｍｅｎｔｓ Ｔｙｐｅ ２９２０）にＬＮＣＡ冷却装置を接続して行った。この冷却装置は、実験後の急速冷却用に液体窒素を用いる。
【０１７０】
試料（４０～５０ｍｇのブレッドクラム）をＤＳＣパン上に据え、手の爪を使って押しつぶした。ＤＳＣパンを圧縮装置で密封した後に試料をＤＳＣ装置内に据えた。
【０１７１】
試料（４０～５０ｍｇのブレッドクラム）を、ステンレス鋼製の密封状態のパン（Ｐｅｒｋｉｎ Ｅｌｍｅｒ）内で２０℃から１６０℃に７．５℃／分の速度で加熱した。１００ｍｇのアルミニウムを充填した中程度の圧力パン（容量１２０ μｌ）を参照用に使用した。
【０１７２】
ミニローフのクラムのエンタルピーを、保存開始から１日後、３日後、７日後、および１４日後に１回測定した。
【０１７３】
以下の実施例は本発明を説明するためのものであって、何ら制限する意図はない。
【０１７４】
実施例１
コムギ植物体の形質転換用ベクターｐＵｂｉＲ１の作製
ベクターｐＵｂｉＲ１を作製するために、最初にベクターｐＵｂｉ．ｃａｓを以下の手順で作製した：ベクターｐＵｂｉ２ａｆｐ（図２参照）を、制限酵素ＰｓｔＩ／ＥｃｏＲＩで部分切断した。結果として得られた４．１９ Ｋｂの断片（ｐＵＣ１９ベクター（Ｙａｎｎｉｓｈ－Ｐｅｒｒｏｎら、１９８５、Ｇｅｎｅ ３３：１０３～１１９）、１．５ Ｋｂのユビキチンプロモーター、ならびにｕｂｉ遺伝子の第一エキソン、および短縮型のユビキチン１イントロン（ＣｌａＩ欠損）（Ｃｈｒｉｓｔｅｎｓｅｎら、Ｐｌａｎｔ Ｍｏｌ．Ｂｉｏｌ． １８、（１９９２）、６７５～６８９）を含む）をゲルから溶出して使用した。

【０１７５】
ｎｏｓターミネーターをＰｓｔＩ／ＥｃｏＲＩ断片として、ベクターｐＡｃｔ．ｃａｓ（Ｐｒｏｄｕｃｔ Ｎｕｍｂｅｒ Ｃａｍｂｉａ ＴＧ００６３、Ｃａｍｂｉａ、ＧＰＯ Ｂｏｘ ３２００、Ｃａｎｂｅｒｒａ ＡＣＴ ２６０１、Ａｕｓｔｒａｌｉａから入手）から単離し、２つの断片を連結させてベクターｐＵｂｉ．ｃａｓを得た。
【０１７６】
次に、ジャガイモのＲ１遺伝子（配列番号：１）のｃＤＮＡを、部分切断断片（ＳｍａＩ／Ａｓｐ７１８断片）としてベクターｐＲＬ２（国際公開公報第９７／１１１８８号、実施例４、Ｇｅｒｍａｎ Ｃｏｌｌｅｃｔｉｏｎ ｆｏｒ ＭｉｃｒｏｏｒｇａｎｉｓｍｓにＤＳＭ番号１０２２５として登録）から単離し、ベクターｐＵｂｉ．ｃａｓ（ＳｍａＩ／Ａｓｐ７１８で切断）に組み込んだ。結果として得られたコンストラクトをｐＵｂｉＲｑと命名した（図３参照）。このコンストラクトは、ジャガイモのＲ１ ｃＤＮＡのコード領域を、ユビキチンプロモーター（Ｃｈｒｉｓｔｅｎｓｅｎら、１９９２、Ｐｌａｎｔ Ｍｏｌ．Ｂｉｏｌ． １８：６７５～６８９）の制御下に含むほか、付加的な調節単位としてｕｂｉ遺伝子（Ｃｈｒｉｓｔｅｎｓｅｎら、Ｐｌａｎｔ Ｍｏｌ．Ｂｉｏｌ． １８、（１９９２）、６７５～６８９）の第一エキソン、および短縮型の第１イントロン（Ｃｈｒｉｓｔｅｎｓｅｎら、Ｐｌａｎｔ Ｍｏｌ．Ｂｉｏｌ． １８、（１９９２）、６７５～６８９）（内部のＣｌａＩ断片が欠損したもの）も含む。
【０１７７】
実施例２
ジャガイモ（Ｓｏｌａｎｕｍ ｔｕｂｅｒｏｓｕｍ）のＲ１遺伝子を発現するコムギ植物体の作製、および同植物体のデンプンのリン酸含量の解析
コムギの形質転換には微粒子銃による形質転換法を用いた（Ｂｅｃｋｅｒら、Ｐｌａｎｔ Ｊ． ５ （２）、（１９９４）、２２９～３０７）。ベクターｐＵｂｉＲ１、および微粒子銃による同時形質転換用にはベクターｐ３５ＳＡｃＳ（図４参照）、またはベクターｐＡｃｔ１－Ｆｎｅｏ／Ｃａｌｇｕｓ （Ｍｕｌｌｅｒら、Ｐｌａｎｔ Ｓｃｉｅｎｃｅ １１４、（１９９６）、７１～８２、図５参照）をＤＮＡ粒子沈殿バッチ中で同一のモル比で使用した。ベクターｐ３５ＳＡｃＳは以下の手順で作製した：土壌細菌の１種（Ｓ．ｖｉｒｉｄｏｃｈｒｏｍｏｇｅｎｅｓ）のｐａｔ遺伝子（Ｗｏｈｌｌｅｂｅｎら、Ｇｅｎｅ ７０、（１９８８）、２５～３７）をポリメラーゼ連鎖反応で増幅した。使用したプライマーは、ＢａｍＨＩ切断部位が増幅産物の両側に生じるように設計した。次にＢａｍＨＩ断片をベクターｐＤＨ５１ （Ｐｉｅｔｚｒａｋら、Ｎｕｃｌｅｉｃ Ａｃｉｄ Ｒｅｓ． １４、（１９８６）、５８５７～５８６８）の３５Ｓプロモーターと３５Ｓターミネーターの間にあるＢａｍＨＩ切断部位にクローン化した。３５Ｓプロモーター、ｐａｔ遺伝子、および３５Ｓターミネーターを含むカセットをＥｃｏＲＩ断片として切り出し、ベクターｐＵＣ１８ （Ｐｉｅｔｚｒａｋら、Ｎｕｃｌｅｉｃ Ａｃｉｄ Ｒｅｓ． １４、（１９８６）、５８５７～５８６８）のＥｃｏＲＩ切断部位にクローン化した。
【０１７８】
１４日齢のコムギ植物体の未熟胚である胚盤を形質転換の標的細胞として使用した。形質転換後に、２ｍｇ／ｌの２，４－Ｄ （＝２，４－ジクロロフェノキシ酢酸）を含むＭＳ⁻培地（ＰＣＴ／ＥＰ９７／０２７９３）でインビトロ培養を行った。形質転換の２週間後に継代培養を同じ培地で行った。同培地には、２ｍｇ／ｌ ホスフィノトリシン、または１５０ｍｇ／ｌ 硫酸カナマイシンを事前に添加しておいた。さらに２週間後に、発生中のカルスを再生用培地（０．１ｍｇ／ｌ ２，４－Ｄ、および２ｍｇ／ｌ ＰＰＴ、または１５０ｍｇ／ｌ カナマイシンを含むＭＳ⁻培地）に移した。発生中のシュートを、２，４－Ｄ、およびホスフィノトリシンまたはカナマイシンを含まない半濃縮状態のＭＳ⁻培地に移した後に、土壌に移植した。土壌に移植してから約１４日後に、それぞれ１５０ｍｇ／ｌおよび２００ｍｇ／ｌのホスフィノトリシン、０．１％ Ｔｗｅｅｎ ２０ （ＩＣＩ Ａｍｅｒｉｃａ、ポリソルベート２０に対応）を含む水溶液を２回噴霧するか、２．５％ 硫酸カナマイシン、０．１％ Ｔｗｅｅｎ ２０を２回噴霧するかしてトランスジ

ェニック植物体を同定した。

【０１７９】

T_0植物体におけるジャガイモのＲ１遺伝子の発現

トランスジェニックコムギのT_0植物体におけるジャガイモのＲ１遺伝子の発現は、ノーザンブロッティング解析およびウェスタンブロッティング解析により、また穎花のデンプンのグルコース単量体のＣ６位におけるリン酸含量の酵素学的測定によって検出した（Ｎｉｅｌｓｅｎら、Ｐｌａｎｔ Ｐｈｙｓｉｏｌ．１０５、（１９９４）、１１１～１１７）。Ｒ１タンパク質は、抗ジャガイモＲ１タンパク質抗体（Ｒｉｔｔｅら、Ｐｌａｎｔ Ｊ．２１（４）、（２０００）、３８７～３９１）を用いてトランスジェニックコムギ植物体で検出した。約２０日齢の未成熟の穎花の胚乳に由来するタンパク質抽出物を対象に、トランスジェニック植物体のスクリーニングを行った。

【０１８０】

トランスジェニックT_0植物体のデンプンを未成熟のコムギ穎花および成熟したコムギ穎花から単離して、Ｃ６リン酸量を決定した。穎花は、乳鉢で摩砕して粉末状にした。１５ｍｌの１００ｍＭ Ｔｒｉｓ緩衝液、ｐＨ ８．０を添加後、懸濁液を１００μｍのフィルターで濾過し、遠心（２６００ｇ、５分、４℃）してデンプンをペレットとして得た。上清は廃棄した。次に、このデンプンのペレットを、２ｍｌの１００ｍＭ Ｔｒｉｓ緩衝液、ｐＨ ８．０に再懸濁し、８ｍｌのパーコール（Ｐｅｒｃｏｌｌ）勾配に移した。デンプンを、１７０ｇで４℃で１５分間遠心してペレットとして得た。次に、このデンプンのペレットを、１０ｍｌの１００ｍＭ Ｔｒｉｓ緩衝液、ｐＨ ８．０で３回洗浄した。最後に、このデンプンをアセトンとインキュベートして脱脂し、乾燥した。

【０１８１】

Ｃ６ Ｐ含量は、光学的－酵素学的試験（Ｎｉｅｌｓｅｎら、Ｐｌａｎｔ Ｐｈｙｓｉｏｌ．１０５、（１９９４）、１１１～１１７）によるグルコース－６－リン酸を測定することにより上述の手順で決定した。

【０１８２】

この試験の結果、サザンブロッティング解析で陽性結果を示すコムギのT_0植物体のうち約５０％の系列では、穎花で、対応する遺伝的に修飾されていないフロリダ種の野生型植物体のデンプンと比較して、グルコース単量体のＣ６位におけるリン酸含量が高くなったデンプンが合成されることがわかった。選択した一部の系列のデータを表１に示す。

【０１８３】

天然の子孫の解析

Ｒ１を発現する親系列から天然に得られた種子を蒔き、分離比を決定した。Ｒ１遺伝子の組込みと、さらには発現の両方を、さまざまな系列のT_1子孫を対象に、サザンブロッティング解析およびノーザンブロッティング解析で検出することができた。これらの系列の、デンプンのＣ６位におけるリン酸含量を決定した。数種の選択された系列のデータを表１に示す。

【表１】

系列番号	C6 P (nmol/mg)	世代	標準偏差
野生型(フロリダ種)	非検出	-	0.0
variety			
19	2.8	T0	0.2
20-25	6.7	T1	0.2
37	1.4	T0	0.3
40A-11-8	10.7	T2	0.7

【０１８４】
実施例３
ジャガイモのＲ１遺伝子を発現するトランスジェニックコムギ植物体のデンプンを対象としたＲＶＡ解析、およびゲル強度の調査
実施例１および２に記載されたコムギ植物体のデンプンを対象にＲＶＡ解析を行った。
【０１８５】
ＲＶＡ解析の結果（実験法参照）、ジャガイモのＲ１遺伝子を発現するトランスジェニックコムギ植物体のデンプンの粘性挙動が、対応する遺伝的に修飾されていない野生型のコムギ植物体（フロリダ種）のデンプンと比較して大きく修飾されていたことがわかる（表２参照）。
【０１８６】
【表２】

系列番号	RVA Max (%)	RVA Min (%)	RVA Fin (%)	RVA Set (%)	RVA T (%)
野生型	100	100	100	100	100
(フロリダ種)					
19 (T0)	98.7	113.8	116.8	120.7	102
20-25 (T1)	155.3	190	193.8	198.8	99
37 (T0)	143.8	156.2	148.8	139	101
40-11-8 (T2)	156.2	185.5	187.7	190.6	97

【０１８７】
レジェンド：
ＲＶＡ＝ラピッドビスコアナライザー（Ｒａｐｉｄ Ｖｉｓｃｏ Ａｎａｌｙｚｅｒ）
Ｍａｘ＝図１参照
Ｍｉｎ＝図１参照
Ｆｉｎ＝最終粘性、図１参照
Ｓｅｔ＝Ｓｅｔｂａｃｋ＝最小値と最終値の差、図１参照
Ｔ＝膠着温度、図１参照
パーセンテージは、野生型（＝１００％）に対する値
【０１８８】
ジャガイモのＲ１遺伝子を発現するトランスジェニックコムギ植物体のデンプンのゲル強

度（ゲル強度の決定：方法参照）は、対応する遺伝的に修飾されていない野生型コムギ植物体（フロリダ種）のデンプンと、後に化学的にリン酸化された野生型のデンプン（フロリダ種）の両方と異なっていた（表３参照）。

【０１８９】

グルコース単量体のＣ６位におけるリン酸含量が同等である化学的にリン酸化されたデンプンとは対照的に、膨着後における、本発明のデンプンのゲルは、野生型植物体のデンプンのゲルと比較して高いゲル強度を示した。この結果とは対照的に、リム（Ｌｉｍ）による方法で作製された化学的にリン酸化されたデンプンは、野生型植物体のデンプンのゲルと同等であった。

【０１９０】

【表３】

系列番号	ゲル強度 (TA) (%)	C6位におけるリン酸含量 (デンプン1g あたりのリン酸含量(μmol))
野生型(フロリダ種)	100	非検出
19 (T0)	167	2.8
20-25 (T1)	192	6.7
37 (T0)	168	1.4
40-11-8 (T2)	224	10.7
化学的にリン酸化された野生型(フロリダ種)のデンプン		
STMP1	84	2.1
STMP6	75	6.5
STMP8	67	11.5

ＴＡ＝テクスチャーアナライザー

パーセンテージは、野生型（＝１００％）に対する値

【０１９１】

実施例４

ジャガイモのＲ１遺伝子を発現するトランスジェニックコムギ植物体のデンプンを対象としたＤＳＣ解析

実施例１および２に記載されたコムギ植物体のデンプンを対象にＤＳＣ解析を実施した。

【０１９２】

ピーク温度は、大容量のカプセルを用いて、Ｐｅｒｋｉｎ Ｅｌｍｅｒから入手した装置（装置番号：ＤＳＣ－７）で決定した（調査対象のカプセルは、デンプンと総含水量を約１：４の比で含み、測定は、１０～１６０℃の温度範囲を対象に１０℃／分の加熱速度で行った）

【０１９３】

ＤＳＣ解析の結果（表４）から、ジャガイモのＲ１遺伝子を発現するトランスジェニックコムギ植物体のデンプンのピーク温度が、対応する遺伝的に修飾されていない野生型コムギ植物体（フロリダ種）のデンプンと比較して、また後に化学的にリン酸化されたグルコース単量体のＣ６位におけるリン酸含量が同等のデンプンとも比較して、低いピーク温度Ｔｐを示すことわかる。

【０１９４】
【表４】

試料	試料の含水量(%)	デンプン(乾燥重量):水(総含水量)	ピーク温度(°C;第一ピーク極大)	他のピーク極大(°C)
野生型(フロリダ種)	16.6	1:4.01	61	101
19 (T0)	10.5	1:3.99	58	100
20-25 (T1)	16.9	1:4.05	55	100
40-11-8 (T2)	16.9	1:3.98	56	100
STMP2*	10.9	1:4.00	60	101
STMP6	10.7	1:4.00	61	101

＊化学的にリン酸化されたデンプンであるＳＴＭＰ２の、Ｃ６位におけるリン酸含量は３．０μｍｏｌ リン酸／ｇ デンプンであった。他のすべてのリン酸に関するデータは表３に示す。

【０１９５】
実施例５
さまざまなデンプンのアミロース成分のＣ６ Ｐ含量の決定
ジャガイモのＲ１遺伝子を発現するトランスジェニックコムギ植物体のデンプンのほかに、２種の化学的にリン酸化されたデンプン（ＳＴＭＰ３およびＳＴＰＰ４）を調べた。化学的リン酸化は、リム（Ｌｉｍ）およびセイブ（Ｓｅｉｂ）（Ｃｅｒｅａｌ Ｃｈｅｍ．７０（２）、（１９９３）、１３７～１４４）に記載された方法で行った（ＳＴＭＰ３は、デンプンをｐＨ ６で２時間かけて反応させて作製し、ＳＴＰＰ４は、ｐＨ ８で３０分間かけて反応させて作製した）。ＳＴＭＰという省略形は、「選択リン酸化試薬（ｓｅｌｅｃｔｅｄ ｐｈｏｓｐｈｏｒｙｌａｔｉｏｎ ｒｅａｇｅｎｔ）すなわちトリメタリン酸ナトリウム」を表し、ＳＴＰＰは、トリポリリン酸ナトリウムを表す。

【０１９６】
さまざまなデンプンのアミロース成分は、上述の手順（水性リーチングによるデンプンのアミロース成分の単離）で単離した。Ｃ６位におけるリン酸含量は、上述の手順（グルコース単量体のＣ６位におけるリン酸含量の決定）で決定した。

【０１９７】
【表５】

試料	C6 P含量(μmol リン酸/g デンプン)	C6 P含量(μmol リン酸/g デンプン)	アミロースのC6 P含量/デンプンのC6 P含量
40-11-8 (T2)	10.7	1.9	0.18
STPP4	20.5	20.4	0.99
STMP3	6.0	5.1	0.85

化学的にリン酸化されたデンプンのＣ６ Ｐ含量に対するアミロースのＣ６ Ｐ含量の比は、ジャガイモのＲ１遺伝子を過剰に発現するトランスジェニックコムギ植物体のデンプン

よりかなり大きいと考えられる。化学的にリン酸化されたデンプンは、リン酸化されたアミロースの量が、トランスジェニックコムギ植物体のデンプンと比べて多い。

【０１９８】

実施例６

さまざまな起源をもつ小麦粉から作製されたマイクロローフのブレッドクラムを対象としたＤＳＣ解析

コムギの野生型植物体（フロリダ種）に由来する小麦粉、およびジャガイモのＲ１遺伝子を過剰に発現する遺伝的に修飾されたコムギ植物体に由来する小麦粉を用いて、以下の手順でマイクロローフを作製した：

小麦粉	１０．００ ｇ
酵母	０．２５ ｇ
塩	０．２０ ｇ
デキストロース	０．１０ ｇ
アスコルビン酸	２０ ｐｐｍ

水　吸水試験で決定され、硬度について補正された水量（方法の「小麦粉を対象とした小規模吸水試験の手順」を参照）

【０１９９】

遺伝的に修飾されたコムギ植物体のパン生地は、混合過程中に非常に粘性が高くなった。適切な取り扱い特性をもつパン生地を得るために少量の水を使用した。パン生地の硬度は、パン生地の堅さの技術的な知覚によって補正した。

【０２００】

パン生地は、プルーフィングキャビネット内で４５分間（２８℃）保つことで酵母のはたらきを調べ、次に手を使って１５分間（２８℃）かけて酵母のはたらきを調べ、型を整えてスズ製のパン型に入れた。次にパン生地を対象に、６０分間（２８℃）かけて最終的に酵母のはたらきを調べた。このローフを、湿らせた木枠にパン型を埋込んだオーブンで１５分間（２００℃）かけて焼いた。このように埋込むことで、パン型が多量の放射熱から保護されるようにした。最後に、ローフを木枠なしで５分間かけて焼き、パン生地を膨らませた。

【０２０１】

差分走査熱量測定（ＤＳＣ）（方法参照）で、ブレッドクラムの熱転移を決定した。ローフのクラムのエンタルピーを、保存開始から１日後、３日後、７日、および１４日後に測定した（図６）。

【０２０２】

図６は、保存開始から１日後、３日後、７日、および１３日後における保存されたブレッドクラムにおけるアミロペクチンの融合のピーク（４０～７０℃）のエンタルピーを示しており、対象が傷んでいることがわかる。野生型（フロリダ種）の小麦粉から作ったローフのブレッドクラムのエンタルピーは、ジャガイモのＲ１遺伝子を過剰に発現する遺伝的に修飾されたコムギ植物体の２種の異なる系列（ＧＭＯ１およびＧＭＯ２）の小麦粉から作った同齢のブレッドクラムより高い。これは、再結晶デンプンのレベルが低く、デンプンの老化が弱く、またパンがそれほど傷んでいないことを意味する。

【０２０３】

配列プロトコル：

配列番号．1 (ジャガイモのR1 cDNA、5061 bpのヌクレオチド配列、コード領域: 216～4607 bp):

gaattgtaatacgactcactatagggcgaattgggtaccgggccccccctcgaggtcgacggtatcgataagctt
gatatcgaattcgcggccgcttttgcttcgtgaattcatcttcatcgaatttctcgacgcttcttcgctaatttcctcgttact
tcactagaaatcgacgtttctagctgaacttgagtgaattaagccagtgggaggatatgagtaattccttagggaat
aacttgctgtaccagggattcctaacctcaacagtgttggaacataaaagtagaatcagtcctccttgtgttggagg
caattctttgtttcaacaacaagtgatctcgaaatcacctttatcaactgagtttcgaggtaacaggttaaaggtgca
gaaaaagaaaatacctatgggaaagaaccgtgctttttctagttctcctcatgctgtacttaccactgatacctcttct
gagctagcagaaaagttcagtctagaagggaatattgagctacaggttgatgttaggcctcccacttcaggtgatg
tgtcctttgtggatttttcaagctacaaatggtagtgataaactgtttttgcactgggggggcagtaaagttcggaaaag
aaacatggtctcttcctaatgatcgtccagatgggaccaaagtgtacaagaacaaagcacttagaactccatttgtt
aaatctggctctaactccatcctgagactggagatacgggacactgctatcgaagctattgagtttctcatatacgat
gaagcctacgataaatggataaagaataatggtggcaattttcgtgtcaaattgtcaagaaaagagatacgagg
cccagatgtttcagttcctgaggagcttgtacagatccaatcatatttgaggtgggagaggaagggaaaacagaa
ttacacccctgagaaagagaaggaggaatatgaggctgctcgaactgagctacaggaggaaatagctcgtggt
gcttccatacaggacattcgagcaaggctaacaaaaactaatgataaaagtcaaagcaaagaagagcctcttc

gccgaactatcctccagaaaagcaaattgaagaactcgaagaagcaagaagagaattgcaacttgagcttga
gaaaggcattacccttgatgagttgcggaaaaagattacaaaaggggagataaaaactaaggcggaaaagc
acgtgaaaagaagctcttttgccgttgaaagaatccaaagaaagaagagagactttgggcagcttattaataagt
atccttccagtcctgcagtacaagtacaaaaggtcttggaagaaccaccagccttatctaaaattaagctgtatgc
caaggagaaggaggagcagattgatgatccgatccttaataaaaagatctttaaggtcgatgatggggagctac
tggtactggtagcaaagtcctctgggaagacaaaagtacatatagctacagatctgaatcagccaattactcttca
ctgggcattatccaaaagtcgtggagagtggatggtaccaccttcaagcatattgcctcctggatcaattattttaga
caaggctgccgaaacacctttttccgccagttcttctgatggtctaacttctaaggtacaatctttggatatagtaattg
aagatggcaattttgtggggatgccatttgttcttttgtctggtgaaaaatggattaagaaccaagggtcggatttctat
gttgacttcagtgctgcatccaaattagcactcaaggctgctggggatggcagtggaactgcaaagtctttactgg
ataaaatagcagatatggaaagtgaggctcagaagtcatttatgcaccggtttaatattgctgctgacttgatagaa
gatgccactagtgctggtgaacttggttttactggaattcttgtatggatgaggttcatggctacaaggcaactgatat
ggaacaaaaactataacgtaaaaccacgtgaaataagcaaggctcaggacagacttacagacttgttgcaga
atgctttcaccagtcaccctcaataccgtgaaattttgcggatgattatgtcaactgttggacgtggaggtgaaggg
gatgtaggacagcgaattagggatgaaattttggtcatccagaggaaaaatgactgcaagggtggtatgatgga
agaatggcatcagaaattgcataataatactagtcctgatgatgttgtgatctgtcaggcattgattgactacatcaa
gagtgattttgatcttggtgtttattggaaaaccctgaatgagaacggaataacaaaagagcgtcttttgagttatga
ccgtgctatccattctgaaccgaattttagaggagatcaaaagaatggtcttttgcgtgatttaggtcactatatgaga
acattgaaggctgttcattcaggtgcagatcttgagtctgctattgcaaactgcatgggctacaaaactgagggag
aaggctttatggttggagtccagataaatcctgtatcaggcttgccatctggctttcagggcctcctccattttgtcttag
accatgtggaagataaaaatgtggaaactcttcttgagggattgctagaggctcgtgaggagcttaggcccttgctt
ctcaaaccaaacaaccgtctaaaggatctgctgtttttggacatagcacttgattctacagttagaacagcagtaga
aaggggatatgaagaattgaacaacgctaatcctgagaaaatcatgtacttcatctccctcgttcttgaaaatctcg
cactctctgtggacgataatgaagatcttgtttattgcttgaagggatggaatcaagctctttcaatgtccaatggtgg
agacaaccattgggctttatttgcaaaagctgtacttgacagaatccgtcttgcacttgcaagcaaggcagagtgg
taccatcacttattgcagccatctgccgaatatctaggatcaatccttggggtggaccaatgggctttgaacatattta
ctgaagaaattatacgtgctggatcagcagcttcattatcctctcttcttaatagactcgatcccgtgcttcggaaaac
tgcaaatctaggaagttggcagattatcagtccagttgaagccgttggatatgttgtcgttgtggatgagttgctttca
gttcagaatgaaatctacaagaagcccacgatcttagtagcaaactctgttaaaggagaggaggaaattcctgat
ggtgctgttgccctgataacaccagacatgccagatgttctttcacatgtttctgttcgagctagaaatgggaaggttt
gctttgctacatgctttgatcccaatatattggctgacctccaagcaaaggaaggaaggattttgctcttaaagccta
caccttcagacataatctatagtgaggtgaatgagattgagctccaaagttcaagtaacttggtagaagctgaaac

10

20

30

40

atggttggagctaaatcacgtaatattgcatatctgaaaggaaaagtgccttcctcggtgggaattcctacgtcagt
agctcttccatttggagtctttgagaaagtactttcagacgacataaatcagggagtggcaaaagagttgcaaattc
tgacgaaaaaactatctgaaggagacttcagcgctcttggtgaaattcgcacaacgattttagatctttcagcacca
gctcaattggtcaaagagctgaaggaaaagatgcagggttctggcatgccttggcctggtgatgaaggtccaaa
gcggtgggaacaagcatggatggccataaaaaaggtgtgggcttcaaaatggaatgagagagcatacttcag
cacaaggaaggtgaaactggatcatgactatctgtgcatggctgtccttgttcaagaaataataaatgctgattatg
catttgtcattcacacaaccaacccatcttccggagacgactcagaaatatatgccgaggtggtcaggggccttg
gggaaacacttgttggagcttacccaggacgtgctttgagttttatctgcaagaaaaaggatctcaactctcctcaa
gtgttaggttacccaagcaaaccgatcggccttttcataaaaagatctatcatcttccgatctgattccaatggggaa
gatttggaaggttatgccggtgctggcctctacgacagtgtaccaatggatgaggaggaaaaagttgtaattgatt
actcttccgacccattgataactgatggtaacttccgccagacaatcctgtccaacattgctcgtgctggacatgcta
tcgaggagctatatggctctcctcaagacatcgagggtgtagtgagggatggaaagatttatgtcgttcagacaag
acctcagatgtgatcatattctcgttgtatgttgttcagagaagaccatagatgtgatcatattctcatggtatcagatct
gtgaccacttacctcccatgaagttgcctgtatgattatacgtgatccaaagccatcacatcatgttcaccttcagcta
ttggaggagaagtgagaagtaggaattgcaatatgaggaataataagaaaaactttgtagaagttaaattagctg
ggtatgatatagggagaaatgtgtaaacattgtactatatatagtatacacacgcattatgtatttgcattatgcactg
aataatatcgcagcatcaaagaagaaatcctttgagtggtttcaattgccgcggccgcgaattcctgcagcccgg
gggatccactagttctagagcggccgccaccgcggtggagctccagcttttgttccctttagtgagggttaatttt

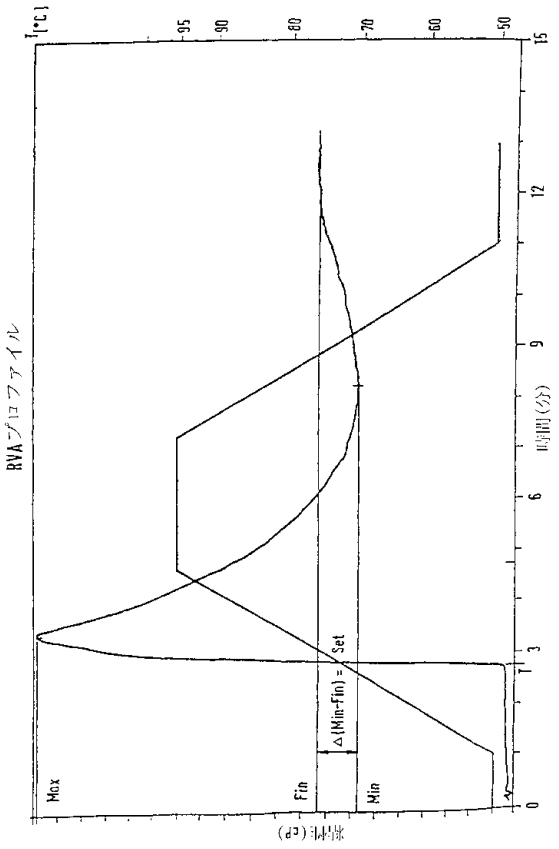
配列番号.2（ジャガイモのR1タンパク質、アミノ酸配列:1464アミノ酸）

MsnslgnnllyqgfltstvlehksrisppcvggnslfqqqviskspIstefrgnrlkvqkkkipMgknrafsssphavl
ttdtsselaekfslegnielqvdvrpptsgdvsfvdfqatngsdklflhwgavkfgketwslpndrpdgtkvyknkal
rtpfvksgsnsilrleirdtaieaiefliydeaydkwiknnggnfrvklsrkeirgpdvsvpeelvqiqsylrwerkgkq
nytpekekeeyeaartelqeeiargasiqdirarltktndksqskeeplhvtkseipddlaqaqayirwekagkpn
yppekqieeleearrelqlelekgitldelrkkitkgeiktkaekhvkrssfaveriqrkkrdfgqlinkypsspavqvq
kvleeppalskiklyakekeeqiddpilnkkifkvddgellvlvakssgktkvhiatdlnqpitlhwalsksrgewMv
ppssilppgsiildkaaetpfsasssdgltskvqsldiviedgnfvgMpfvllsgekwiknqgsdfyvdfsaasklal
kaagdgsgtakslldkiadMeseaqksfMhrfniaadliedatsagelgftgilvwMrfMatrqliwnknynvkpr
eiskaqdrltdllqnaftshpqyreilrMiMstvgrggegdvgqrirdeilviqrkndckggMMeewhqklhnnts
pddvvicqalidyiksdfdlgvywktlnengitkerllsydraihsepnfrgdqkngllrdlghyMrtlkavhsgadle
saiancMgyktegegfMvgvqinpvsglpsgfqgllhfvldhvedknvetllegllearеelrplllkpnnrlkdllfld

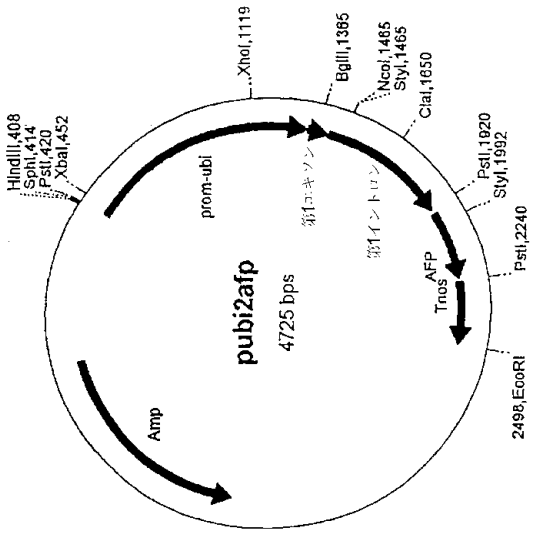
drirlalaskaewyhhllqpsaeylgsilgvdqwalnifteeiiragsaaslssllnrldpvlrktanlgswqiispveav
gyvvvvdellsvqneiykkptilvansvkgeeeipdgavalitpdMpdvlshvsvrarngkvcfatcfdpniladlq
akegrilllkptpsdiiysevneielqsssnlveaetsatlrlvkkqfggcyaisadeftseMvgaksrniaylkgkvp
ssvgiptsvalpfgvfekvlsddinqgvakelqiltkklsegdfsalgeirttildlsapaqlvkelkekMqgsgMpw
pgdegpkrweqawMaikkvwaskwnerayfstrkvkldhdylcMavlvqeiinadyafvihttnpssgddsei
yaevvrglgetlvgaypgralsfickkkdlnspqvlgypskpiglfikrsiifrsdsngedlegyagaglydsvpMde
eekvvidyssdplitdgnfrqtilsniaraghaieelygspqdiegvvrdgkiyvvqtrpqM

【図面の簡単な説明】
【図１】ＲＶＡプロファイルの略図である。
【図２】ベクターｐＵｂｉ２ａｆｐのプラスミドマップである。
【図３】ベクターｐＵｂｉＲ１のプラスミドマップである。
【図４】ベクターｐ３５ＳＡｃＳのプラスミドマップである（ｐＵＣ１８の誘導体（Ｐｉｅｔｒｚａｋ Ｍ.ら、Ｎｕｃｌｅｉｃ Ａｃｉｄｓ Ｒｅｓ. １４、（１９８６）、５８５７～５８６８）：ＣａＭＶ ３５Ｓプロモーターの制御下に、ストレプトマイセス・ビリドクロモジェネス（Ｓｔｒｅｐｔｏｍｙｃｅｓ ｖｉｒｉｄｏｃｈｒｏｍｏｇｅｎｅｓ）に由来するｐａｔ遺伝子を含む（Ｗｏｈｌｌｅｂｅｎら、Ｇｅｎｅ ７０、（１９８８）、２５～３７）。
【図５】ベクターｐＡｃｔ１－Ｆｎｅｏ／Ｃａｌｇｕｓのプラスミドマップである（ｐＵＣ１９誘導体（Ｙａｎｎｉｓｈ－Ｐｅｒｒｏｎら、１９８５、Ｇｅｎｅ ３３：１０３～１１９）：ｐＡｃｔ１－Ｆｎｅｏ（Ｍｕｌｌｅｒら、Ｐｌａｎｔ Ｓｃｉｅｎｃｅ １１４、（１９９６）、７１～８２）、およびＣａＭＶ ３５Ｓプロモーターを含むｐＣａｌｇｕｓ（例えば米国特許Ａ－５，３５２，６０５号を参照）、トウモロコシのＡｄｈ１のイントロン（Ｇｅｎｅｓ Ｄｅｖ １９８７ Ｄｅｃ；１（１０）：１１８３～２００）、ならびにベーターグルクロニダーゼ（ＧＵＳ）遺伝子（植物遺伝子発現研究用ツールとしてのＧＵＳレポーターシステム（Ｔｈｅ ＧＵＳ Ｒｅｐｏｒｔｅｒ Ｓｙｓｔｅｍ ａｓ ａ Ｔｏｏｌ ｔｏ Ｓｔｕｄｙ Ｐｌａｎｔ Ｇｅｎｅ ｅｘｐｒｅｓｓｉｏｎ）：「ＧＵＳプロトコル：遺伝子発現のレポーターとしてＧＵＳ遺伝子を使用（ＧＵＳ Ｐｒｏｔｏｃｏｌｓ：Ｕｓｉｎｇ ｔｈｅ ＧＵＳ Ｇｅｎｅ ａｓ ａ Ｒｅｐｏｒｔｅｒ ｏｆ Ｇｅｎｅ ｅｘｐｒｅｓｓｉｏｎ）」、Ａｃａｄｅｍｉｃ Ｐｒｅｓｓ（１９９２）、２３～４３）から作製）。
【図６】保存開始から１日後、３日後、７日後、および１３日後の間におけるブレッドクラムのエンタルピーを示す。

【図１】


RVAプロファイル
T[°C]
95
90
80
70
60
50
15
12
9
6
3
0
時間(分)
Max
Fin
Min
Δ(Min-Fin) = Set
粘性(cP)

【図２】


HindIII,408
SphI,414
PstI,420
XbaI,452
XhoI,1119
BglII,1385
NcoI,1465
StyI,1465
ClaI,1650
PstI,1920
StyI,1992
PstI,2240
2498,EcoRI
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AFP
Tnos
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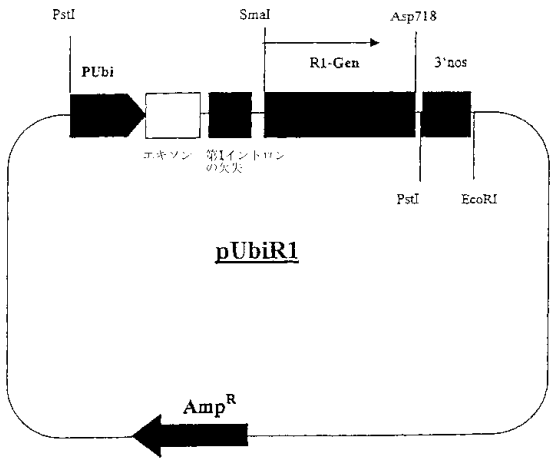
【図３】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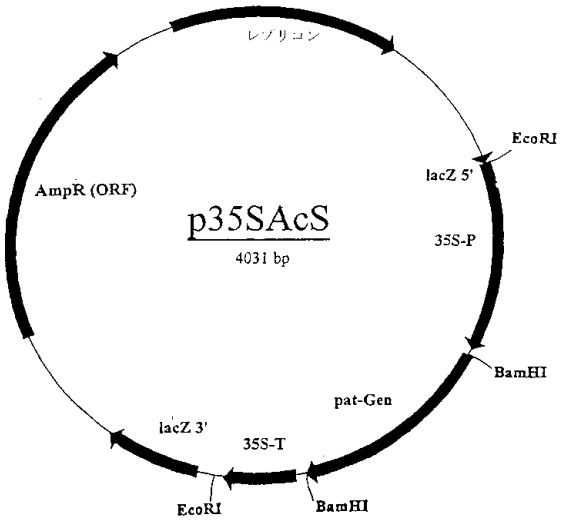
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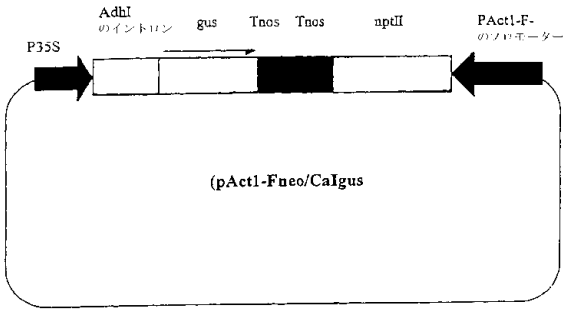
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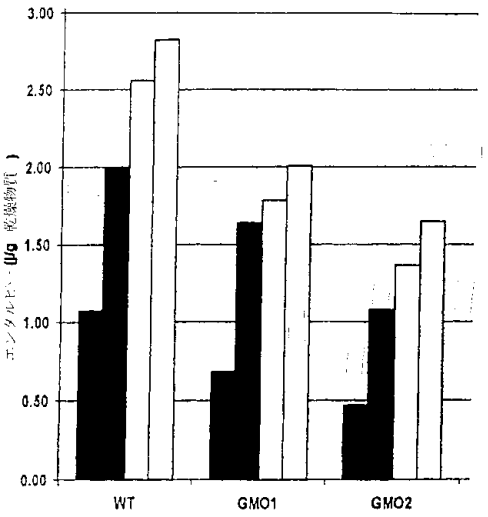
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【国際公開パンフレット】

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(54) Title: MONOCOTYLEDON PLANT CELLS AND PLANTS WHICH SYNTHESISE MODIFIED STARCH

(57) Abstract: The present invention relates to monocotyledon plant cells and plants which are genetically modified, wherein the genetic modification consists of the introduction of an extraneous nucleic acid molecule which codes for a protein with the biological activity of an R1 protein. The present invention further relates to means and methods for the production thereof. Plant cells and plants of this type synthesise a modified starch, which is characterised in that is has an increased phosphate content and/or a modified phosphorylation pattern and/or an increased final viscosity in an RVA profile and/or a reduced peak temperature in DSC analysis and/or an increased gel strength in the texture analysis compared with starch from corresponding non genetically modified monocotyledon plants. Therefore, the present invention also relates to the starch which is synthesised from the plant cells and plants according to the invention, and to methods of producing said starch. The present invention further relates to wheat flours which contain said modified starches, and to food products and bakery products which contain said wheat flours and/or starch.

Description

Monocotyledon plant cells and plants which synthesise modified starch

The present invention relates to monocotyledon plant cells and plants which are genetically modified, wherein the genetic modification consists of the introduction of an extraneous nucleic acid molecule which codes for a protein with the biological activity of an R1 protein. The present invention further relates to means and methods for the production thereof. Plant cells and plants of this type synthesise a modified starch which is characterised in that it has an increased phosphate content and/or a modified phoshorylation pattern and/or an increased final viscosity in an RVA profile and/or a reduced peak temperature in DSC analysis and/or an increased gel strength in the texture analysis compared with starch from corresponding non-genetically modified monocotyledon plants. Therefore, the present invention also relates to the starch which is synthesised from the plant cells and plants according to the invention, and to methods of producing said starch. The present invention further relates to wheat flours which contain said modified starches, and to food products and bakery products which contain said wheat flours and/or starch.

With regard to the increasing importance which has recently been attached to substances of plant content as renewable sources of raw materials, one of the tasks of biotechnological research is to endeavour to adapt these plant raw materials to the requirements of the industry which processes them. To facilitate the use of renewable raw materials in as many fields of use as possible, it is also necessary to provide a considerable multiplicity of substances.

Apart from oils, fats and proteins, polysaccharides constitute the important renewable raw materials from plants. In addition to cellulose, starch, which is one

of the most important storage materials in higher plants, assumes a central position in polysaccharides.

Polysaccharide starch is a polymer of chemically uniform basic components, namely glucose molecules. However, it is a very complex mixture of different forms of molecules, which differ with regard to their degree of polymerisation and the occurrence of branched region in the glucose chains. Starch therefore does not constitute a uniform raw material. A distinction is made between two chemically different components of starch: amylose and amylopectin. In typical plants which are used for starch production, such as maize, wheat or potatoes, the synthesised starch consists of up to about 20% - 30% of amylose starch and up to about 70% - 80% of amylopectin starch.

Amylose was for a long time considered to be a linear polymer consisting of α-1,4-glycosidically bonded α-D-glucose monomers. In more recent studies, however, the presence of about 0.1% of α-1,6-glycosidic branching sites has been detected (Hizukuri and Takagi, Carbohydr. Res. 134, (1984), 1-10; Takeda et al., Carbohydr. Res. 132, (1984), 83-92). Basically, however, achieving a complete separation of amylose from amylopectin very difficult, so that the quality of the amylose is strongly dependent on the type of separation method selected.

In contrast to amylose, amylopectin is more strongly branched and comprises about 4% of branching sites which are formed due to the occurrence of additional α-1,6-glycoside linkages. Amylopectin constitutes a complexes mixture of differently branched glucose chains.

A further significant difference between these two molecules is their molecular weight. Whereas amylose, depending on the origin of the starch, has a molecular weight of $5x10^5 - 10^6$ Da, that of amylopectin ranges between 10^7 and 10^8 Da. These two macromolecules can be by distinguished by their molecular weight and by their different physicochemical properties, which are most readily manifested by their different iodine bonding properties.

A further significant difference between amylose and amylopectin is the relative amounts of trace substances which can exist in association with these macromolecules. Amylose has a high affinity for hydrophobic molecules. In

cereals in particular, amylose can be complexed with relatively high amounts of lipids (Morrison, Cereal Foods World 40, (1995), 437-446). On the other hand, amylopectin can contain covalently bonded inorganic phosphate in the form of starch phosphate monoesters, which has not hitherto been described for amylose. High contents of phosphate monoesters are found in particular in starches which are obtained from tubers. Amongst commercially available starches, potato starch has the highest phosphate content, which can range between 10-30 nmol mg^{-1} starch In some types of Curcuma the phosphate content can even be 2 to 4 times higher (Bay-Smidt et al., 5th ISPMP Meeting Proceedings, (1997), 741), whilst it is about 100 times less in cereals (Kasemsuwan and Jane, Cereal Chem. 73, (1996), 702-707). In contrast to starches from tubers, roots and legumes, the detectable phosphate in cereal starches (monocotyledon plants) rarely occurs in the form of starch monoester derivatives, but mainly occurs in the form of phospholipids (Jane et al., Cereal Foods World 41, (1996), 827-832).

Apart from its amylose/amylopectin ratio and phosphate content, the functional properties of starch are influenced by its molecular weight, its pattern of side chain distribution, its content of ions, its lipid and protein content, etc. Important functional properties which should be cited here are the solubility, the retrogradation behaviour, the water absorption capacity, the film-forming properties, the viscosity, the conglutination properties, the freeze-thaw stability, the stability in relation to acids, the gel strength, etc. The starch grain size can also be important for various applications.

In principle, the phosphate content can be modified either by genetic engineering approaches or by the subsequent chemical phoshorylation of native starches (see, for example: Starch Chemistry and Technology. Eds. R. L. Whistler, J. N. BeMiller and E. F. Paschall. Academic Press, New York, 1988, 349-364). Chemical modifications are generally costly and time-consuming, however, and result in starches, the physicochemical properties of which can differ from those of starches modified in vivo.

Since starches from monocotyledon wild-type plants, particularly from cereal plants (wheat, rice, maize, oats, millet, rye), only have a very low content of

71, (1994), 488), one object of the present invention is to provide monocotyledon plants which synthesise starches with an increased phosphate content (content of starch phosphate monoesters) and modified physicochemical properties compared with corresponding wild-type plant cells and plants.

The underlying object of the present invention is thus to provide genetically modified monocotyledon plant cells and plants which synthesise starches with modified structural and/or functional properties compared with corresponding non-genetically modified wild-type plant cells and plants, and is also to provide starch, the structural and/or functional properties of which differ from those of starch from corresponding non-genetically modified wild-type plant cells and plants and from those of chemically modified starch, and which is thus more suitable for general and/or special industrial purposes of use.

This object is achieved by the provision of the embodiments described in the claims, because it has surprisingly been found that the introduction of an extraneous nucleic acid molecule into the genome of monocotyledon plant cells and plants results in a modification of the structural and/or functional properties of the starch which is synthesised in said monocotyledon plant cells and plants.

Expression of the extraneous nucleic acid molecule is primarily advantageous in starch-storing organs of monocotyledon plants, particularly of wheat plants, and leads to an increase of the phosphate content and modification of the viscosity properties of the starch which can be isolated from the starch-storing organs compared with starches which can be isolated from starch-storing organs of corresponding non-genetically modified wild-type plants, particularly wheat plants. Moreover, the starches according to the invention are distinguished from chemically phosphorylated starches by a modified phoshorylation pattern and modified viscosity properties, and after conglutination of the starches and gel formation are also distinguished by modified gel strengths.

Thus the present invention relates to monocotyledon genetically modified plant

extraneous nucleic acid molecule and the extraneous nucleic acid molecule is selected from the group consisting of:

a) nucleic acid molecules which comprise the coding region of the nucleotide sequence represented in Seq ID No. 1;
b) nucleic acid molecules which encode an R1 protein from Solanum tuberosum with the amino acid sequence given in Seq ID No. 2;
c) nucleic acid molecules which constitute a derivative of the nucleotide sequence given in Seq ID No. 1; and
d) nucleic acid molecules which constitute fragments of the nucleic acid molecules cited in (a), (b) or (c).

In the sense of the present invention, the term "genetically modified" means that the genetic information of the plant cell is modified by the introduction of an extraneous nucleic acid molecule and that the presence or expression of the extraneous nucleic acid molecule results in a phenotypic modification. The term "phenotypic modification" preferably means a measurable modification of one or more functions of the cells. For example, the genetically modified plant cells according to the invention exhibit a modified expression pattern. In the sense of the present invention, the term "genetically modified" means that the monocotyledon plant cell according to the invention contains at least one extraneous nucleic acid molecule which is integrated in the genome in a stable manner.

In the sense of the present invention, the term "extraneous nucleic acid molecule" is to be understood to mean a nucleic acid molecule which codes for a protein with the biological activity of an R1 protein, preferably an R1 protein from Solanum tuberosum, and which does not occur naturally in corresponding non-genetically modified wild-type plant cells. The extraneous nucleic acid molecule is preferably a recombinant molecule which consists of different elements, the combination or specific spatial arrangement of which does not occur naturally in plant cells. The monocotyledon plant cells according to the invention contain at least one extraneous nucleic acid molecule, wherein the latter is preferably linked

to regulatory DNA elements which ensure transcription in plant cells, particularly with a promoter.

An example of an extraneous nucleic acid molecule which codes for an R1 protein from Solanum tuberosum is represented in SEQ ID No.1. Nucleotide sequences which code for an R1 protein from Solanum tuberosum have also been described in WO 97/11188 A1 and by Lorberth et al. (Nature Biotech. 16, (1998), 473-477).

The important characteristics of of R1 proteins from Solanum tuberosum are i) their amino acid sequence (see SEQ ID No. 2, for example); ii) their location in the stroma of plastids of plant cells, wherein they can exist there both in a form bound to starch grains and in soluble form; and iii) their capacity for influencing the degree of phoshorylation of starch in plants. For example, inhibition of the R1 gene which codes for an R1 protein from potatoes in transgenic potato plants results in a reduction of the phosphate content of the starch which can be isolated from the potato tubers. Furthermore, Lorberth et al. showed that the R1 protein from Solanum tuberosum is capable of phosphorylating bacterial glycogen if the corresponding R1 cDNA is expressed in E. coli (Lorberth et al., Nature Biotech. 16, (1998), 473-477).
Ritte et al. (Plant J. 21, (2000), 387-391) showed that the R1 protein from Solanum tuberosum binds reversibly to starch grains in potato plants, wherein the strength of binding to the starch grain depends on the metabolic status of the plant. In starch grain-bound form, the protein in potato plants mainly occurs in leaves which are kept in the dark. After the leaves are illuminated, however, the protein is mainly present in a soluble form which is not bound to starch grains. Moreover, inhibiting the expression of the R1 gene from potatoes in transgenic potato plants results in a "starch-excess" phenotype, i.e. the leaves of corresponding plants have an increased content of starch (Lorberth et al., Nature Biotech. 16, (1998), 473-477). In addition, the tubers of such potato plants are distinguished in that after cold storage they exhibit reduced "cold-induced sweetening" (Lorberth et al., Nature Biotech. 16, (1998), 473-477).

In principle, the extraneous nucleic acid molecule which codes for an R1 protein can originate from any kind of potato or potato plant, preferably from potatoes of the Tomensa, Desiree, Tempora and Thomana varieties.

In one preferred embodiment, the extraneous nucleic acid molecule has the nucleotide sequence represented in SEQ ID No. 1.

In a further preferred embodiment the invention comprises the extraneous nucleic acid molecule of the coding region of the nucleotide sequence represented in SEQ ID No.1.

In a further preferred embodiment of the invention, the extraneous nucleic acid molecule encodes an R1 protein from Solanum tuberosum which has the amino acid sequence given in SEQ ID No. 2.

In yet another embodiment of the invention, the extraneous nucleic acid molecules comprise the coding region of the mature (without a plastidary signal peptide) protein (bp 447-bp 4607 of the nucleotide sequence given in SEQ ID No. 1). Instead of the plastidary N-terminal signal peptide of the R1 protein from potatoes (amino acids 1 to 77 coded by nucleotides 216 to 446 of the nucleotide sequence given in SEQ ID No.1), in this embodiment of the invention the extraneous nucleic acid molecules comprise a heterologous plastidary signal sequence, i.e. a signal sequence which does not exist naturally in association with the mature R1 protein. Plastidary signal sequences are known to one skilled in the art.
The signal sequence of Ferrodoxin:$NADP^+$ oxidoreductase (FNR) from spinate can be used as a plastidary signal sequence, for example. This sequence contains the 5'-non-translated region as well as the flanking transit peptide sequence of the cDNA of the plastidary protein Ferrodoxin:$NADP^+$ oxidoreductase from spinate (nucleotides -171 to + 165; Jansen et al., Current Genetics 13, (1988), 517-522).

In addition, for example, the transit peptide of the waxy protein from maize plus the first 34 amino acids of the mature waxy protein can be used as a signal sequence (Klösgen et al., Mol. Gen. Genet. 217, (1989). 155-161). Furthermore, the transit peptide of the waxy protein from maize can be used without the first 34 amino acids of the mature waxy protein.

In a further preferred embodiment of the invention, the extraneous nucleic acid molecule constitutes a derivative of the nucleotide sequence given in SEQ ID No.1.

In the sense of the present invention, the expression "derivative" means that the sequences of these molecules differ at one or more positions from the nucleotide sequence given in SEQ ID No. 1 and exhibit a high degree of homology with the coding region of the nucleotide sequence given in SEQ ID No. 1. In addition, derivatives are distinguished in that they code for a protein with the biological activity of an R1 protein, preferably of an R1 protein from *Solanum tuberosum*. In the sense of the present invention, "homology" means a sequence identity of nucleotide sequences of at least 65 %, particularly of at least 85 %, more particularly an identity of at least 90 %, preferably more than 95 % and most preferably more than 98 %. Differences from the nucleic acid molecules described above can occur due to deletion, addition, substitution, insertion or recombination.

The degree of homology is preferably determined by comparing the sequence of the respective nucleotide sequence with the coding region of SEQ ID No.1, particularly with region of SEQ ID No.1 (bp447-bp4607) which codes for the mature protein of the nucleotide sequence given in SEQ ID No. 1). If the sequences to be compared are of different lengths, the degree of homology preferably refers to the percentage of nucleotides in the shorter nucleotide sequence which are identical to the nucleotides of the longer sequence, i.e. the sequence identity is determined for the region in which the respective nucleotide sequences overlap. Sequence comparisons can be made using known computer

Research 22, (1994), 4673-4680), which is distributed by Julie Thompson (Thompson@EMBL-Heidelberg.DE) and Toby Gibson (Gibson@EMBL-Heidelberg.DE; European Molecular Biology Laboratory, Meyerhofstrasse 1, D 69117 Heidelberg, Germany). Clustal W can also be downloaded from various internet sites, e.g. that of the IGBMC (Institut de Génétique et de Biologie Moléculaire et Cellulaire, B.P.163, 67404 Illkirch Cedex, France; ftp://ftp-igbmc.u-strasbg.fr/pub/) and that of the EBI (European Bioinformatics Institute) (ftp://ftp.ebi.ac.uk/pub/software/), as well as from internet sites with links to the EBI (European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SD, UK).

When using the ClustalW program (Version 1.8), the default values of the various parameters are employed. In the case of DNA sequence comparisons, these parameters have the following values: KTUPLE=2, TOPDIAGS=4, PAIRGAP=5, DNAMATRIX:IUB, GAPOPEN=10, GAPEXT=5, MAXDIV=40, TRANSITIONS: unweighted.

In the case of protein sequence comparisons the default values of the parameters of the ClustalW program are likewise used. These parameters have the following values: KTUPLE=1, TOPDIAG=5, WINDOW=5, PAIRGAP=3, GAPOPEN=10, GAPEXTEND=0.05, GAPDIST=8, MAXDIV=40, MATRIX=GONNET, ENDGAPS(OFF), NOPGAP, NOHGAP.

The degree of homology can be determined, for example, using known computer programs such as Mview (http://www.sacseeucsf.edu/documentation/seqsoftware/mview; Brown, N.P., Leroy C., Sander C. (1998). MView: A Web compatible database search or multiple alignment viewer. *Bioinformatics*,14(4):380-381).

In the sense of the present invention, "homology" also means that there is functional and/or structural equivalence between the respective nucleic acid molecules or the proteins which they encode. The nucleic acid molecules which are homologous with the nucleic acid molecules described in SEQ ID No.1 and which constitute derivatives of these molecules are variations of these molecules which constitute modifications which perform the same biological function, and

monocotyledon plants. These can be either naturally occurring variations, for example sequences from other types of potatoes, or mutations, wherein said mutations may have been formed naturally or may have been introduced by targeted mutagenesis.

In a further embodiment of the invention, the derivative of SEQ ID No.1 comprises allelic variants of the nucleic acid molecule given in SEQ ID No.1.
These allelic variants are naturally occurring variants which are capable of increasing the phosphate content of starches.

In a further embodiment of the invention, the extraneous nucleic acid molecule constitutes a fragment of the extraneous nucleic acid molecule which is defined according to the invention.

In the sense of the present invention, the term "fragment" denotes parts of the extraneous nucleic acid molecule which code for a biologically active, preferably enzymatically active, part of the R1 protein.
A biologically active part of an R1 protein is distinguished in that *in planta* it can cause an increase of the phosphate content with superexpression of the extraneous nucleic acid molecule and/or that after expression in E. coli it facilitates phoshorylation of glycogen (see WO 97/11188 A1, Example 9).

The proteins which are encoded by the different variants (fragments, derivatives, allelic variants) of the extraneous nucleic acid molecule exhibit defined general characteristics. Examples of the latter include the biological activity, the enzymatic activity, or a similar primary structure which can be investigated by means of the protein homology comparisons described above. The amino acid sequences of the proteins exhibit a mutual homology of at least 60%, preferably at least 85%, particularly at least 95% and most preferably at least 97%.

In addition to the characteristics described above, the extraneous nucleic acid molecules may be characterised in that they code for proteins with the biological

particularly at least 10 and most preferably all of the following characteristic peptide motifs:
DKAAET (SEQ ID No. 3), IADME (SEQ ID No. 4), VWMRFM (SEQ ID No. 5), MQEWHQ (SEQ ID No. 6), LGHYM (SEQ ID No. 7), ERGYEE (SEQ ID No.8), KAVLDR (SEQ ID No. 9), LSSLL (SEQ ID No. 10), IPDGAV (SEQ ID No.11), KVCFAT (SEQ ID No. 12), ISADEF (SEQ ID No. 13), PFGVFE (SEQ ID No. 14), SSGDD (SEQ ID No. 15), SFICKK (SEQ ID No. 16).

Amongst other differences, the plant cells according to the invention can also be distinguished from naturally occurring plant cells in that they contain an extraneous nucleic acid molecule which does not occur naturally in these cells, or that a molecule such as this is integrated at a place in the genome of the cell at which it does not occur naturally, i.e. in a different genomic environment. Moreover, transgenic plant cells of this type according to the invention can be distinguished from naturally occurring plant cells in that they contain at least one copy of the extraneous nucleic acid molecule which is integrated in a stable manner in their genome contain, optionally in addition to copies of a molecule such as this which occur naturally in the cells. If the nucleic acid molecules which are introduced into the cells are additional copies of molecules which already occur naturally in the cells, the plant cells according to the invention can be distinguished in particular from naturally occurring plant cells in that these additional copies are located at sites in the genome at which they do not occur naturally. This can be detected subsequently by means of Southern blotting analysis, for example.
Furthermore, the plant cells according to the invention are preferably distinguished from naturally occurring plant cells by at least one of the following features: if the nucleic acid molecule which is introduced is heterologous with respect to the plant the transgenic plant cells according to the invention comprise transcripts of the nucleic acid molecules which are introduced, in fact even in those organs in which no transcripts can be detected in wild-type plants. Plant cells according to the invention preferably contain transcripts of the extraneous nucleic acid molecules. These can be detected by Northern blotting analysis, for example. Plant cells according to the invention preferably contain a protein which

is encoded by an introduced extraneous nucleic acid molecule. This additional protein can be detected by immunological methods for example, particularly by Western blotting analysis.

In one preferred embodiment, the plant cells and plants according to the invention exhibit an increased biological activity of the R1 protein compared with corresponding non-genetically modified wild-type plant cells and wild-type-plants. In connection with the present invention, an "increased biological activity of the R1 protein" can be determined, for example, by measuring the phosphate content of the starches which are synthesised in the plant cells and plants according to the invention. Compared with starches from corresponding non-genetically modified wild-type plant cells, starches from the plant cells according to the invention, which exhibit increased biological activity of the R1 protein, are distinguished in that they synthesise a starch with an increased phosphate content, preferably an increased phosphate content in the C6 position of the glucose monomer.
Moreover, an increased biological activity of the R1 protein can be determined by measuring the amount of R1-transcripts, e.g. by Northern blotting analysis, compared with the amount of R1-transcripts of corresponding non-genetically modified wild-type plants.
Furthermore, an increased biological activity of the R1 protein can be determined by measuring the amount of R1 protein, e.g. by Western blotting analysis, compared with the amount of R1 protein of corresponding non-genetically modified wild-type plants.

It has been found that the monocotyledon plant cells according to the invention synthesise a starch with an increased phosphate content in the C6 position of the glucose monomer and/or a modified phoshorylation pattern and/or modified viscosity properties compared with starch from corresponding non-genetically modified plant cells of wild-type-plants.

Therefore, the present invention preferably relates to plant cells according to the

C6 position of the glucose monomer and/or a modified phoshorylation pattern and/or modified viscosity properties compared with starch from corresponding non-genetically modified plant cells of wild-type-plants.

In the sense of the present invention, the term "phosphate content" denotes the content of phosphate which is covalently bonded in the form of starch phosphate monoesters.

In the sense of the present invention, the term "C6 position" is to be understood to mean phosphate groups, which are bonded to the glucose monomer of the starch at carbon atom position "6".

In connection with the present invention, the expression "increased phosphate content in the C6 position" is to be understood to mean that glucose monomer phosphate groups can be detected in the in the C6 position by means of an optical-enzymatic test (Nielsen et al., Plant Physiol. 105, (1994), 111-117, see methods). The expression "increased phosphate content in the C6 position" is preferably understood to mean an increase in the phosphate content of the starch in the C6 position of the glucose monomer by at least 20%, preferably by at least 50% and most preferably by at least 100%, compared with the phosphate content of starch from corresponding non-genetically modified wild-type-plants.
In principle, the C2, C3 and C6 positions of the glucose units can be phosphorylated in the starch in vivo. In connection with the present invention, the phosphate content in the C6 position (= C6 P content) can be determined by a glucose-6-phosphate determination by means of an optical-enzymatic test (Nielsen et al., Plant Physiol. 105, (1994), 111-117) (see methods).

In the sense of the present invention, the term "modified phoshorylation pattern means that the total phosphate content of phosphate groups which are covalently bonded to the starch within the various layers of the starch grain is modified compared with chemically phosphorylated starches which are produced from starches from corresponding non-genetically modified plants. Chemically

starch grains, wherein the outer layers are generally more strongly phosphorylated than are the inner layers. In contrast to this, the starches according to the invention are distinguished in that the phosphate groups are distributed differently over the various layers of the starch grain, i.e. the starches according to the invention can be characterised in that they do not comprise the gradients from the outside to the inside which are typical of chemically phosphorylated starches.

Methods of investigatingh the phoshorylation pattern of starch are known to one skilled in the art (see Jane and Shen, Carbohydrate Research 247, (1993), 279-290; Gough and Pybus, Staerke 25, (1973), 123-130, for example). These methods are based on step-wise chemical conglutination of the various layers of the starch grains, in which the conglutinated layers of the starch grains are mechanically removed in steps. This shelling procedure is followed by a determination of the total phosphate content of the various starch grain layers by standard methods.

In the sense of the present invention, the term "chemically phosphorylated starch" is to be understood to mean a starch which is produced by the chemical phoshorylation of native starch from corresponding non-genetically modified plant cells and/or plants. In connection with the present invention, chemically phosphorylated starch is preferably distinguished by an amylose content which is comparable with the amylose content of the starches according to the invention. During the chemical phoshorylation of native starches, the phosphate content in the C6 position is adjusted by selecting suitable test conditions so that the phosphate content in the C6 position of the chemically phosphorylated starch is identical to and/or comparable with the phosphate content in the C6 position of the glucose monomer of the starches according to the invention.

In connection with the present invention, the term "modified viscosity properties" is to be understood in particular to mean an increase in the final viscosity in an RVA profile and/or a lowering of the peak temperature in DSC analysis.

In a further preferred embodiment, the plant cells according to the invention synthesise a starch which has an increased final viscosity compared with starch from corresponding non-genetically modified wild-type plants.

In connection with the present invention, the term "final viscosity" is to be understood to mean the viscosity which can be determined from a viscosity profile (see Figure 1) and which is denoted there as "final viscosity = Fin". The viscosity profile can be obtained by means of a Rapid Visco Analyzer (RVA) (Newport Scientific Pty Ltd, Investment Support Group, Warriewood, NSW 2102, Australia). In the analysis of wheat starches, the viscosity profile is determined by the following procedure: 2.5 g starch (dry substance) are taken up in 25 ml H_2O and used for analysis in a Rapid Visco Analyzer (Newport Scientific Pty Ltd., Investment Support Group, Warriewood NSW 2102, Australia). The instrument is operated according to the manufacturer's instructions. The complete temperature programme is illustrated schematically in Figure 1. Performing an RVA analysis is described in detail below (see methods).

In the sense of the present invention, the term "increased final viscosity" means that the final viscosity is increased by at least 10%, preferably by at least 30%, particularly by at least 50% and most preferably by at least 80% compared with starches from corresponding non-genetically modified wild-type plant cells, wherein the final viscosity can be increased by 1000% at most, preferably by 500% at most, particularly by 250% at most, compared with starches from corresponding non-genetically modified wild-type plant cells.

In a further preferred embodiment, the plant cells according to the invention synthesise a starch which has an identical or comparable phosphate content in the C6 position of the glucose monomer and/or a reduced peak temperature compared with starch from corresponding non-genetically modified wild-type plants and/or compared with chemically phosphorylated starches.

In the context of the present invention, the term "peak temperature" should be

differential scanning calorimetry (DSC), which is known to one skilled in the art. The peak temperature is the temperature which can generally be assigned to the first peak maximum of the DSC curve. It can be determined, for example, using an instrument supplied by Perkin Elmer (instrument designation: DSC-7) using capsules of large volume, wherein the sample to be investigated comprises a ratio of starch to total water content of about 1:4 and measurements are made over a temperature range from 10°C to 160°C at a heating rate of 10°C/min (see Example 4).

The term "reduced peak temperature" Tp means that compared with starches from corresponding non-genetically modified wild-type plant cells the peak temperature Tp is reduced by at least 1.5°C, preferably by at least 2.5°C, particularly by at least 4°C and most preferably by at least 6°C, and is reduced at most by 5°C, by 12°C or by 9°C.

In a further preferred embodiment, the present invention relates to plant cells according to the invention which synthesise a starch which after conglutination forms a gel, which has an increased gel strength compared with a gel formed from starch from corresponding non-genetically modified wild-type plant cells.

In the sense of the present invention, the term "increased gel strength" is to be understood to mean an increase gel strength by at least 20%, particularly by at least 50%, preferably by at least 80% and most preferably by at least 100%, with the maximum reduction being 500% at most or 250% at most compared with the gel strength of starch from corresponding non-genetically modified wild-type plant cells.
In connection with the present invention, the gel strength can be determined by means of a texture analyser under the conditions described below (see methods).

In a further embodiment of the invention, the increase in the phosphate content of the starches can relate to the amylopectin component of the starch.
Therefore, the present invention also relates to plant cells according to the

phosphorylated and the amylose component of which has a reduced total phosphate content compared with the amylose component of corresponding chemically phosphorylated starches with the same starch phosphate content in the C6 position of the glucose monomer.
Therefore, in contrast to chemically phosphorylated starches with the same phosphate content in the C6 position of the glucose monomer, the starches of the plant cells according to the invention are distinguished in that that the amylose component of the starch from the plant cells according to the invention has a reduced total phosphate content and/or a reduced phosphate content in the C6 position of the glucose monomer compared with the amylose component of chemically phosphorylated starch with the same phosphate content in the C6 position of the glucose monomer, which is produced from starch, which preferably has a comparable amylose content, from corresponding non-genetically modified plants.

In connection with the present invention, the term "total phosphate content" is to be understood to mean the content of phosphate which is covalently bound in the form of starch phosphate monoesters in the C2, C3 and C6 positions of the glucose units. According to the invention, the content of phosphorylated non-glucans, such as phospholipids, is not included under the term "total phosphate content". Phosphorylated non-glucans therefore have to be quantitatively separated before the determination of the total phosphate content.
Methods of separating phosphorylated non-glucans (e.g. phospholipids) from starch are known to one skilled in the art.

In the sense of the present invention, the term "reduced total phosphate content" is to be understood to mean a reduction of the total phosphate content by at least 5%, particularly by at least 20%, preferably by at least 50% and most preferably by at least 80%, compared with the total phosphate content of the amylose component of a chemically phosphorylated starch with the same C6 phosphate content which is produced from starch from corresponding non-genetically modified plants.

Methods of determining of the total phosphate content are known to one skilled in the art and are described below (see methods).

In the sense of the present invention, the term "reduced phosphate content in the C6 position of the glucose monomer" should be understood to mean a reduction of the phosphate content in the C6 position of the glucose monomer of the amylose component by at least 10%, particularly by at least 20%, preferably by at least 50% and most preferably by at least 80% compared with the phosphate content in the C6 position of the glucose monomer of the amylose component of a chemically phosphorylated starch of the same C6 phosphate content which is produced from starch from corresponding non-genetically modified plants. Methods of determining of the phosphate content in the C6 position of the glucose monomer are known to one skilled in the art and are described below (see methods).

In the sense of the present invention the term "amylopectin component" is to be understood to mean the amylopectin of the starch.

In the sense of the present invention, term "amylose component" is to be understood to mean the amylose of the starch.

Methods of separating amylose and amylopectin are known to one skilled in the art. For example, the amylose component can be obtained by aqueous leaching of the starch, as described, for example, by Roger & Colonna (International Journal of Biological Macromolecules 19, (1996), 51-61).

In a further preferred embodiment of the present invention, the starches according to the invention are additionally characterised in that the ratio of the C6 phosphate content of the glucose monomer in the C6 position of the amylose component to the C6 phosphate content of the glucose monomer in the C6 position of the starch is less than 0.75, particularly less than 0.5, preferably less than 0.25 and most preferably less than 0.20. In contrast to chemically

distinguished in that the major part of the phosphate in the C6 position of the glucose monomer is detected in the amylopectin component of the starch and not in the amylose component.

The plant cells according to the invention originate from monocotyledon plants. They are preferably plant cells from plants which are used in agriculture, i.e. from plants, which are cultivated by humans for the purpose of nourishment or for technical purposes, particularly industrial purposes. Thus the present invention preferably relates to plant cells from starch-synthesising or starch-storing plants, e.g. rye, barley, oats, wheat, millet, rice or maize.

In one preferred embodiment of the present invention, the plant cells according to the invention originate from a plant of the group consisting of wheat, rice, barley, oats, rye and maize. Plant cells from wheat, rice and maize plants are preferred; plant cells from wheat plants are particularly preferred.

In one particularly preferred embodiment, the plant cells according to the invention synthesise a starch which in the C6 position of the glucose monomer has a phosphate content of at least 0.1 nmol C6 P mg^{-1} starch, particularly at least 0.5 nmol C6 P mg^{-1}, preferably at least 1 nmol C6 P mg^{-1} starch, more preferably at least 2 nmol C6 P mg^{-1} starch, most preferably at least 5 nmol C6 P mg^{-1} starch, most particularly preferably at least 10 nmol C6 P mg^{-1} starch, wherein the plant cells according to the invention synthesise a starch which in the C6 position of the glucose monomer has a phosphate content of 100 nmol C6 P mg^{-1} starch at most, particularly 50 nmol C6 P mg^{-1} starch at most, and most particularly 25 nmol C6 P mg^{-1} starch at most.

In a further embodiment of the invention, the plant cells according to the invention can synthesise a starch which in the C6 position of the glucose monomer has a phosphate content of at least 15 nmol C6 P mg^{-1} starch, wherein the plant cells according to the invention synthesise a starch which in the C6 position of the glucose monomer have a phosphate content of 100 nmol C6 P mg^{-1} starch at

most, particularly 50 nmol C6 P mg^{-1} starch at most, and most particularly 25 nmol C6 P mg^{-1} starch at most.

In a further embodiment, the present invention relates to a method of producing a plant cell according to the invention, wherein a cell of a monocotyledon plant is genetically modified, and wherein the genetic modification consists of the introduction of at least one extraneous nucleic acid molecule.

There is a multiplicity of techniques which are available for the transfer of DNA into a plant host cell. These techniques include the transformation of plant cells with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as a transformation agent, fusion of protoplasts, injection, electroporation of DNA, the introduction of DNA by means of biolistic techniques and other possible techniques.

The use of the transformation of plant cells facilitated by agrobacteria has been intensively investigated and is satisfactorily described in EP 120516; Hoekema, IN: The Binary Plant Vector System Offsetdrukkerij Kanters B.V., Alblasserdam (1985), Chapter V; Fraley et al., Crit. Rev. Plant Sci. 4, 1-46 and An et al. EMBO J. 4, (1985), 277-287. For the transformation of potatoes, see Rocha-Sosa et al., EMBO J. 8, (1989), 29-33.), for example.

The transformation of monocotyledon plants has in the meantime been routinely accomplished by means of a biolistic technique and by means of agrobacteria (Komari et al., (1998), *Advances in cereal gene transfer*; Current Opinion in Plant Biotechnology 1, page 161 et seq.; Bilang et al. (1999), Transformation of Cereals, Genetic Engineering, 12, pages 113-148, edited by: JK Setlow, Kluwer Academic / Plenum Publisher, New York). Vectors based on agrobacteria have been described (Chan et al., Plant Mol. Biol. 22, (1993), 491-506; Hiei et al., Plant J. 6, (1994) 271-282; Deng et al, Science in China 33, (1990), 28-34; Wilmink et al., Plant Cell Reports 11, (1992), 76-80; May et al., Bio/Technology 13, (1995), 486-492; Conner and Domisse, Int. J. Plant Sci. 153 (1992), 550-555; Ritchie et al, Transgenic Res. 2, (1993), 252-265). Alternative systems for the transformation of monocotyledon plants include transformation by means of a

al., Bio/Technology 11 (1993), 1553-1558; Ritala et al., Plant Mol. Biol. 24, (1994), 317-325; Spencer et al., Theor. Appl. Genet. 79, (1990), 625-631), protoplast transformation, electroporation of partially permeabilised cells, and the introduction of DNA by means of glass fibres. In particular, the transformation of maize has been described many times in the literature (see WO 95/06128, EP 0513849, EO 0465875, EP 292435; Fromm et al., Biotechnology 8, (1990), 833-844; Gordon-Kamm et al., Plant Cell 2, (1990), 603-618; Koziel et al., Biotechnology 11 (1993), 194-200; Moroc et al., Theor. Appl. Genet. 80, (1990), 721-726, for example).

The successful transformation of other types of cereals has also been described, e.g. for barley (Wan and Lemaux, see above; Ritala et al., see above; Krens et al., Nature 296, (1982), 72-74) and for wheat (Becker et al., Plant J. 5 (2), (1994), 229-307; Nehra et al., Plant J. 5, (1994), 285-297).

Various transformation methods have been described for rice, e.g. transformation facilitated by agrobacteria (Hiei et al., Plant J. 6 (1994), 271-282; Hiei et al., Plant Mol. Biol. 35 (1997), 205-218; Park et al., J. Plant Biol. 38 (1995), 365-371), protoplast transformation (Datta, In "Gene transfer to plants", Potrykus, Spangenberg (Eds.), Springer-Verlag, Berlin, Heidelberg, 1995, 66-75; Datta et al., Plant Mol. Biol. 20 (1992), 619-629; Sadasivam et al., Plant Cell Rep. 13 (1994), 394-396), a biolistic technique for plant transformation (Li et al., Plant Cell Rep. 12 (1993), 250-255; Cao et al., Plant Cell Rep. 11 (1992), 586-591; Christou, Plant Mol. Biol. (1997), 197-203), and electroporation (Xu et al., In "Gene transfer to plants", Potrykus, Spangenberg (Eds.), Springer-Verlag, Berlin, Heidelberg, 1995, 201-208).

The present invention further relates to plants containing the plant cells according to the invention and/or which can be produced by regeneration from plant cells according to the invention. These plants are preferably useful monocotyledon plants, e.g. rye, barley, oats, wheat, millet, rice and maize. Wheat, rice and maize are preferred; wheat is particularly preferred.

The plants according to the invention synthesise a modified starch, the phosphate

properties of which differ from those of starches from corresponding non-genetically modified wild-type-plants, as described in connection with the plant cells according to the invention.

Therefore, the present invention also relates to plants according to the invention which synthesise a starch, which has an increased phosphate content in the C6 position of the glucose monomer and/or exhibits a modified phoshorylation pattern and/or modified viscosity properties compared with starch from corresponding non-genetically modified plants.
The statements made in connection with the plant cells according to the invention are applicable with regard to the increase of the phosphate content in the C6 position of the glucose monomer of the starch, the modification of the phoshorylation pattern and the modification of the viscosity properties of the starch.
The present invention preferably relates to plants according to the invention which synthesise a starch after conglutination forms a gel, which has an increased gel strength compared with a gel formed from starch from corresponding non-genetically modified wild-type-plants.

The term "increased gel strength" was defined in connection with the plant cells according to the invention.

In a further preferred embodiment of the invention, the plants according to the invention, preferably wheat plants, synthesise a starch, preferably wheat starch, which in the C6 position of the glucose monomer has a phosphate content of at least 0.1 nmol C6 P mg^{-1}starch, particularly at least 0.5 nmol C6 P mg^{-1}, preferably at least 1 nmol C6 P mg^{-1} starch, most preferably at least 2 nmol C6 P mg^{-1} starch, particularly at least 5 nmol C6 P mg^{-1} starch, quite most preferably at least 10 nmol C6 P mg^{-1} starch, wherein the plants according to the invention synthesise a starch which in the C6 position of the glucose monomer has a phosphate content of 100 nmol C6 P mg^{-1} starch at most, particularly 50 nmol C6 P mg^{-1} starch at most, or 25 nmol C6 P mg^{-1} starch at most.

In a further embodiment of the invention, the plants according to the invention can synthesise a starch which in the in the C6 position of the glucose monomer has a phosphate content of at least 15 nmol C6 P mg^{-1} starch, wherein the plants according to the invention synthesise a starch which in the C6 position of the glucose monomer has a phosphate content of 100 nmol C6 P mg^{-1} starch at most, particularly 50 nmol C6 P mg^{-1} starch at most or 25 nmol C6 P mg^{-1} starch at most.

In a further preferred embodiment of the invention, the plants according to the invention synthesise the starch according to the invention in the starch-storing organs of the plant according to the invention.

In a most preferred embodiment, the plants according to the invention synthesises a starch in its starch-storing organs which has an increased phosphate content in the C6 position of the glucose monomer and/or a modified phosphorylation pattern and/or modified viscosity properties compared with starch from starch-storing organs of corresponding non-genetically modified wild-type plants.

In this connection, the expression "starch-storing organs" is to be understood to mean those organs, e.g. the grains of maize, rice and wheat plants, which incorporate stored starch, in contrast to those organs, such as leaves, which only synthesise starch transiently.

Expression of the extraneous nucleic acid molecule is particularly advantageous in starch-storing organs of monocotyledon plants, especially in wheat plants, and results in an increase of the phosphate content of the starches which can be isolated from the starch-storing organs compared with starches which can be isolated from the starch-storing organs of corresponding non-genetically modified wild-type plants, particularly wheat plants.

Expression of the extraneous nucleic acid molecule in starch-storing organs of monocotyledon plants can be achieved firstly by the use of constitutive promoters,

5,352,605, for example) and the ubiquitin promoter of maize (see US-A-5,614,399, for example).

Promoters are preferably used which are specific for starch-storing organs, e.g. endosperm-specific promoters, such as the glutelin promoter (Leisy et al., Plant Mol. Biol. 14, (1990), 41-50; Zheng et al., Plant J. 4, (1993), 357-366; Yoshihara et al., FEBS Lett. 383, (1996), 213-218), the shrunken-1 promoter (Werr et al., EMBO J. 4, (1985), 1373-1380), the HMW promoter of wheat (Anderson, Theoretical and Applied Genetics 96, (1998), 568-576, Thomas, Plant Cell 2 (12), (1990), 1171-1180), the USP promoter, the phaseolin promoter (Sengupta-Gopalan, Proc. Natl. Acad. Sci. USA 82 (1985), 3320-3324, Bustos, Plant Cell 1 (9) (1989), 839-853) or promoters of zein genes from maize (Pedersen et al., Cell 29, (1982), 1015-1026; Quatroccio et al., Plant Mol. Biol. 15 (1990), 81-93) or caryopsis-specific promoters of GBSSI (granule bound starch synthase I) (DE10041861.9) and of SSII (soluble starch synthase II) from wheat (DE10032379.0).

In one particularly preferred embodiment, the plants according to the invention exhibit R1 gene expression in the starch-storing organs of said plants.

R1 gene expression can be determined, for example, by measuring the amount of R1 transcripts, e.g. by Northern blotting analysis, compared with the amount of R1 transcripts of wild-type plants.

Moreover, in connection with the present invention, R1 gene expression can be determined by measuring the amount of R1 protein, e.g. by Western blotting analysis. The amount of R1 protein is preferably determined by means of protein extracts which are isolated from plant cells of endosperm.

In a further embodiment of the invention, the plants according to the invention exhibit R1 gene expression in the starch-storing organs of said plants, which is increased compared with R1 gene expression in starch-storing organs of corresponding non-genetically modified wild-type-plants, preferably by at least

50%, most preferably by at least 100%, particularly by at least 250% and most particularly by at least 500%.

In one most preferred embodiment, the plants according to the invention exhibit a organ-specific expression of the extraneous nucleic acid molecule in the starch-storing organs of the plants according to the invention.

In the sense of the present invention, the term "organ-specific" is to be understood to mean that the selected promoter favours expression of the extraneous nucleic acid molecule in the starch-storing organs compared with mature leaves, and results in significantly increased degree of expression, such as a level of expression which is increased at least 2- to 5 times, preferably 5- to 10 times, most preferably 10- to 100 times.
In this embodiment of the invention, the plants according to the invention are distinguished in that due to the organ-specific expression of the extraneous nucleic acid molecule they exhibit increased R1 gene expression in the starch-storing organs compared with the gene expression of the endogeneous R1 gene in the starch-storing organs of a corresponding non-genetically modified wild-type-plant. In this embodiment of the invention, an increase in R1 gene expression in the leaves of the plants according to the invention cannot be detected to the same extent as that in the starch-storing organs. The increase in R1 gene expression is preferably related solely to the starch-storing organs.

In a preferred embodiment of the invention, the plants according to the invention are plants from the group consisting of wheat, rice, barley, millet, oats, rye and maize. Wheat, rice and maize plants are preferred; wheat plants are particularly preferred.

The invention also relates to a method of producing a plant as defined according to the invention, wherein

(a) a cell of a monocotyledon plant is genetically modified, wherein the genetic modification consists of the introduction of at least one extraneous nucleic

(b) a plant is regenerated from the cell according to step (a); and optionally
(c) further plants are produced from the plant produced according to step (b).

The present invention further relates to a method of producing a plant as defined according to the invention, which in the starch-storing organs of said plant synthesises a starch with an increased phosphate content and/or a modified phoshorylation pattern and/or an increased final viscosity and/or a reduced peak temperature compared with starch from starch-storing organs of wild-type-plants, wherein

(a) a cell of a monocotyledon plant is genetically modified, wherein the genetic modification consists of the introduction of at least one extraneous nucleic acid molecule as defined according to the invention;
(b) a plant is regenerated from the cell according to step (a); and optionally
(c) further plants are produced from the plant produced according to step (b).

The same applies to the genetic modification which is introduced according to step (a) as that which has already been explained above in connection with the plant cells and plants according to the invention.
The regeneration of plants according to step (b) can be accomplished by methods known to one skilled in the art.
The production of further plants according to step (c) of the method according to the invention can be accomplished, for example, by vegetative propagation) e.g. via cuttings, tubers or via callus cultivation and the regeneration of whole plants) or by sexual propagation. Sexual propagation preferably occurs in a controlled manner, i.e. selected plants with defined properties are crossed and propagated with each other.

In one preferred embodiment of the invention, the methods according to the invention are distinguished in that the extraneous nucleic acid molecule which is introduced into the plant cell according to step (a) is selected from the group consisting of:

(i) nucleic acid molecules which comprise the coding region of the nucleotide sequence represented in Seq ID No. 1;

(ii) nucleic acid molecules which encode an R1 protein from Solanum tuberosum with the amino acid sequence given in Seq ID No. 2;
(iii) nucleic acid molecules which constitute a derivative of the nucleotide sequence given in Seq ID No. 1; and
(iv) nucleic acid molecules which constitute fragments of the nucleic acid molecules cited in (i), (ii) or (iii).

In one particularly preferred embodiment, the methods according to the invention are distinguished in that the extraneous nucleic acid molecule is under the control of a promoter, preferably an endosperm-specific and/or caryopsis-specific promoter, which organ-specifically facilitates R1 gene expression in starch-storing tissue.

Promoters of this type, such as endosperm-specific and caryopsis-specific promoters, have been exemplified above in connection with the plants according to the invention.

In a further embodiment, the methods according to the invention relate to useful monocotyledon plants, e.g. rye, barley, oats, wheat, millet, rice and maize. Wheat, rice and maize are preferred; wheat is particularly preferred.

The present invention also relates to the plants which can be obtained by the method according to the invention.

The present invention also relates to propagation material for plants containing plant cells according to the invention, and to the plants produced by the method according to the invention. In the sense of the present invention, the term "propagation material" comprises any constituents of the plant which are suitable for production or propagation by a vegetative or generative route. Cuttings, callus cultures, rhizomes or tubers are suitable for vegetative propagation, for example. Other propagation material comprises fruit, seeds, seedlings, protoplasts, cell cultures etc., for example. Seeds are the preferred propagation material.

In one preferred embodiment, the present invention relates to maize grains.

In a most preferred embodiment, the present invention relates to wheat grains.

The maize and wheat grains according to the invention are particularly suitable for the production of fodder and of food products.

The present invention further relates to the use of extraneous nucleic acid molecules as defined according to the invention for the production of plants according to the invention, preferably of wheat, maize and rice plants, most preferably wheat plants, or for the production of monocotyledon plant cells according to the invention.

The plant cells and plant according to the invention synthesise, particularly in their starch-storing organs, a starch, the physicochemical properties of which, particularly the phosphate content of and/or the viscosity behaviour of which and/or the phoshorylation pattern of which is modified compared with that of starch synthesised in wild-type-plants and compared with chemically phosphorylated starches.

Therefore, the present invention also relates to starch which can be obtained from the plant cells, plants and/or propagation material according to the invention.

In one preferred embodiment of the invention, the starches according to the invention are characterised in that they have an increased phosphate content in the C6 position of the glucose monomer and/or a modified phoshorylation pattern and/or an increased final viscosity and/or a reduced peak temperature compared with starch from corresponding non-genetically modified wild-type plants.
The present invention also relates starch which is characterised in that it has an increased phosphate content in the C6 position of the glucose monomer and/or a modified phoshorylation pattern and/or an increased final viscosity and/or a reduced peak temperature compared with starch from corresponding non-

The statements made in connection with the plant cells according to the invention are applicable with regard to the increase of the phosphate content in the C6 position, the modification of the phoshorylation pattern and the modification of the viscosity properties (final viscosity, peak temperature).

In a further embodiment, the invention relates to starches, preferably wheat starches, which are characterised in that in the C6 position of the glucose monomer they have a phosphate content of at least 0.1 nmol C6 P mg^{-1} starch, particularly at least 0.5 nmol C6 P mg^{-1} starch, preferably at least 1 nmol C6 P mg^{-1} starch, most preferably at least 2 nmol C6 P mg^{-1} starch, most particularly at least 5 nmol C6 P mg^{-1} starch, quite most preferably at least 10 nmol C6 P mg^{-1} starch and/or an increased final viscosity and/or a reduced peak temperature, wherein in the C6 position of the glucose monomer the starch according to the invention has a phosphate content of 100 nmol C6 P mg^{-1} starch at most, particularly 50 nmol C6 P mg^{-1} starch at most, or 25 nmol C6 P mg^{-1} starch at most.

In a further embodiment of the invention, the starches according to the invention can be characterised in that in the C6 position of the glucose monomer they have a phosphate content of at least 15 nmol C6 P mg^{-1} starch, wherein in the C6 position of the glucose monomer the starch according to the invention has a phosphate content of 100 nmol C6 P mg^{-1} starch at most, particularly 50 nmol C6 P mg^{-1} starch at most, or 25 nmol C6 P mg^{-1} starch at most.

In a further preferred embodiment, the starches according to the invention, preferably wheat starches, are characterised in that after conglutination the starches according to the invention form gels which exhibit an increased gel strength compared with gels of corresponding chemically phosphorylated starches of the same phosphate content and/or compared with gels of starches from corresponding non-genetically modified plants.

In a further preferred embodiment of the invention, the starches according to the invention can be characterised in that the major part of the phosphate groups are bonded to the amylopectin component of the starch, whilst the amylose only has a very low content of covalently bonded starch monophosphate esters. Compared with chemically phosphorylated starches of comparable C-6 phosphate content, the starches according to the invention from the plant cells according to the invention are distinguished in that the amylose of the starches according to the invention is less strongly phosphorylated than is the amylopectin component.

In a further embodiment of the invention, the starches according to the invention can be characterised in that the amylose component of said starch has a reduced total phosphate content in the amylose component compared with the amylose component of chemically phosphorylated starch, which preferably has the same phosphate content in the C6 position of the glucose monomer.

Methods of determining of the total phosphate content are known to one skilled in the art and are described below (see methods).

In a further embodiment the invention, the starch according to the invention can be characterised in that the total phosphate content, which can be detected by 31P NMR (Kasemsuwan and Jane (Cereal Chemistry 73 (6), (1996), 702-707) of the amylose component of said starch is reduced by at least 5%, preferably by at least 20%, particularly by at least 50% and most preferably by at least 80%, compared with the amylose component of chemically phosphorylated starch with the same phosphate content in the C6 position of the glucose monomer which is produced from starch of corresponding non-genetically modified plants.
In one preferred embodiment of the invention, the starches according to the invention can be characterised in that they exhibit a modified phoshorylation pattern compared with chemically phosphorylated starches.
The term "modified phoshorylation pattern" has already been defined in connection with the plant cells according to the invention.

In one preferred embodiment of the invention, the starches are starches from useful monocotyledon plants, such as rye-, barley, oats, wheat, millet, rice and maize starch. Wheat, rice and maize starch are preferred; wheat starch is particularly preferred.

The present invention further relates to a method of producing the starch according to the invention, comprising the steps of extracting the starch from a plant or plant cell as described above and/or from starch-storing parts of plant such as this and/or from the propagation material according to the invention. A method such as this also preferably comprises the step of harvesting the cultivated plants and/or starch-storing parts of said plants before the extraction of the starch, and most preferably further comprises the step of cultivating plants according to the invention and/or of propagation materials according to the invention before harvesting. Methods of extracting starch from plants or from starch-storing parts of plants are known to one skilled in the art. Methods of extracting starch from various starch-storing plants are also described, for example, in "Starch: Chemistry and Technology (edited by: Whistler, BeMiller and Paschall (1994), 2nd Edition, Academic Press Inc. London Ltd; ISBN 0-12-746270-8; see Chapter XII, pages 412-468, for example: Maize 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; and Chapter XVI, pages 507 to 528: Rice starch: production and uses; by Rohmer and Klem; and see Maize starch: Eckhoff et al., Cereal Chem. 73 (1996) 54-57. The extraction of maize starch on an industrial scale is generally effected by what is termed "wet milling"). Apparatuses which are customarily used for methods of extracting starch from plant material include mills, separators, decanters, hydrocyclones, spray driers and fluidised-bed driers.

The present invention also relates to starch according to the invention which can be obtained by the methods according to the invention which were described

The starches according to the invention can be subsequently modified by methods known to one skilled in the art, and in their unmodified or modified form are suitable for various uses in the food or non-food sectors.

Therefore, the present invention also relates to starches as defined according to the invention which have been subsequently chemically and/or physically modified.

Various options which can be used for chemical and/or physical modification are described in detail below.

The production of modified starches by means of genetic engineering operations on a plant can firstly modify the properties of the starch obtained from the plant so that further modification by means of chemical or physical methods no longer appear to be necessary. Secondly, starches which have been modified by genetic engineering methods can be subjected to further chemical and/or physical modifications, which results in additional improvements in quality for some of the areas of use described above. These chemical and physical modifications are known in principle. In particular, they comprise modifications by means of:

- heat treatment,
- treatment with acids,
- production of starch ethers,
  starch alkyl ethers, O-allyl ethers, hydroxylalkyl ethers, O-carboxylmethyl ethers, N-containing starch ethers, P containing starch ethers, S-containing starch ethers,
- production of crosslinked starches,
- production of starch graft polymers,
- oxidation, and
- esterification operations which are used for the production of phosphate, nitrate, sulphate, xanthate, acetate and citrate starches. Other organic acids can also be used for esterification.

In a further embodiment, the present invention relates to the use of the starches according to the invention in industry, preferably for the production of foodstuffs.

In a further embodiment, the invention relates to wheat flour which contains the starches according to the invention. Amongst its other properties, the wheat flour according to the invention is distinguished by modified baking properties compared with conventional wheat flours. Methods of producing wheat flour from wheat grains are known to one skilled in the art. By means of these methods, the wheat flour according to the invention can be isolated from the plant cells and plants according to the invention and from the propagation material according to the invention.

Thus the present invention also relates to wheat flour which can be obtained from the plant cells and plants according to the invention and from the propagation material according to the invention.

Compared with flours from corresponding non-genetically modified wild-type plants, the flours according to the invention are distinguished in particular by their enhanced water absorption capacity. Methods of determining water absorption capacity are known to one skilled in the art and are described in detail below (see methods).

In one preferred embodiment of the invention, the wheat flours exhibit a water absorption capacity which is increased by at least 5%, particularly by at least 15%, preferably by at least 20% and most preferably by at least 25% compared with wheat flours from corresponding non-genetically modified wheat plants.

Due to their high water absorption capacity, the flours according to the invention have the advantage for the industrial production of dough that less water has to be supplied to the flour during the dough preparation stage.
In a further embodiment, the present invention relates to the use of the wheat flour according to the invention and/or of the starch according to the invention for

In a further embodiment, the present invention relates the use of the wheat flour according to the invention and/or of the starch according to the invention for the coating of packages.

In a particularly preferred embodiment, the present invention relates to a baking mixture which contains the wheat flour according to the invention and/or the starch according to the invention. In this connection, the term "baking mixture" is to be understood to mean any mixture which can be used for the production of dough (e.g. yeast dough, pasta dough, shortcrust pastry, sour dough etc.) and/or of bakery products (e.g. bread, cakes, rolls, croissants, etc.).

In a further particularly preferred embodiment, the present invention relates to a food product, preferably bakery and dough products (e.g. pasta) which is produced using the wheat flour according to the invention and/or the baking mixture according to the invention and/or the starch according to the invention.

Compared with bakery products which are produced from wheat flours from corresponding non-genetically modified wheat wild-type wheat plants, the bakery products and/or food products according to the invention are distinguished on storage by a slowing down of the ageing process which the bakery products undergo.

The ageing process of a bakery product is closely linked to the degree of retrogradation (recrystallisation) of the amylopectin component of the starch. It is assumed that the ageing process of a bakery product and/or food product has progressed further, the higher is the degree of retrogradation of the amylopectin component The enthalpies of fusion of the amylopectin which can be determined by DSC provide information on the degree of retrogradation of the amylopectin. The degree of ageing of a bakery product can thus be determined by determining the enthalpy of fusion of the amylopectin at a given point in time by means of DSC (=differential scanning calorimetry) analysis (see methods).

If the enthalpy of fusion of the amylopectin of the bakery products and/or food products according to the invention is determined after storage for three and/or seven and/or thirteen days, for example, it is found that the enthalpy of fusion of the amylopectin is reduced compared with the enthalpy of fusion of the amylopectin of bakery products and/or food products which are stored for the same length of time and which are based on wheat flours of corresponding non-genetically modified wild-type plants. This shows that the bakery products and food products according to the invention are distinguished by a slower ageing process compared with conventional products.

The Figures are as follows:

Figure 1: is a schematic illustration of an RVA profile;

Figure 2: is a plasmid map of the vector pUbi2afp

Figure 3: is a plasmid map of the vector pUbiR1

Figure 4: is a plasmid map of the vector p35SAcS (a derivative of pUC18 (Pietrzak M. et al., Nucleic Acids Res.14, (1986), 5857-5868): contains the pat gene from Streptomyces viridochromogenes (Wohlleben et al., Gene 70, (1988), 25-37) under the control of the CaMV35S promoter.

Figure 5: is a plasmid map of the vector pAct1-Fneo/Calgus (pUC19-derivative (Yannish-Perron et al., 1985, Gene 33: 103-119): formed from pAct1-Fneo (Müller et al., Plant Science 114, (1996), 71-82) and pCalgus, which contains the CaMV35S promoter (see US-A-5,352,605, for example), the Adh1 intron from maize (Genes Dev 1987 Dec;1(10):1183-200) and the beta-glucuronidase (GUS) gene (The GUS Reporter System as a Tool to Study Plant Gene expression in: GUS Protocols: Using the GUS Gene as a Reporter of Gene expression, Academic Press (1992), 23-43).

Figure 6: shows the enthalpy of breadcrumbs during storage after 1, 3, 7 and 13 days.

Methods:

Determination of the phosphate content in the C6 position of the glucose monomer (= C6 P content) of the starch (Nielsen et al. Plant Physiol. 105, (1994), 111-117):

In order to determine the C6 P content of the starch, 50 mg starch were hydrolysed in 500 µl 0.7 M HCl for 4 hours at 95°C. The batches were subsequently centrifuged for 10 min at 15500 g and the supernatants were taken off. 7µl of the supernatants were mixed with 193 µl imidazole buffer (100 mM imidazole, pH 7.4; 5 mM $MgCl_2$, 1 mM EDTA and 0.4 mM NAD). Measurements were made in a photometer at 340 nm. After establishing the base absorption, the enzyme reaction was initiated by adding 2U glucose-6-phosphate dehydrogenase (from Leuconostoc mesenteroides, Boehringer Mannheim). The change in absorption was directly proportional to the concentration of the C-6 P content of the starch.

Determination of the total phosphate content of the starch:

Before determining the total phosphate content of the starch, the starch had to be completely separated from phosphorylated non-glucans such as phospholipids. The Determination of the total phosphate content was determined by the method of Ames (Methods in Enzymology VIII, (1966), 115-118), as follows:
About 50 mg starch were treated with 30 µl of 10% ethanolic magnesium nitrate solution and were ignited for three hours in a muffle furnace. The residue was treated with 500 µl 0.5 M hydrochloric acid and incubated for 30 min at 60°C. A 20 µl aliquot was then made up to 300 µl with 0,5 M hydrochloric acid, was added to a mixture of 100 µl of 10% ascorbic acid and 600 µl of 0.42% ammonium molybdate in 1 M sulphuric acid, and was incubated for 20 min at 45°C. This was followed by a photometric determination at 820nm using a phosphate calibration series as a standard.

Determination of the gel strength (texture analyser):

2.5 g starch (TS) were conglutinated in 25 ml H_2O in an RVA instrument (see determination of the viscosity properties by means of a Rapid Visco Analyzer (RVA)) and were subsequently stored for 24 hours at room temperature. The

samples were fixed under the sensor (a cylindrical plunger with a planar surface) of a TA-XT2 texture analysers supplied by Stable Micro Systems (Surrey, UK) and the gel strength was determined using the following instrument settings:

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

Determination of the viscosity properties (e.g. final viscosity) by means of a Rapid Visco Analyzer (RVA):

2.5 g starch (dry weight) were taken up in 25 ml H_2O and used for analysis in a Rapid Visco Analyzer (Newport Scientific Pty Ltd., Investment Support Group, Warriewod NSW 2102, Australia). The instrument was operated according to the manufacturer's instructions. In order to determine the viscosity of the aqueous solution of the starch, the starch suspension was first heated for one minute to a temperature of 50°C and was then heated from 50°C to 95°C at a rate of 12°C per minute. The temperature was subsequently held for 2.5 minutes at 95°C. Thereafter, the solution was cooled from 95°C to 50°C at a rate of 12°C per minute. Finally, the temperature was held for a further 6 minutes at 50°C. The viscosity was determined over the entire duration of the test.

Isolation of the amylose component of the starch by aqueous leaching:

It is known that amylose can be obtained from starch by aqueous leaching (Roger and Colonna (International Journal of Biological Macromolecules 19, (1996), 51-61).

In orde to isolate the amylose component of the starch, 25ml of an aqueous starch suspension (10% w/v) were prepared and heated in an RVA (Newport Scientific) with stirring, using the following profile:

Time [min]	Type	Value [°C] or [rpm]
0	Temp	30°
0	Speed	960 rpm

0.5	Speed	300 rpm
10	Temp	95°

Directly after the viscosity of the suspension or solution exceeded 200 cP, the programme was terminated and the starch suspension or solution was transferred into a cold, 50ml reaction vessel, mixed with 25ml of cold water and cooled for 10 min in an ice bath to about room temperature (RT). Thereafter, the undissolved glucan was formed into a pellet by centrifugation (20min at 2300g at RT) and the clear supernatant was treated with 10mg NaCl/ml. The dissolved glucan was then precipitated on to ice from 80% ethanol, was washed with ethanol again, and was dried for 3 days at 37°C. The pellet was subsequently ground (15s 30Hz with tungsten carbide balls in a in a vibrating mill supplied by Retsch, Germany), and 25mg of the pellet was suspended in 500µl of 0.7M HCl hydrolysed at 95 °C for 4 hours. Undissolved particles were then removed by centrifugation and the content of glucose-6P was determined enzymatically (see above: "Determination of the phosphate content in the C6 position of the glucose monomer").

Procedure for the small scale water absorption test on wheat flour:

Materials

Eppendorf cups (2 ml) with screw cap from Sarsted.

Eppendorf centrifuge (Sigma 202)

Vortex (Wilten)

Analytical Balance (Mettler)

Method

Measure the weight of the epp.cup to four decimal places using the analytical balance.

Fill the cup with 100 to 110 mg flour and note the exact weight.

Put the cup with flour on the vortex and add 1.0 ml water during vortexing.

Keep on vortexing for 10 seconds.

Add 0.9 ml water and close the cup with the screw cap.

Wait ten minutes.

Put the cups in the 6*10 rotor (swing out) in the centrifuge.

Run the centrifuge for 30 minutes at 10,000 rpm (about 8600*g).

Pour off the water from the cup.
Dry the inside of the cup with filter paper without disturbing the wet residue.
Measure the weight of the Eppendorf cup with the wet flour to four decimal places using the analytical balance.
Calculate the weight of the absorbed water.
Determine the waterabsorption of each flour ten times and calculate the average.

Differential Scanning Calorimetry :{tc "Differential Scanning Calorimetry " \l 2}

Differential Scanning Calorimetry (DSC) is a technique studying thermal transitions, in this case of breadcrumbs.
With this technique the heat flow into a sample is determined at a continuous (relatively low) heating rate. Either a peak (offset from the base line (endotherm)) or a step in the base line (glass transition) determines transitions.
Integration of the peak area with respect to the base line gives the enthalpy of the transition. For breadcrumbs, an amylopectin heat of fusion between 40 and 70°C (60°C peak) and an amylose-lipid decomplexation at about 120°C are generally observed.
Differences in the heat of fusion of amylopectin between the various flour samples indicate differences in retrogradation behaviour, and hence indicate that the bread is becoming stale. The enthalpy of fusion of the amylopectin crystals increases with storage time after baking. An increase in the enthalpy of fusion during storage is a measure of an increase in the amount of recrystallised amylopectin with time. The enthalpies (j/g) were calculated on a dry matter basis in order to correct for differences in moisture content.

DSC measurements were made using a TA Instruments Type 2920 calorimeter with LNCA cooling unit. This cooling unit uses liquid nitrogen for fast cooling after the experiment.
Samples (40 - 50 mg breadcrumbs) were placed in a DSC pan and compressed by hand with a nail. After sealing the DSC pan with a compression unit the sample was placed in the DSC-apparatus.

Samples (40 - 50 mg breadcrumbs) were heated from 20°C to 160°C at a rate of 7.5 C/min in stainless steel hermetically sealed pans (Perkin Elmer). A medium pressure pan (120µl volume), filled with 100 mg aluminium, was used as a reference.
The enthalpies of the crumbs of the mini-loaves were measured after 1, 3, 7 and 14 days of storage in single measurements.

The following examples serve to explain the invention without limiting it in any respect:

Example 1

Production of vector pUbiR1 for the transformation of wheat plants

In order to produce vector pUbiR1, vector pUbi.cas was first produced as follows:
Vector pUbi2afp (see Figure 2) was partially cut with restriction enzymes PstI/EcoRI. The 4.19 Kb fragment which resulted therefrom, consisting of the pUC19 vector (Yannish-Perron et al., 1985, Gene 33: 103-119), the 1.5 Kb ubiquitin promoter and of the first exon of the ubi gene and a shortened ubiquitin1 intron (ClaI deletion) (Christensen et al., Plant Mol. Biol. 18, (1992), 675-689) was used further after gel elution.
The nos terminator was isolated as a PstI/EcoRI fragment from the vector pAct.cas (Product Number Cambia TG0063, supplied by Cambia, GPO Box 3200, Canberra ACT 2601, Australia) and ligation of the two fragments was effected to give the vector pUbi.cas.
The cDNA of the potato R1 gene (SEQ ID No.1) was subsequently isolated as a partial digest (SmaI/Asp718 fragment) from the vector pRL2 (WO97/11188, Example 4, deposited at the German Collection for Microorganisms with the number DSM 10225) and was integrated in the vector pUbi.cas (restriction with SmaI/Asp718). The resulting construct was designated as pUbiR1 (see Figure 3).

This construct comprises the coding region of the R1 cDNA from potatoes, which is under the control of the ubiquitin promoter (Christensen et al., 1992, Plant Mol. Biol. 18: 675-689), and also comprises, as additional regulation units, the first exon of the ubi gene (Christensen et al., Plant Mol. Biol. 18, (1992), 675-689) and the shortened first intron (Christensen et al., Plant Mol. Biol. 18, (1992), 675-689), the internal ClaI fragment being deleted).

Example 2

Production of wheat plants which express an R1 gene from Solanum tuberosum, and analysis of the phosphate content of the starch of these plants

The biolistic transformation method was used for the transformation of wheat (Becker et al., Plant J. 5 (2), (1994), 229-307). The vector pUbiR1 and, for biolistic cotransformation, the vectors p35SAcS (see Fig. 4) or pAct1-Fneo/Calgus (Müller et al., Plant Science 114, (1996), 71-82, see Fig. 5) were used in identical molar ratios in the DNA particle precipitation batch.
Vector p35SAcS was produced as follows: The pat gene from S. *viridochromogenes* (Wohlleben et al., Gene 70, (1988), 25-37) was amplified via a polymerase chain reaction. The primers used were designed so that a *Bam*HI cleavage site was created on both sides of the amplificate. The *Bam*HI fragment was subsequently cloned into the *Bam*HI cleavage site placed between the 35S promoter and the 35S terminator of the vector pDH51 (Pietzrak et al., Nucleic Acids Res. 14, (1986), 5857-5868). The cassette containing the 35S promoter, the *pat* gene and the 35S terminator was cut as an EcoRI fragment and was cloned into in the EcoRI cleavage site of the vector pUC18 (Pietzrak et al., Nucleic Acids Res. 14, (1986), 5857-5868).
Scutelli from 14-day old, unripe embryos of wheat plants were used as target cells for transformation. Transformation was followed by *in vitro* culture on MS medium

(PCT/EP97/02793) containing 2 mg/l 2,4-D (=2,4-dichlorophenoxyacetic acid). Two weeks after transformation, subculture was effected on the same medium, to which 2 mg/l phosphinotricin or 150 mg/l kanamycin sulphate had been added. After a further two weeks, the developing calli were transplanted on to a regeneration medium (MS$^-$ medium with 0.1 mg/l 2,4-D and 2 mg/l PPT or 150 mg/l kanamycin). The developing shoots were transferred to semi-concentrated MS$^-$ medium without 2,4-D and phosphinotricin or kanamycin and were subsequently transferred into the ground. About 14 days after their establishment in the ground, the transgenic plants were identified by spraying twice with an aqueous solution containing 150 and 200 mg/l phosphinotricin, respectively, 0.1% Tween 20 (ICI America, corresponding to polysorbate 20) or by spraying twice with 2.5% kanamycin sulphate, 0.1% Tween 20.

Expression of the potato R1 gene in T_0 plants

Expression of the potato R1 gene in transgenic wheat T_0 plants was detected by Northern and Western blotting analyses and by the enzymatic determination of the phosphate content in the C6 position of the glucose monomer of the starch from caryopses (Nielsen et al., Plant Physiol. 105, (1994), 111-117).
R1 protein was detected in the transgenic wheat plants with the aid of an anti-potato R1 protein antibody (Ritte et al., Plant J. 21(4), (2000), 387-391).
Protein extracts from the endosperm of unripe caryopses about 20 days old was used for screening the transgenic plants.
Starch from transgenic T_0 plants was isolated from unripe and ripe wheat caryopses in order to determine C6 phosphate. The caryopses were triturated in a mortar to form a powder. After adding 15 ml of 100 mM Tris buffer, pH 8.0, the suspension was filtered through a 100 µm sieve and the starch was pelletised by centrifugation (2600 g, 5 min, 4°C). The supernatant was discarded. The starch pellet was subsequently re-suspended in 2 ml of 100 mM Tris buffer, pH 8.0 and transferred to an 8 ml Percoll gradient. The starch was palletised by centrifugation for 15 minutes at 170 g and 4°C. The starch pellet was subsequently washed three times with 10 ml of 100 Tris buffer, pH 8.0. Finally, the starch was degreased by acetone incubation and was dried.

The C6 P content was determined by glucose-6-phosphate determination by means of an optical-enzymatic test (Nielsen et al., Plant Physiol. 105, (1994), 111-117) in the manner described above.

The tests showed that of the wheat T_0 plants which gave a positive result in Southern blotting analysis about 50% of the lines in the caryopses synthesised a starch which had an increased phosphate content in the C6 position of the glucose monomer compared with starch from corresponding non-genetically modified wild-type-plants of the Florida variety. Table 1 gives the data for some selected lines.

Analysis of natural descendants

Seeds which were obtained naturally from R1-expressing parental lines was sown and the segregation ratios were determined. It was possible to detect both the integration and also the expression of the R1 gene in T_1descendants of different lines by Southern and Northern blotting analyses. The phosphate content of these lines in the C6 position of the starch was determined. Table 1 shows the data for some selected lines.

Table 1:

Line No.	C6 P in nmol/mg	Gene-ration	Standard deviation
Wild-type Florida variety	Not detectable	-	0.0
19	2.8	T0	0.2
20-25	6.7	T1	0.2
37	1.4	T0	0.3
40A-11-8	10.7	T2	0.7

Example 3

RVA analysis of the starch of plants from transgenic wheat plants which express an R1 gene from Solanum tuberosum, and investigation of the gel strength

The starches from the wheat plants described in Examples 1 and 2 were subjected to an RVA analysis.
The results of the RVA analysis (experimental: see methods) showed that the viscosity behaviour of the starches from the transgenic wheat plants which express an R1 gene from Solanum tuberosum is significantly modified compared with starches from corresponding non-genetically modified wild-type-wheat plants (Florida variety) (see Table 2).

Table 2:

Line No.	RVA Max (%)	RVA Min (%)	RVA Fin (%)	RVA Set (%)	RVA T (%)
Wild-type (Florida)	100	100	100	100	100
19 (T0)	98.7	113.8	116.8	120.7	102
20-25 (T1)	155.3	190	193.8	198.8	99
37 (T0)	143.8	156.2	148.8	139	101
40-11-8 (T2)	156.2	185.5	187.7	190.6	97

Legend:

RVA = Rapid Visco Analyzer

Max = see Figure 1

Min = see Figure 1

Fin = final viscosity, see Figure 1

Set = Setback = Difference between Min and Fin, see Figure 1

T = Conglutination temperature, see Figure 1

The percentages are given with respect to the wild-type (= 100 %).

The gel strengths of the starches (determination of the gel strength: see methods) from the transgenic wheat plants which express an R1 gene from Solanum tuberosum differed both from those of starches from corresponding non-genetically modified wild-type-wheat plants (Florida variety) and from those of wild-type-starches (Florida variety), which had subsequently been chemically phosphorylated (see Table 3).

In contrast to chemically phosphorylated starches with a comparable phosphate content in the C6 position of the glucose monomer, after conglutination the gels of the starches according to the invention exhibited an increased gel strength compared with gels of starches of wild-type-plants. In contrast to this, the

chemically phosphorylated starches which had been produced by the method described by Lim compared with gels of starches of wild-type plants.

Table 3:

Line No.	Gel strength (TA) (%)	Phosphate content in the C6 position in µmol phosphate/g starch
Wild-type (Florida)	100	Not detectable
19 (T0)	167	2.8
20-25 (T1)	192	6.7
37 (T0)	168	1.4
40-11-8 (T2)	224	10.7
Chemically phosphorylated wild-type (Florida) starch		
STMP1	84	2.1
STMP6	75	6.5
STMP8	67	11.5

TA = texture analyzer
The percentages are given with respect to the wild-type (= 100 %).

Example 4

DSC analysis of the starch of plants from transgenic wheat plants which express an R1 gene from Solanum tuberosum

The starches from the wheat plants described in Examples 1 and 2 were subjected to DSC analysis.

The peak temperature was determined using an instrument supplied by Perkin Elmer (instrument designation: DSC-7) using large volume capsules, wherein the sample to be investigated had a ratio of starch to total water content of about 1:4

and measurements were made over the temperature range from 10°C to 160°C at a heating rate of 10°C/min.

The results of the DSC analysis (Table 4) show that the peak temperature of the starches from the transgenic wheat plants which express an R1 gene from Solanum tuberosum exhibit a reduced peak temperature Tp compared with starches from corresponding non-genetically modified wild-type-wheat plants (Florida variety) and also compared with starches of comparable phosphate content in the C6 position of the glucose monomer which were subsequently chemically phosphorylated.

Table 4:

Sample	Water content of sample (%)	Starch (dry weight): water (total water content)	Peak temperature in °C (first peak maximum)	Other peak maxima in °C
Wild-type (Florida)	16.6	1 : 4.01	61	101
19 (T0)	10.5	1 : 3.99	58	100
20-25 (T1)	16.9	1 : 4.05	55	100
40-11-8 (T2)	16.9	1 : 3.98	56	100
STMP2*	10.9	1 : 4.00	60	101
STMP6	10.7	1 : 4.00	61	101

* The chemically phosphorylated starch STMP2 had a phosphate content in the C6 position of 3.0 µmol phosphate/g starch. All the other phosphate data are given in Table 3.

Example 5

Determination of the C6 P content of the amylose component of various starches

Apart from starch from transgenic wheat plants which express an R1 gene from Solanum tuberosum, two chemically phosphorylated starches (STMP3 and STPP4) were investigated. Chemical phoshorylation was effected by the method described by Lim and Seib (Cereal Chem. 70 (2), (1993), 137-144), wherein STMP3 was produced by reacting the starch for 2 hours at pH 6, and STPP4 was produced by reaction for 30 minutes at pH 8. The abbreviation STMP represents the selected phoshorylation reagent, namely sodium trimetaphosphate; STPP represents sodium tripolyphosphate.

The amylose component of various starches was isolated as described above (isolation of the amylose component of the starch by aqueous leaching). The phosphate content in the C6 position was determined as described above (determination of the phosphate content in the C6 position of the glucose monomer).

Table 5:

Sample	C6 P content in µmol phosphate/g starch	C6 P content in µmol phosphate/g amylose	C6 P content of amylose/ C6 P content of starch
40-11-8 (T2)	10.7	1.9	0.18
STPP4	20.5	20.4	0.99
STMP3	6.0	5.1	0.85

It can be seen that the ratio of the C6 P content of the amylose to the C6 P content of the starch in the chemically phosphorylated starches is considerably higher than in the starches from the transgenic wheat plants which superexpress

an R1 Gen from potatoes. The chemically phosphorylated starches have a higher content of phosphorylated amylose than do the starches from the transgenic wheat plants.

Example 6

DSC analysis of breadcrumbs from micro-loaves produced from flour of different origins

Wheat flour from wheat wild-type plants (Florida variety) and from genetically modified wheat plants which superexpress the R1 gene from potato was used to produce micro-loaves, using the following recipe:

Wheat flour	10.00 g
Yeast	0.25 g
Salt	0.20 g
Dextrose	0.10 g
Ascorbic acid	20 ppm
Water	the amount of water as determined in the water absorption test and corrected for consistency (see method "Procedure for small scale water absorption test on wheat flour")

The dough of the genetically modified wheat plants became very sticky during the mixing process. To obtain doughs with correct handling properties a smaller amount of water was used. The dough consistency was corrected by technical sensory perception of the stiffness of the dough.

The dough was proofed in a proofing cabinet for 45 minutes (28°C), manually worked through, proofed for 15 minutes (28°C) for a second time, formed and put in a baking tin. Subsequently, the dough was given a final proof for 60 minutes (28°C). The loaves were baked in an oven for 15 minutes (200°C), with the tins embedded in a moist wooden frame. This embedding was done to shield the baking tin from high amounts of radiative heat. Finally, the loaves were baked for 5 minutes, without the wooden frame, to allow the crust to brown.

Differential scanning calorimetry (DSC) (see methods) was used to determine thermal transitions of the breadcrumbs. The enthalpies of crumbs from the loaves were measured after 1, 3, 7 and after 14 days of storage (Figure 6).

Figure 6 shows the enthalpy of the amylopectin fusion peak (40°C-70°C) for stored bread crumbs after 1, 3, 7 and 13 days of storage, giving an indication of staling effects. The breadcrumbs from loaves made from wild-type (Florida) flour have a higher enthalpy compared with breadcrumbs of the same age made from flour from two different lines (GMO1 and GMO2) of genetically modified wheat plants which superexpress the R1 gene from potato. This indicates a lower level of recrystallised starch, less retrogradation of starch and less staling of the bread.

Sequence protocol:

SEQ ID No. 1 (R1 cDNA potato, nucleotide sequence 5061 bp, coding region: bp 216 to bp 4607):

gaattgtaatacgactcactatagggcgaattgggtaccgggccccccctcgaggtcgacggtatcgataagctt
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Claims

1. A genetically modified monocotyledon plant cell, wherein the genetic modification consists of the introduction of at least one extraneous nucleic acid molecule and the extraneous nucleic acid molecule is selected from the group consisting of:

a) nucleic acid molecules which comprise the coding region of the nucleotide sequence represented in Seq ID No. 1;

b) nucleic acid molecules which encode an R1 protein from Solanum tuberosum with the amino acid sequence given in Seq ID No. 2;

c) nucleic acid molecules which constitute a derivative of the nucleotide sequence given in Seq ID No. 1; and

d) nucleic acid molecules which constitute fragments of the nucleic acid molecules cited in (a), (b) or (c).

2. A plant cell according to claim 1, which exhibits an increased biological activity of R1 protein compared with a corresponding non-genetically modified wild-type plant cell.

3. A plant cell according to one or more of claims 1 or 2, which synthesises a starch which has an increased phosphate content in the C6 position of the glucose monomer compared with starch from corresponding non-genetically modified wild-type plant cells.

4. A plant cell according to one or more of claims 1 to 3, which synthesises a starch which has an increased final viscosity and/or a reduced peak temperature compared with starch from corresponding non-genetically modified wild-type plant cells.

5. A plant cell according to one or more of claims 1 to 4, which synthesises a starch which after conglutination forms a gel which has an increased gel strength compared with a gel of starch from corresponding non-genetically modified wild-

6. A plant cell according to one or more of claims 1 to 5, which originates from a plant of the group consisting of wheat, rice, barley, oats, millet, rye and maize.

7. A plant cell according to one or more of claims 1 to 5, which originates from a wheat plant.

8. A plant cell according to claim 7, which synthesises a starch which in the C6 position of the glucose monomer has a phosphate content of at least 0.1 nmol C6 P mg^{-1} starch.

9. A method of producing a plant cell according to one or more of claims 1 to 8, wherein a cell of a monocotyledon plant is genetically modified, and wherein the genetic modification consists of the introduction of at least one extraneous nucleic acid molecule.

10. A plant containing plant cells according to one or more of claims 1 to 8.

11. A plant according to claim 10, which in its starch-storing organs synthesises a starch which has an increased phosphate content in the C6 position of the glucose monomer compared with starch from the starch-storing organs of corresponding non-genetically modified wild-type-plants.

12. A plant according to one or more of claims 10 to 11, which is a rice, wheat or maize plant.

13. A method of producing a plant according to one or more of claims 10 to 12, wherein

(a) a cell of a monocotyledon plant is genetically modified, wherein the genetic modification consists of the introduction of at least one extraneous nucleic acid molecule;

(b) a plant is regenerated from the cell according to step (a); and optionally

14. A method according to claim 13, wherein the extraneous nucleic acid molecule is under the control of a promoter which organ-specifically facilitates R1 gene expression in starch-storing tissues.

15. A method according to one or more of claims 13 to 14, which relates to a wheat plant.

16. A propagation material for a plant according to one or more of claims 10 to 12 containing plant cells according to one or more of claims 1 to 8.

17. Use of nucleic acid molecules as defined in claim 1 for the production of monocotyledon plants according to one or more of claims 10 to 12 or of monocotyledon plant cells according to one or more of claims 1 to 8.

18. A starch obtainable from a plant cell according to one or more of claims 1 to 8 or a plant according to one or more of claims 10 to 12.

19. A starch according to claim 18, characterised in that it has an increased phosphate content in the C6 position of the glucose monomer and/or an increased final viscosity and/or a reduced peak temperature compared with starch from corresponding wild-type plants.

20. A starch, characterised in that it has an increased phosphate content in the C6 position of the glucose monomer and/or an increased final viscosity and/or a reduced peak temperature compared with starch from corresponding wild-type plants.

21. A starch, characterised in that in the C6 position of the glucose monomer it has a phosphate content of at least 0.1 nmol C6 P mg^{-1} starch.

22. A starch according to claim 21, characterised in that it has a final viscosity which is increased by at least 50% and/or a peak temperature which is reduced by at least 1.5°C.

23. A starch according to one or more of claims 18 to 22, characterised in that after conglutination said starch forms gels which exhibit an increased gel strength compared with gels of corresponding chemically phosphorylated starches with the same phosphate content in the C6 position of the glucose monomer and/or compared with gels of starches from corresponding non-genetically modified plants.

24. A starch according to one or more of claims 18 to 23, characterised in that the amylose component of said starch has a reduced total phosphate content in its amylose component compared with the amylose component of chemically phosphorylated starch.

25. A starch according to one or more of claims 18 to 24, characterised in that it exhibits a modified phoshorylation pattern compared with chemically phosporylated starch.

26. A starch according to one or more of claims 18 to 25, characterised in that it is a wheat starch.

27. A method of producing a starch according to one or more of claims 18 to 26 comprising the extraction of the starch from a plant according to one or more of claims 10 to 12.

28. Use of a starch according to one or more of claims 18 to 26 in the industrial field, preferably for the production of foodstuffs.

29. Wheat flour,containing a starch according to one or more of claims 18 to 26.

30. Wheat flour obtainable from a plant cell according to one or more of claims 1 to 8 or from a plant according to one or more of claims 10 to 12 or from propagation material according to claim 16.

31. Use of the wheat flour according to claim 29 or 30 or of the starch according to one or more of claims 18 to 26 for the production of a baking mixture and/or for the production of a food product.

32. A baking mixture which contains the wheat flour according to claims 29 or 30 or the starch according to one or more of claims 18 to 26.

33. A food product which has been produced using the wheat flour according to claims 29 or 30 and/or using the baking mixture according to claim 32 and/or using the starch according to one or more of claims 18 to 26.

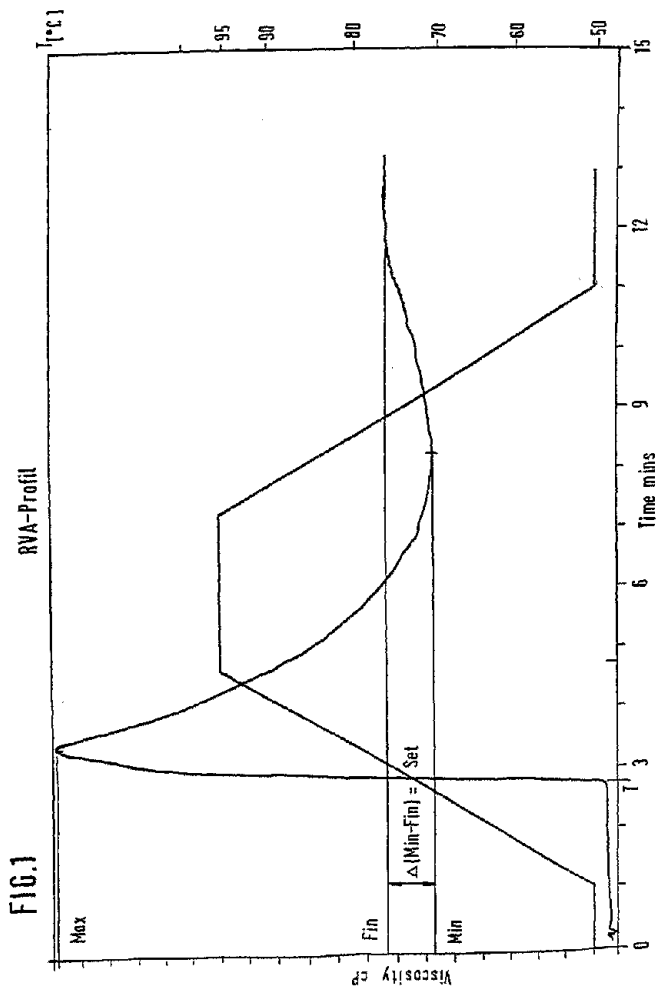
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FIG.1

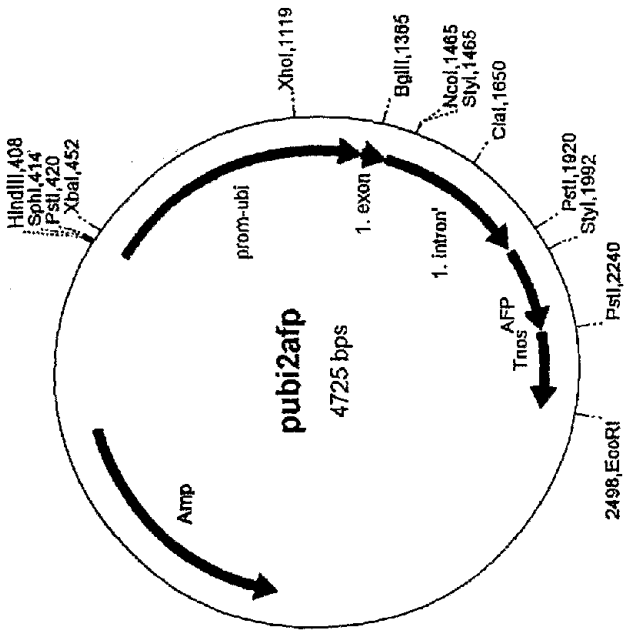
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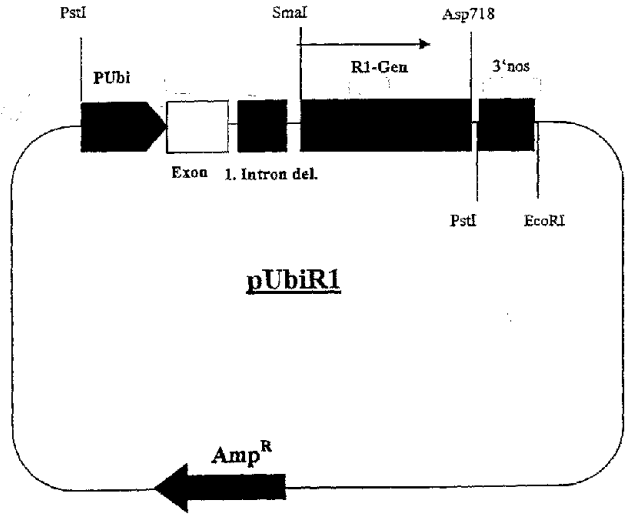
Fig.2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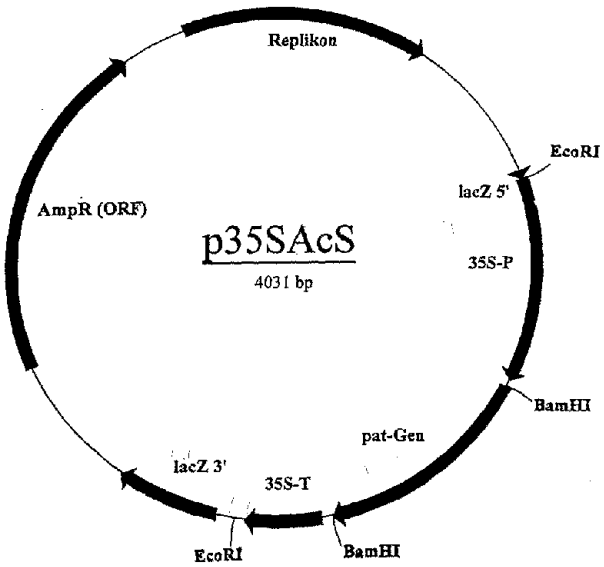
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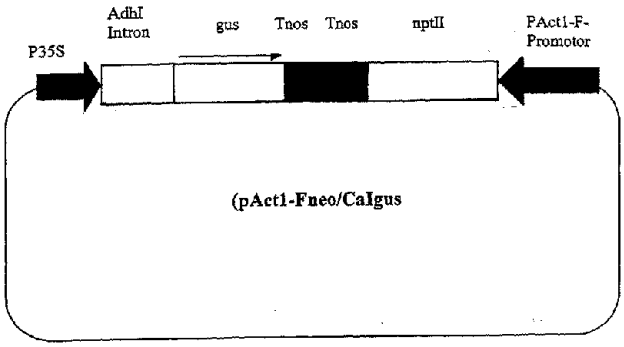
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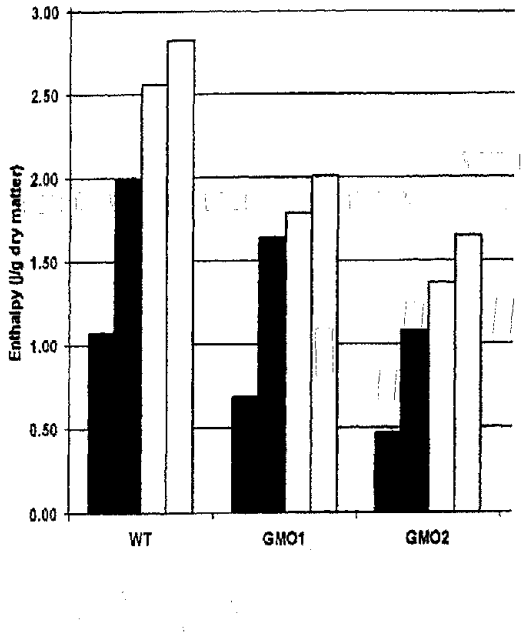
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Figure 6

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Leu Tyr Ala Lys Glu Lys Glu Glu Gln Ile Asp Asp Pro Ile Leu Asn
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aaa aag atc ttt aag gtc gat gat ggg gag cta ctg gta ctg gta gca  1529
Lys Lys Ile Phe Lys Val Asp Asp Gly Glu Leu Leu Val Leu Val Ala
        425                 430                 435

aag tcc tct ggg aag aca aaa gta cat ata gct aca gat ctg aat cag  1577
Lys Ser Ser Gly Lys Thr Lys Val His Ile Ala Thr Asp Leu Asn Gln
    440                 445                 450

cca att act ctt cac tgg gca tta tcc aaa agt cgt gga gag tgg atg  1625
Pro Ile Thr Leu His Trp Ala Leu Ser Lys Ser Arg Gly Glu Trp Met
455                 460                 465                 470

gta cca cct tca agc ata ttg cct cct gga tca att att tta gac aag  1673
Val Pro Pro Ser Ser Ile Leu Pro Pro Gly Ser Ile Ile Leu Asp Lys
                475                 480                 485

gct gcc gaa aca cct ttt tcc gcc agt tct tct gat ggt cta act tct  1721
Ala Ala Glu Thr Pro Phe Ser Ala Ser Ser Ser Asp Gly Leu Thr Ser
            490                 495                 500

aag gta caa tct ttg gat ata gta att gaa gat ggc aat ttt gtg ggg  1769
Lys Val Gln Ser Leu Asp Ile Val Ile Glu Asp Gly Asn Phe Val Gly
        505                 510                 515

atg cca ttt gtt ctt ttg tct ggt gaa aaa tgg att aag aac caa ggg  1817
Met Pro Phe Val Leu Leu Ser Gly Glu Lys Trp Ile Lys Asn Gln Gly
    520                 525                 530

tcg gat ttc tat gtt gac ttc agt gct gca tcc aaa tta gca ctc aag  1865
Ser Asp Phe Tyr Val Asp Phe Ser Ala Ala Ser Lys Leu Ala Leu Lys
535                 540                 545                 550
```

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gct gct ggg gat ggc agt gga act gca aag tct tta ctg gat aaa ata   1913
Ala Ala Gly Asp Gly Ser Gly Thr Ala Lys Ser Leu Leu Asp Lys Ile
                555                 560                 565

gca gat atg gaa agt gag gct cag aag tca ttt atg cac cgg ttt aat   1961
Ala Asp Met Glu Ser Glu Ala Gln Lys Ser Phe Met His Arg Phe Asn
            570                 575                 580

att gct gct gac ttg ata gaa gat gcc act agt gct ggt gaa ctt ggt   2009
Ile Ala Ala Asp Leu Ile Glu Asp Ala Thr Ser Ala Gly Glu Leu Gly
        585                 590                 595

ttt act gga att ctt gta tgg atg agg ttc atg gct aca agg caa ctg   2057
Phe Thr Gly Ile Leu Val Trp Met Arg Phe Met Ala Thr Arg Gln Leu
    600                 605                 610

ata tgg aac aaa aac tat aac gta aaa cca cgt gaa ata agc aag gct   2105
Ile Trp Asn Lys Asn Tyr Asn Val Lys Pro Arg Glu Ile Ser Lys Ala
615                 620                 625                 630

cag gac aga ctt aca gac ttg ttg cag aat gct ttc acc agt cac cct   2153
Gln Asp Arg Leu Thr Asp Leu Leu Gln Asn Ala Phe Thr Ser His Pro
                635                 640                 645

caa tac cgt gaa att ttg cgg atg att atg tca act gtt gga cgt gga   2201
Gln Tyr Arg Glu Ile Leu Arg Met Ile Met Ser Thr Val Gly Arg Gly
            650                 655                 660

ggt gaa ggg gat gta gga cag cga att agg gat gaa att ttg gtc atc   2249
Gly Glu Gly Asp Val Gly Gln Arg Ile Arg Asp Glu Ile Leu Val Ile
        665                 670                 675

cag agg aaa aat gac tgc aag ggt ggt atg atg gaa gaa tgg cat cag   2297
Gln Arg Lys Asn Asp Cys Lys Gly Gly Met Met Glu Glu Trp His Gln
    680                 685                 690

aaa ttg cat aat aat act agt cct gat gat gtt gtg atc tgt cag gca   2345
Lys Leu His Asn Asn Thr Ser Pro Asp Asp Val Val Ile Cys Gln Ala
695                 700                 705                 710

ttg att gac tac atc aag agt gat ttt gat ctt ggt gtt tat tgg aaa   2393
Leu Ile Asp Tyr Ile Lys Ser Asp Phe Asp Leu Gly Val Tyr Trp Lys
                715                 720                 725

acc ctg aat gag aac gga ata aca aaa gag cgt ctt ttg agt tat gac   2441
Thr Leu Asn Glu Asn Gly Ile Thr Lys Glu Arg Leu Leu Ser Tyr Asp
            730                 735                 740

cgt gct atc cat tct gaa ccg aat ttt aga gga gat caa aag aat ggt   2489
Arg Ala Ile His Ser Glu Pro Asn Phe Arg Gly Asp Gln Lys Asn Gly
        745                 750                 755

ctt ttg cgt gat tta ggt cac tat atg aga aca ttg aag gct gtt cat   2537
Leu Leu Arg Asp Leu Gly His Tyr Met Arg Thr Leu Lys Ala Val His
    760                 765                 770

tca ggt gca gat ctt gag tct gct att gca aac tgc atg ggc tac aaa   2585
Ser Gly Ala Asp Leu Glu Ser Ala Ile Ala Asn Cys Met Gly Tyr Lys
775                 780                 785                 790
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act gag gga gaa ggc ttt atg gtt gga gtc cag ata aat cct gta tca  2633
Thr Glu Gly Glu Gly Phe Met Val Gly Val Gln Ile Asn Pro Val Ser
                795                 800                 805

ggc ttg cca tct ggc ttt cag ggc ctc ctc cat ttt gtc tta gac cat  2681
Gly Leu Pro Ser Gly Phe Gln Gly Leu Leu His Phe Val Leu Asp His
            810                 815                 820

gtg gaa gat aaa aat gtg gaa act ctt ctt gag gga ttg cta gag gct  2729
Val Glu Asp Lys Asn Val Glu Thr Leu Leu Glu Gly Leu Leu Glu Ala
        825                 830                 835

cgt gag gag ctt agg ccc ttg ctt ctc aaa cca aac aac cgt cta aag  2777
Arg Glu Glu Leu Arg Pro Leu Leu Leu Lys Pro Asn Asn Arg Leu Lys
    840                 845                 850

gat ctg ctg ttt ttg gac ata gca ctt gat tct aca gtt aga aca gca  2825
Asp Leu Leu Phe Leu Asp Ile Ala Leu Asp Ser Thr Val Arg Thr Ala
855                 860                 865                 870

gta gaa agg gga tat gaa gaa ttg aac aac gct aat cct gag aaa atc  2873
Val Glu Arg Gly Tyr Glu Glu Leu Asn Asn Ala Asn Pro Glu Lys Ile
                875                 880                 885

atg tac ttc atc tcc ctc gtt ctt gaa aat ctc gca ctg tct gtg gac  2921
Met Tyr Phe Ile Ser Leu Val Leu Glu Asn Leu Ala Leu Ser Val Asp
            890                 895                 900

gat aat gaa gat ctt gtt tat tgc ttg aag gga tgg aat caa gct ctt  2969
Asp Asn Glu Asp Leu Val Tyr Cys Leu Lys Gly Trp Asn Gln Ala Leu
        905                 910                 915

tca atg tcc aat ggt gga gac aac cat tgg gct tta ttt gca aaa gct  3017
Ser Met Ser Asn Gly Gly Asp Asn His Trp Ala Leu Phe Ala Lys Ala
    920                 925                 930

gta ctt gac aga atc cgt ctt gca ctt gca agc aag gca gag tgg tac  3065
Val Leu Asp Arg Ile Arg Leu Ala Leu Ala Ser Lys Ala Glu Trp Tyr
935                 940                 945                 950

cat cac tta ttg cag cca tct gcc gaa tat cta gga tca atc ctt ggg  3113
His His Leu Leu Gln Pro Ser Ala Glu Tyr Leu Gly Ser Ile Leu Gly
                955                 960                 965

gtg gac caa tgg gct ttg aac ata ttt act gaa gaa att ata cgt gct  3161
Val Asp Gln Trp Ala Leu Asn Ile Phe Thr Glu Glu Ile Ile Arg Ala
            970                 975                 980

gga tca gca gct tca tta tcc tct ctt ctt aat aga ctc gat ccc gtg  3209
Gly Ser Ala Ala Ser Leu Ser Ser Leu Leu Asn Arg Leu Asp Pro Val
        985                 990                 995

ctt cgg aaa act gca aat cta gga agt tgg cag att atc agt cca gtt  3257
Leu Arg Lys Thr Ala Asn Leu Gly Ser Trp Gln Ile Ile Ser Pro Val
   1000                1005                1010

gaa gcc gtt gga tat gtt gtc gtt gtg gat gag ttg ctt tca gtt cag  3305
Glu Ala Val Gly Tyr Val Val Val Val Asp Glu Leu Leu Ser Val Gln
1015                1020                1025                1030
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aat gaa atc tac aag aag ccc acg atc tta gta gca aac tct gtt aaa  3353
Asn Glu Ile Tyr Lys Lys Pro Thr Ile Leu Val Ala Asn Ser Val Lys
                1035                1040                1045

gga gag gag gaa att cct gat ggt gct gtt gcc ctg ata aca cca gac  3401
Gly Glu Glu Glu Ile Pro Asp Gly Ala Val Ala Leu Ile Thr Pro Asp
            1050                1055                1060

atg cca gat gtt ctt tca cat gtt tct gtt cga gct aga aat ggg aag  3449
Met Pro Asp Val Leu Ser His Val Ser Val Arg Ala Arg Asn Gly Lys
        1065                1070                1075

gtt tgc ttt gct aca tgc ttt gat ccc aat ata ttg gct gac ctc caa  3497
Val Cys Phe Ala Thr Cys Phe Asp Pro Asn Ile Leu Ala Asp Leu Gln
    1080                1085                1090

gca aag gaa gga agg att ttg ctc tta aag cct aca cct tca gac ata  3545
Ala Lys Glu Gly Arg Ile Leu Leu Leu Lys Pro Thr Pro Ser Asp Ile
1095                1100                1105                1110

atc tat agt gag gtg aat gag att gag ctc caa agt tca agt aac ttg  3593
Ile Tyr Ser Glu Val Asn Glu Ile Glu Leu Gln Ser Ser Ser Asn Leu
                1115                1120                1125

gta gaa gct gaa act tca gca aca ctt aga ttg gtg aaa aag caa ttt  3641
Val Glu Ala Glu Thr Ser Ala Thr Leu Arg Leu Val Lys Lys Gln Phe
            1130                1135                1140

ggt ggt tgt tac gca ata tca gca gat gaa ttc aca agt gaa atg gtt  3689
Gly Gly Cys Tyr Ala Ile Ser Ala Asp Glu Phe Thr Ser Glu Met Val
        1145                1150                1155

gga gct aaa tca cgt aat att gca tat ctg aaa gga aaa gtg cct tcc  3737
Gly Ala Lys Ser Arg Asn Ile Ala Tyr Leu Lys Gly Lys Val Pro Ser
    1160                1165                1170

tcg gtg gga att cct acg tca gta gct ctt cca ttt gga gtc ttt gag  3785
Ser Val Gly Ile Pro Thr Ser Val Ala Leu Pro Phe Gly Val Phe Glu
1175                1180                1185                1190

aaa gta ctt tca gac gac ata aat cag gga gtg gca aaa gag ttg caa  3833
Lys Val Leu Ser Asp Asp Ile Asn Gln Gly Val Ala Lys Glu Leu Gln
                1195                1200                1205

att ctg acg aaa aaa cta tct gaa gga gac ttc agc gct ctt ggt gaa  3881
Ile Leu Thr Lys Lys Leu Ser Glu Gly Asp Phe Ser Ala Leu Gly Glu
            1210                1215                1220

att cgc aca acg att tta gat ctt tca gca cca gct caa ttg gtc aaa  3929
Ile Arg Thr Thr Ile Leu Asp Leu Ser Ala Pro Ala Gln Leu Val Lys
        1225                1230                1235

gag ctg aag gaa aag atg cag ggt tct ggc atg cct tgg cct ggt gat  3977
Glu Leu Lys Glu Lys Met Gln Gly Ser Gly Met Pro Trp Pro Gly Asp
    1240                1245                1250

gaa ggt cca aag cgg tgg gaa caa gca tgg atg gcc ata aaa aag gtg  4025
Glu Gly Pro Lys Arg Trp Glu Gln Ala Trp Met Ala Ile Lys Lys Val
1255                1260                1265                1270
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tgg gct tca aaa tgg aat gag aga gca tac ttc agc aca agg aag gtg   4073
Trp Ala Ser Lys Trp Asn Glu Arg Ala Tyr Phe Ser Thr Arg Lys Val
                1275                1280                1285

aaa ctg gat cat gac tat ctg tgc atg gct gtc ctt gtt caa gaa ata   4121
Lys Leu Asp His Asp Tyr Leu Cys Met Ala Val Leu Val Gln Glu Ile
            1290                1295                1300

ata aat gct gat tat gca ttt gtc att cac aca acc aac cca tct tcc   4169
Ile Asn Ala Asp Tyr Ala Phe Val Ile His Thr Thr Asn Pro Ser Ser
        1305                1310                1315

gga gac gac tca gaa ata tat gcc gag gtg gtc agg ggc ctt ggg gaa   4217
Gly Asp Asp Ser Glu Ile Tyr Ala Glu Val Val Arg Gly Leu Gly Glu
    1320                1325                1330

aca ctt gtt gga gct tac cca gga cgt gct ttg agt ttt atc tgc aag   4265
Thr Leu Val Gly Ala Tyr Pro Gly Arg Ala Leu Ser Phe Ile Cys Lys
1335                1340                1345                1350

aaa aag gat ctc aac tct cct caa gtg tta ggt tac cca agc aaa ccg   4313
Lys Lys Asp Leu Asn Ser Pro Gln Val Leu Gly Tyr Pro Ser Lys Pro
                1355                1360                1365

atc ggc ctt ttc ata aaa aga tct atc atc ttc cga tct gat tcc aat   4361
Ile Gly Leu Phe Ile Lys Arg Ser Ile Ile Phe Arg Ser Asp Ser Asn
            1370                1375                1380

ggg gaa gat ttg gaa ggt tat gcc ggt gct ggc ctc tac gac agt gta   4409
Gly Glu Asp Leu Glu Gly Tyr Ala Gly Ala Gly Leu Tyr Asp Ser Val
        1385                1390                1395

cca atg gat gag gag gaa aaa gtt gta att gat tac tct tcc gac cca   4457
Pro Met Asp Glu Glu Glu Lys Val Val Ile Asp Tyr Ser Ser Asp Pro
    1400                1405                1410

ttg ata act gat ggt aac ttc cgc cag aca atc ctg tcc aac att gct   4505
Leu Ile Thr Asp Gly Asn Phe Arg Gln Thr Ile Leu Ser Asn Ile Ala
1415                1420                1425                1430

cgt gct gga cat gct atc gag gag cta tat ggc tct cct caa gac atc   4553
Arg Ala Gly His Ala Ile Glu Glu Leu Tyr Gly Ser Pro Gln Asp Ile
                1435                1440                1445

gag ggt gta gtg agg gat gga aag att tat gtc gtt cag aca aga cct   4601
Glu Gly Val Val Arg Asp Gly Lys Ile Tyr Val Val Gln Thr Arg Pro
            1450                1455                1460

cag atg tgatcatatt ctcgttgtat gttgttcaga gaagaccata gatgtgatca    4657
Gln Met

tattctcatg gtatcagatc tgtgaccact tacctcccat gaagttgcct gtatgattat 4717

acgtgatcca aagccatcac atcatgttca ccttcagcta ttggaggaga agtgagaagt 4777

aggaattgca atatgaggaa taataagaaa aactttgtag aagttaaatt agctgggtat 4837

gatataggga gaaatgtgta aacattgtac tatatatagt atacacacgc attatgtatt 4897

tgcattatgc actgaataat atcgcagcat caaagaagaa atcctttgag tggtttcaat 4957
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tgccgcggcc gcgaattcct gcagcccggg ggatccacta gttctagagc ggccgccacc 5017

gcggtggagc tccagctttt gttccctta gtgagggtta attt 5061

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Met Ser Asn Ser Leu Gly Asn Asn Leu Leu Tyr Gln Gly Phe Leu Thr
1 5 10 15

Ser Thr Val Leu Glu His Lys Ser Arg Ile Ser Pro Pro Cys Val Gly
20 25 30

Gly Asn Ser Leu Phe Gln Gln Gln Val Ile Ser Lys Ser Pro Leu Ser
35 40 45

Thr Glu Phe Arg Gly Asn Arg Leu Lys Val Gln Lys Lys Lys Ile Pro
50 55 60

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

Thr Asp Thr Ser Ser Glu Leu Ala Glu Lys Phe Ser Leu Glu Gly Asn
85 90 95

Ile Glu Leu Gln Val Asp Val Arg Pro Pro Thr Ser Gly Asp Val Ser
100 105 110

Phe Val Asp Phe Gln Ala Thr Asn Gly Ser Asp Lys Leu Phe Leu His
115 120 125

Trp Gly Ala Val Lys Phe Gly Lys Glu Thr Trp Ser Leu Pro Asn Asp
130 135 140

Arg Pro Asp Gly Thr Lys Val Tyr Lys Asn Lys Ala Leu Arg Thr Pro
145 150 155 160

Phe Val Lys Ser Gly Ser Asn Ser Ile Leu Arg Leu Glu Ile Arg Asp
165 170 175

Thr Ala Ile Glu Ala Ile Glu Phe Leu Ile Tyr Asp Glu Ala Tyr Asp
180 185 190

Lys Trp Ile Lys Asn Asn Gly Gly Asn Phe Arg Val Lys Leu Ser Arg
195 200 205

Lys Glu Ile Arg Gly Pro Asp Val Ser Val Pro Glu Glu Leu Val Gln
210 215 220

Ile Gln Ser Tyr Leu Arg Trp Glu Arg Lys Gly Lys Gln Asn Tyr Thr
225 230 235 240

Pro Glu Lys Glu Lys Glu Glu Tyr Glu Ala Ala Arg Thr Glu Leu Gln
245 250 255

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Glu Glu Ile Ala Arg Gly Ala Ser Ile Gln Asp Ile Arg Ala Arg Leu
            260                 265                 270

Thr Lys Thr Asn Asp Lys Ser Gln Ser Lys Glu Glu Pro Leu His Val
        275                 280                 285

Thr Lys Ser Glu Ile Pro Asp Asp Leu Ala Gln Ala Gln Ala Tyr Ile
    290                 295                 300

Arg Trp Glu Lys Ala Gly Lys Pro Asn Tyr Pro Pro Glu Lys Gln Ile
305                 310                 315                 320

Glu Glu Leu Glu Glu Ala Arg Arg Glu Leu Gln Leu Glu Leu Glu Lys
                325                 330                 335

Gly Ile Thr Leu Asp Glu Leu Arg Lys Lys Ile Thr Lys Gly Glu Ile
            340                 345                 350

Lys Thr Lys Ala Glu Lys His Val Lys Arg Ser Ser Phe Ala Val Glu
        355                 360                 365

Arg Ile Gln Arg Lys Lys Arg Asp Phe Gly Gln Leu Ile Asn Lys Tyr
    370                 375                 380

Pro Ser Ser Pro Ala Val Gln Val Gln Lys Val Leu Glu Glu Pro Pro
385                 390                 395                 400

Ala Leu Ser Lys Ile Lys Leu Tyr Ala Lys Glu Lys Glu Glu Gln Ile
                405                 410                 415

Asp Asp Pro Ile Leu Asn Lys Lys Ile Phe Lys Val Asp Asp Gly Glu
            420                 425                 430

Leu Leu Val Leu Val Ala Lys Ser Ser Gly Lys Thr Lys Val His Ile
        435                 440                 445

Ala Thr Asp Leu Asn Gln Pro Ile Thr Leu His Trp Ala Leu Ser Lys
    450                 455                 460

Ser Arg Gly Glu Trp Met Val Pro Pro Ser Ser Ile Leu Pro Pro Gly
465                 470                 475                 480

Ser Ile Ile Leu Asp Lys Ala Ala Glu Thr Pro Phe Ser Ala Ser Ser
                485                 490                 495

Ser Asp Gly Leu Thr Ser Lys Val Gln Ser Leu Asp Ile Val Ile Glu
            500                 505                 510

Asp Gly Asn Phe Val Gly Met Pro Phe Val Leu Leu Ser Gly Glu Lys
        515                 520                 525

Trp Ile Lys Asn Gln Gly Ser Asp Phe Tyr Val Asp Phe Ser Ala Ala
    530                 535                 540

Ser Lys Leu Ala Leu Lys Ala Ala Gly Asp Gly Ser Gly Thr Ala Lys
545                 550                 555                 560

Ser Leu Leu Asp Lys Ile Ala Asp Met Glu Ser Glu Ala Gln Lys Ser
                565                 570                 575
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Phe Met His Arg Phe Asn Ile Ala Ala Asp Leu Ile Glu Asp Ala Thr
            580                 585                 590

Ser Ala Gly Glu Leu Gly Phe Thr Gly Ile Leu Val Trp Met Arg Phe
        595                 600                 605

Met Ala Thr Arg Gln Leu Ile Trp Asn Lys Asn Tyr Asn Val Lys Pro
    610                 615                 620

Arg Glu Ile Ser Lys Ala Gln Asp Arg Leu Thr Asp Leu Leu Gln Asn
625                 630                 635                 640

Ala Phe Thr Ser His Pro Gln Tyr Arg Glu Ile Leu Arg Met Ile Met
                645                 650                 655

Ser Thr Val Gly Arg Gly Gly Glu Gly Asp Val Gly Gln Arg Ile Arg
            660                 665                 670

Asp Glu Ile Leu Val Ile Gln Arg Lys Asn Asp Cys Lys Gly Gly Met
        675                 680                 685

Met Glu Glu Trp His Gln Lys Leu His Asn Asn Thr Ser Pro Asp Asp
    690                 695                 700

Val Val Ile Cys Gln Ala Leu Ile Asp Tyr Ile Lys Ser Asp Phe Asp
705                 710                 715                 720

Leu Gly Val Tyr Trp Lys Thr Leu Asn Glu Asn Gly Ile Thr Lys Glu
                725                 730                 735

Arg Leu Leu Ser Tyr Asp Arg Ala Ile His Ser Glu Pro Asn Phe Arg
            740                 745                 750

Gly Asp Gln Lys Asn Gly Leu Leu Arg Asp Leu Gly His Tyr Met Arg
        755                 760                 765

Thr Leu Lys Ala Val His Ser Gly Ala Asp Leu Glu Ser Ala Ile Ala
    770                 775                 780

Asn Cys Met Gly Tyr Lys Thr Glu Gly Glu Gly Phe Met Val Gly Val
785                 790                 795                 800

Gln Ile Asn Pro Val Ser Gly Leu Pro Ser Gly Phe Gln Gly Leu Leu
                805                 810                 815

His Phe Val Leu Asp His Val Glu Asp Lys Asn Val Glu Thr Leu Leu
            820                 825                 830

Glu Gly Leu Leu Glu Ala Arg Glu Glu Leu Arg Pro Leu Leu Leu Lys
        835                 840                 845

Pro Asn Asn Arg Leu Lys Asp Leu Leu Phe Leu Asp Ile Ala Leu Asp
    850                 855                 860

Ser Thr Val Arg Thr Ala Val Glu Arg Gly Tyr Glu Glu Leu Asn Asn
865                 870                 875                 880

Ala Asn Pro Glu Lys Ile Met Tyr Phe Ile Ser Leu Val Leu Glu Asn
                885                 890                 895
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Leu Ala Leu Ser Val Asp Asp Asn Glu Asp Leu Val Tyr Cys Leu Lys
            900                 905                 910

Gly Trp Asn Gln Ala Leu Ser Met Ser Asn Gly Gly Asp Asn His Trp
        915                 920                 925

Ala Leu Phe Ala Lys Ala Val Leu Asp Arg Ile Arg Leu Ala Leu Ala
    930                 935                 940

Ser Lys Ala Glu Trp Tyr His His Leu Leu Gln Pro Ser Ala Glu Tyr
945                 950                 955                 960

Leu Gly Ser Ile Leu Gly Val Asp Gln Trp Ala Leu Asn Ile Phe Thr
                965                 970                 975

Glu Glu Ile Ile Arg Ala Gly Ser Ala Ala Ser Leu Ser Ser Leu Leu
            980                 985                 990

Asn Arg Leu Asp Pro Val Leu Arg Lys Thr Ala Asn Leu Gly Ser Trp
        995                1000                1005

Gln Ile Ile Ser Pro Val Glu Ala Val Gly Tyr Val Val Val Val Asp
    1010                1015                1020

Glu Leu Leu Ser Val Gln Asn Glu Ile Tyr Lys Lys Pro Thr Ile Leu
1025                1030                1035                1040

Val Ala Asn Ser Val Lys Gly Glu Glu Glu Ile Pro Asp Gly Ala Val
                1045                1050                1055

Ala Leu Ile Thr Pro Asp Met Pro Asp Val Leu Ser His Val Ser Val
            1060                1065                1070

Arg Ala Arg Asn Gly Lys Val Cys Phe Ala Thr Cys Phe Asp Pro Asn
        1075                1080                1085

Ile Leu Ala Asp Leu Gln Ala Lys Glu Gly Arg Ile Leu Leu Leu Lys
    1090                1095                1100

Pro Thr Pro Ser Asp Ile Ile Tyr Ser Glu Val Asn Glu Ile Glu Leu
1105                1110                1115                1120

Gln Ser Ser Ser Asn Leu Val Glu Ala Glu Thr Ser Ala Thr Leu Arg
                1125                1130                1135

Leu Val Lys Lys Gln Phe Gly Gly Cys Tyr Ala Ile Ser Ala Asp Glu
            1140                1145                1150

Phe Thr Ser Glu Met Val Gly Ala Lys Ser Arg Asn Ile Ala Tyr Leu
        1155                1160                1165

Lys Gly Lys Val Pro Ser Ser Val Gly Ile Pro Thr Ser Val Ala Leu
    1170                1175                1180

Pro Phe Gly Val Phe Glu Lys Val Leu Ser Asp Asp Ile Asn Gln Gly
1185                1190                1195                1200

Val Ala Lys Glu Leu Gln Ile Leu Thr Lys Lys Leu Ser Glu Gly Asp
                1205                1210                1215
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Phe Ser Ala Leu Gly Glu Ile Arg Thr Thr Ile Leu Asp Leu Ser Ala
          1220                1225                1230

Pro Ala Gln Leu Val Lys Glu Leu Lys Glu Lys Met Gln Gly Ser Gly
       1235                1240                1245

Met Pro Trp Pro Gly Asp Glu Gly Pro Lys Arg Trp Glu Gln Ala Trp
   1250                1255                1260

Met Ala Ile Lys Lys Val Trp Ala Ser Lys Trp Asn Glu Arg Ala Tyr
1265                1270                1275                1280

Phe Ser Thr Arg Lys Val Lys Leu Asp His Asp Tyr Leu Cys Met Ala
                1285                1290                1295

Val Leu Val Gln Glu Ile Ile Asn Ala Asp Tyr Ala Phe Val Ile His
          1300                1305                1310

Thr Thr Asn Pro Ser Ser Gly Asp Asp Ser Glu Ile Tyr Ala Glu Val
       1315                1320                1325

Val Arg Gly Leu Gly Glu Thr Leu Val Gly Ala Tyr Pro Gly Arg Ala
   1330                1335                1340

Leu Ser Phe Ile Cys Lys Lys Lys Asp Leu Asn Ser Pro Gln Val Leu
1345                1350                1355                1360

Gly Tyr Pro Ser Lys Pro Ile Gly Leu Phe Ile Lys Arg Ser Ile Ile
                1365                1370                1375

Phe Arg Ser Asp Ser Asn Gly Glu Asp Leu Glu Gly Tyr Ala Gly Ala
          1380                1385                1390

Gly Leu Tyr Asp Ser Val Pro Met Asp Glu Glu Glu Lys Val Val Ile
       1395                1400                1405

Asp Tyr Ser Ser Asp Pro Leu Ile Thr Asp Gly Asn Phe Arg Gln Thr
   1410                1415                1420

Ile Leu Ser Asn Ile Ala Arg Ala Gly His Ala Ile Glu Glu Leu Tyr
1425                1430                1435                1440

Gly Ser Pro Gln Asp Ile Glu Gly Val Val Arg Asp Gly Lys Ile Tyr
                1445                1450                1455

Val Val Gln Thr Arg Pro Gln Met
          1460
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Met Gln Glu Trp His Gln
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<220>
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1 5

<210> 8
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<213> Artificial Sequence

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1 5

<210> 9
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<213> Artificial Sequence

<220>
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<400> 9
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1 5

<210> 10
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<212> PRT
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<223> Description of Artificial Sequence: Teilsequenz

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<210> 11
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<223> Description of Artificial Sequence: Teilsequenz

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<223> Description of Artificial Sequence: Teilsequenz

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<223> Description of Artificial Sequence: Teilsequenz

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<223> Description of Artificial Sequence: Teilsequenz

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<223> Description of Artificial Sequence: Teilsequenz

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(57) Abstract: The present invention relates to monocotyledon plant cells and plants which are genetically modified, wherein the genetic modification consists of the introduction of an extraneous nucleic acid molecule which codes for a protein with the biological activity of an R1 protein. The present invention further relates to means and methods for the production thereof. Plant cells and plants of this type synthesise a modified starch, which is characterised in that is has an increased phosphate content and/or a modified phosphorylation pattern and/or an increased final viscosity in an RVA profile and/or a reduced peak temperature in DSC analysis and/or an increased gel strength in the texture analysis compared with starch from corresponding non-genetically modified monocotyledon plants. Therefore, the present invention also relates to the starch which is synthesised from the plant cells and plants according to the invention, and to methods of producing said starch. The present invention further relates to wheat flours which contain said modified starches, and to food products and bakery products which contain said wheat flours and/or starch.

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(74) Agent: LEDERER & KELLER; Prinzregentenstrasse 16, 80538 München (DE).

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(57) Abstract: The present invention relates to monocotyledon plant cells and plants which are genetically modified, wherein the genetic modification consists of the introduction of an extraneous nucleic acid molecule which codes for a protein with the biological activity of an R1 protein. The present invention further relates to means and methods for the production thereof. Plant cells and plants of this type synthesise a modified starch, which is characterised in that is has an increased phosphate content and/or a modified phosphorylation pattern and/or an increased final viscosity in an RVA profile and/or a reduced peak temperature in DSC analysis and/or an increased gel strength in the texture analysis compared with starch from corresponding non genetically modified monocotyledon plants. Therefore, the present invention also relates to the starch which is synthesised from the plant cells and plants according to the invention, and to methods of producing said starch. The present invention further relates to wheat flours which contain said modified starches, and to food products and bakery products which contain said wheat flours and/or starch.

Description

Monocotyledon plant cells and plants which synthesise modified starch

The present invention relates to monocotyledon plant cells and plants which are genetically modified, wherein the genetic modification consists of the introduction of an extraneous nucleic acid molecule which codes for a protein with the biological activity of an R1 protein. The present invention further relates to means and methods for the production thereof. Plant cells and plants of this type synthesise a modified starch which is characterised in that it has an increased phosphate content and/or a modified phoshorylation pattern and/or an increased final viscosity in an RVA profile and/or a reduced peak temperature in DSC analysis and/or an increased gel strength in the texture analysis compared with starch from corresponding non-genetically modified monocotyledon plants. Therefore, the present invention also relates to the starch which is synthesised from the plant cells and plants according to the invention, and to methods of producing said starch. The present invention further relates to wheat flours which contain said modified starches, and to food products and bakery products which contain said wheat flours and/or starch.

With regard to the increasing importance which has recently been attached to substances of plant content as renewable sources of raw materials, one of the tasks of biotechnological research is to endeavour to adapt these plant raw materials to the requirements of the industry which processes them. To facilitate the use of renewable raw materials in as many fields of use as possible, it is also necessary to provide a considerable multiplicity of substances.

Apart from oils, fats and proteins, polysaccharides constitute the important renewable raw materials from plants. In addition to cellulose, starch, which is one

of the most important storage materials in higher plants, assumes a central position in polysaccharides.

Polysaccharide starch is a polymer of chemically uniform basic components, namely glucose molecules. However, it is a very complex mixture of different forms of molecules, which differ with regard to their degree of polymerisation and the occurrence of branched region in the glucose chains. Starch therefore does not constitute a uniform raw material. A distinction is made between two chemically different components of starch: amylose and amylopectin. In typical plants which are used for starch production, such as maize, wheat or potatoes, the synthesised starch consists of up to about 20% - 30% of amylose starch and up to about 70% - 80% of amylopectin starch.

Amylose was for a long time considered to be a linear polymer consisting of α-1,4-glycosidically bonded α-D-glucose monomers. In more recent studies, however, the presence of about 0.1% of α-1,6-glycosidic branching sites has been detected (Hizukuri and Takagi, Carbohydr. Res. 134, (1984), 1-10; Takeda et al., Carbohydr. Res. 132, (1984), 83-92). Basically, however, achieving a complete separation of amylose from amylopectin very difficult, so that the quality of the amylose is strongly dependent on the type of separation method selected.

In contrast to amylose, amylopectin is more strongly branched and comprises about 4% of branching sites which are formed due to the occurrence of additional α-1,6-glycoside linkages. Amylopectin constitutes a complexes mixture of differently branched glucose chains.

A further significant difference between these two molecules is their molecular weight. Whereas amylose, depending on the origin of the starch, has a molecular weight of $5x10^5 - 10^6$ Da, that of amylopectin ranges between 10^7 and 10^8 Da. These two macromolecules can be by distinguished by their molecular weight and by their different physicochemical properties, which are most readily manifested by their different iodine bonding properties.

A further significant difference between amylose and amylopectin is the relative amounts of trace substances which can exist in association with these macromolecules. Amylose has a high affinity for hydrophobic molecules. In

cereals in particular, amylose can be complexed with relatively high amounts of lipids (Morrison, Cereal Foods World 40, (1995), 437-446). On the other hand, amylopectin can contain covalently bonded inorganic phosphate in the form of starch phosphate monoesters, which has not hitherto been described for amylose. High contents of phosphate monoesters are found in particular in starches which are obtained from tubers. Amongst commercially available starches, potato starch has the highest phosphate content, which can range between 10-30 nmol mg^{-1} starch In some types of Curcuma the phosphate content can even be 2 to 4 times higher (Bay-Smidt et al., 5th ISPMP Meeting Proceedings, (1997), 741), whilst it is about 100 times less in cereals (Kasemsuwan and Jane, Cereal Chem. 73, (1996), 702-707). In contrast to starches from tubers, roots and legumes, the detectable phosphate in cereal starches (monocotyledon plants) rarely occurs in the form of starch monoester derivatives, but mainly occurs in the form of phospholipids (Jane et al., Cereal Foods World 41, (1996), 827-832).

Apart from its amylose/amylopectin ratio and phosphate content, the functional properties of starch are influenced by its molecular weight, its pattern of side chain distribution, its content of ions, its lipid and protein content, etc. Important functional properties which should be cited here are the solubility, the retrogradation behaviour, the water absorption capacity, the film-forming properties, the viscosity, the conglutination properties, the freeze-thaw stability, the stability in relation to acids, the gel strength, etc. The starch grain size can also be important for various applications.

In principle, the phosphate content can be modified either by genetic engineering approaches or by the subsequent chemical phoshorylation of native starches (see, for example: Starch Chemistry and Technology. Eds. R. L. Whistler, J. N. BeMiller and E. F. Paschall. Academic Press, New York, 1988, 349-364). Chemical modifications are generally costly and time-consuming, however, and result in starches, the physicochemical properties of which can differ from those of starches modified in vivo.

Since starches from monocotyledon wild-type plants, particularly from cereal plants (wheat, rice, maize, oats, millet, rye), only have a very low content of phosphate in the form of starch phosphate monoesters (Lim et al. Cereal Chem.

71, (1994), 488), one object of the present invention is to provide monocotyledon plants which synthesise starches with an increased phosphate content (content of starch phosphate monoesters) and modified physicochemical properties compared with corresponding wild-type plant cells and plants.

The underlying object of the present invention is thus to provide genetically modified monocotyledon plant cells and plants which synthesise starches with modified structural and/or functional properties compared with corresponding non-genetically modified wild-type plant cells and plants, and is also to provide starch, the structural and/or functional properties of which differ from those of starch from corresponding non-genetically modified wild-type plant cells and plants and from those of chemically modified starch, and which is thus more suitable for general and/or special industrial purposes of use.

This object is achieved by the provision of the embodiments described in the claims, because it has surprisingly been found that the introduction of an extraneous nucleic acid molecule into the genome of monocotyledon plant cells and plants results in a modification of the structural and/or functional properties of the starch which is synthesised in said monocotyledon plant cells and plants.

Expression of the extraneous nucleic acid molecule is primarily advantageous in starch-storing organs of monocotyledon plants, particularly of wheat plants, and leads to an increase of the phosphate content and modification of the viscosity properties of the starch which can be isolated from the starch-storing organs compared with starches which can be isolated from starch-storing organs of corresponding non-genetically modified wild-type plants, particularly wheat plants. Moreover, the starches according to the invention are distinguished from chemically phosphorylated starches by a modified phoshorylation pattern and modified viscosity properties, and after conglutination of the starches and gel formation are also distinguished by modified gel strengths.

Thus the present invention relates to monocotyledon genetically modified plant cells, wherein the genetic modification consists of the introduction of at least one

extraneous nucleic acid molecule and the extraneous nucleic acid molecule is selected from the group consisting of:

a) nucleic acid molecules which comprise the coding region of the nucleotide sequence represented in Seq ID No. 1;
b) nucleic acid molecules which encode an R1 protein from Solanum tuberosum with the amino acid sequence given in Seq ID No. 2;
c) nucleic acid molecules which constitute a derivative of the nucleotide sequence given in Seq ID No. 1; and
d) nucleic acid molecules which constitute fragments of the nucleic acid molecules cited in (a), (b) or (c).

In the sense of the present invention, the term "genetically modified" means that the genetic information of the plant cell is modified by the introduction of an extraneous nucleic acid molecule and that the presence or expression of the extraneous nucleic acid molecule results in a phenotypic modification. The term "phenotypic modification" preferably means a measurable modification of one or more functions of the cells. For example, the genetically modified plant cells according to the invention exhibit a modified expression pattern. In the sense of the present invention, the term "genetically modified" means that the monocotyledon plant cell according to the invention contains at least one extraneous nucleic acid molecule which is integrated in the genome in a stable manner.

In the sense of the present invention, the term "extraneous nucleic acid molecule" is to be understood to mean a nucleic acid molecule which codes for a protein with the biological activity of an R1 protein, preferably an R1 protein from Solanum tuberosum, and which does not occur naturally in corresponding non-genetically modified wild-type plant cells. The extraneous nucleic acid molecule is preferably a recombinant molecule which consists of different elements, the combination or specific spatial arrangement of which does not occur naturally in plant cells. The monocotyledon plant cells according to the invention contain at least one extraneous nucleic acid molecule, wherein the latter is preferably linked

to regulatory DNA elements which ensure transcription in plant cells, particularly with a promoter.

An example of an extraneous nucleic acid molecule which codes for an R1 protein from Solanum tuberosum is represented in SEQ ID No.1. Nucleotide sequences which code for an R1 protein from Solanum tuberosum have also been described in WO 97/11188 A1 and by Lorberth et al. (Nature Biotech. 16, (1998), 473-477).

The important characteristics of of R1 proteins from Solanum tuberosum are i) their amino acid sequence (see SEQ ID No. 2, for example); ii) their location in the stroma of plastids of plant cells, wherein they can exist there both in a form bound to starch grains and in soluble form; and iii) their capacity for influencing the degree of phoshorylation of starch in plants. For example, inhibition of the R1 gene which codes for an R1 protein from potatoes in transgenic potato plants results in a reduction of the phosphate content of the starch which can be isolated from the potato tubers. Furthermore, Lorberth et al. showed that the R1 protein from Solanum tuberosum is capable of phosphorylating bacterial glycogen if the corresponding R1 cDNA is expressed in E. coli (Lorberth et al , Nature Biotech. 16, (1998), 473-477).
Ritte et al. (Plant J. 21, (2000), 387-391) showed that the R1 protein from Solanum tuberosum binds reversibly to starch grains in potato plants, wherein the strength of binding to the starch grain depends on the metabolic status of the plant. In starch grain-bound form, the protein in potato plants mainly occurs in leaves which are kept in the dark. After the leaves are illuminated, however, the protein is mainly present in a soluble form which is not bound to starch grains. Moreover, inhibiting the expression of the R1 gene from potatoes in transgenic potato plants results in a "starch-excess" phenotype, i.e. the leaves of corresponding plants have an increased content of starch (Lorberth et al., Nature Biotech. 16, (1998), 473-477). In addition, the tubers of such potato plants are distinguished in that after cold storage they exhibit reduced "cold-induced sweetening" (Lorberth et al., Nature Biotech. 16, (1998), 473-477).

In principle, the extraneous nucleic acid molecule which codes for an R1 protein can originate from any kind of potato or potato plant, preferably from potatoes of the Tomensa, Desiree, Tempora and Thomana varieties.

In one preferred embodiment, the extraneous nucleic acid molecule has the nucleotide sequence represented in SEQ ID No. 1.

In a further preferred embodiment the invention comprises the extraneous nucleic acid molecule of the coding region of the nucleotide sequence represented in SEQ ID No.1.

In a further preferred embodiment of the invention, the extraneous nucleic acid molecule encodes an R1 protein from Solanum tuberosum which has the amino acid sequence given in SEQ ID No. 2.

In yet another embodiment of the invention, the extraneous nucleic acid molecules comprise the coding region of the mature (without a plastidary signal peptide) protein (bp 447-bp 4607 of the nucleotide sequence given in SEQ ID No. 1). Instead of the plastidary N-terminal signal peptide of the R1 protein from potatoes (amino acids 1 to 77 coded by nucleotides 216 to 446 of the nucleotide sequence given in SEQ ID No.1), in this embodiment of the invention the extraneous nucleic acid molecules comprise a heterologous plastidary signal sequence, i.e. a signal sequence which does not exist naturally in association with the mature R1 protein. Plastidary signal sequences are known to one skilled in the art.
The signal sequence of Ferrodoxin:$NADP^+$ oxidoreductase (FNR) from spinate can be used as a plastidary signal sequence, for example. This sequence contains the 5'-non-translated region as well as the flanking transit peptide sequence of the cDNA of the plastidary protein Ferrodoxin:$NADP^+$ oxidoreductase from spinate (nucleotides -171 to + 165; Jansen et al., Current Genetics 13, (1988), 517-522).

In addition, for example, the transit peptide of the waxy protein from maize plus the first 34 amino acids of the mature waxy protein can be used as a signal sequence (Klösgen et al., Mol. Gen. Genet. 217, (1989), 155-161). Furthermore, the transit peptide of the waxy protein from maize can be used without the first 34 amino acids of the mature waxy protein.

In a further preferred embodiment of the invention, the extraneous nucleic acid molecule constitutes a derivative of the nucleotide sequence given in SEQ ID No.1.

In the sense of the present invention, the expression "derivative" means that the sequences of these molecules differ at one or more positions from the nucleotide sequence given in SEQ ID No. 1 and exhibit a high degree of homology with the coding region of the nucleotide sequence given in SEQ ID No. 1. In addition, derivatives are distinguished in that they code for a protein with the biological activity of an R1 protein, preferably of an R1 protein from Solanum tuberosum. In the sense of the present invention, "homology" means a sequence identity of nucleotide sequences of at least 65 %, particularly of at least 85 %, more particularly an identity of at least 90 %, preferably more than 95 % and most preferably more than 98 %. Differences from the nucleic acid molecules described above can occur due to deletion, addition, substitution, insertion or recombination.

The degree of homology is preferably determined by comparing the sequence of the respective nucleotide sequence with the coding region of SEQ ID No.1, particularly with region of SEQ ID No 1 (bp447-bp4607) which codes for the mature protein of the nucleotide sequence given in SEQ ID No. 1). If the sequences to be compared are of different lengths, the degree of homology preferably refers to the percentage of nucleotides in the shorter nucleotide sequence which are identical to the nucleotides of the longer sequence, i.e. the sequence identity is determined for the region in which the respective nucleotide sequences overlap. Sequence comparisons can be made using known computer programs, such as the ClustalW program (Thompson et al., Nucleic Acids

Research 22, (1994), 4673-4680), which is distributed by Julie Thompson (Thompson@EMBL-Heidelberg.DE) and Toby Gibson (Gibson@EMBL-Heidelberg.DE; European Molecular Biology Laboratory, Meyerhofstrasse 1, D 69117 Heidelberg, Germany). Clustal W can also be downloaded from various internet sites, e.g. that of the IGBMC (Institut de Génétique et de Biologie Moléculaire et Cellulaire, B.P.163, 67404 Illkirch Cedex, France; ftp://ftp-igbmc.u-strasbg.fr/pub/) and that of the EBI (European Bioinformatics Institute) (ftp://ftp.ebi.ac.uk/pub/software/), as well as from internet sites with links to the EBI (European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SD, UK).

When using the ClustalW program (Version 1.8), the default values of the various parameters are employed. In the case of DNA sequence comparisons, these parameters have the following values: KTUPLE=2, TOPDIAGS=4, PAIRGAP=5, DNAMATRIX:IUB, GAPOPEN=10, GAPEXT=5, MAXDIV=40, TRANSITIONS: unweighted.

In the case of protein sequence comparisons the default values of the parameters of the ClustalW program are likewise used. These parameters have the following values: KTUPLE=1, TOPDIAG=5, WINDOW=5, PAIRGAP=3, GAPOPEN=10, GAPEXTEND=0.05, GAPDIST=8, MAXDIV=40, MATRIX=GONNET, ENDGAPS(OFF), NOPGAP, NOHGAP.

The degree of homology can be determined, for example, using known computer programs such as Mview (http://www.sacseeucsf.edu/documentation/seqsoftware/mview; Brown, N.P., Leroy C., Sander C. (1998). MView: A Web compatible database search or multiple alignment viewer. *Bioinformatics*,14(4):380-381).

In the sense of the present invention, "homology" also means that there is functional and/or structural equivalence between the respective nucleic acid molecules or the proteins which they encode. The nucleic acid molecules which are homologous with the nucleic acid molecules described in SEQ ID No.1 and which constitute derivatives of these molecules are variations of these molecules which constitute modifications which perform the same biological function, and which in vivo are also capable of increasing the phosphate content of starches in

monocotyledon plants. These can be either naturally occurring variations, for example sequences from other types of potatoes, or mutations, wherein said mutations may have been formed naturally or may have been introduced by targeted mutagenesis.

In a further embodiment of the invention, the derivative of SEQ ID No.1 comprises allelic variants of the nucleic acid molecule given in SEQ ID No.1.
These allelic variants are naturally occurring variants which are capable of increasing the phosphate content of starches.

In a further embodiment of the invention, the extraneous nucleic acid molecule constitutes a fragment of the extraneous nucleic acid molecule which is defined according to the invention.

In the sense of the present invention, the term "fragment" denotes parts of the extraneous nucleic acid molecule which code for a biologically active, preferably enzymatically active, part of the R1 protein.
A biologically active part of an R1 protein is distinguished in that *in planta* it can cause an increase of the phosphate content with superexpression of the extraneous nucleic acid molecule and/or that after expression in E. coli it facilitates phoshorylation of glycogen (see WO 97/11188 A1, Example 9).

The proteins which are encoded by the different variants (fragments, derivatives, allelic variants) of the extraneous nucleic acid molecule exhibit defined general characteristics. Examples of the latter include the biological activity, the enzymatic activity, or a similar primary structure which can be investigated by means of the protein homology comparisons described above. The amino acid sequences of the proteins exhibit a mutual homology of at least 60%, preferably at least 85%, particularly at least 95% and most preferably at least 97%.

In addition to the characteristics described above, the extraneous nucleic acid molecules may be characterised in that they code for proteins with the biological activity of R1 proteins, which comprise at least one, preferably at least five,

particularly at least 10 and most preferably all of the following characteristic peptide motifs:
DKAAET (SEQ ID No. 3), IADME (SEQ ID No. 4), VWMRFM (SEQ ID No. 5), MQEWHQ (SEQ ID No. 6), LGHYM (SEQ ID No. 7), ERGYEE (SEQ ID No.8), KAVLDR (SEQ ID No. 9), LSSLL (SEQ ID No. 10), IPDGAV (SEQ ID No.11), KVCFAT (SEQ ID No. 12), ISADEF (SEQ ID No. 13), PFGVFE (SEQ ID No. 14), SSGDD (SEQ ID No. 15), SFICKK (SEQ ID No. 16).

Amongst other differences, the plant cells according to the invention can also be distinguished from naturally occurring plant cells in that they contain an extraneous nucleic acid molecule which does not occur naturally in these cells, or that a molecule such as this is integrated at a place in the genome of the cell at which it does not occur naturally, i.e. in a different genomic environment. Moreover, transgenic plant cells of this type according to the invention can be distinguished from naturally occurring plant cells in that they contain at least one copy of the extraneous nucleic acid molecule which is integrated in a stable manner in their genome contain, optionally in addition to copies of a molecule such as this which occur naturally in the cells. If the nucleic acid molecules which are introduced into the cells are additional copies of molecules which already occur naturally in the cells, the plant cells according to the invention can be distinguished in particular from naturally occurring plant cells in that these additional copies are located at sites in the genome at which they do not occur naturally. This can be detected subsequently by means of Southern blotting analysis, for example.
Furthermore, the plant cells according to the invention are preferably distinguished from naturally occurring plant cells by at least one of the following features: if the nucleic acid molecule which is introduced is heterologous with respect to the plant the transgenic plant cells according to the invention comprise transcripts of the nucleic acid molecules which are introduced, in fact even in those organs in which no transcripts can be detected in wild-type plants. Plant cells according to the invention preferably contain transcripts of the extraneous nucleic acid molecules. These can be detected by Northern blotting analysis, for example. Plant cells according to the invention preferably contain a protein which

is encoded by an introduced extraneous nucleic acid molecule. This additional protein can be detected by immunological methods for example, particularly by Western blotting analysis.

In one preferred embodiment, the plant cells and plants according to the invention exhibit an increased biological activity of the R1 protein compared with corresponding non-genetically modified wild-type plant cells and wild-type-plants. In connection with the present invention, an "increased biological activity of the R1 protein" can be determined, for example, by measuring the phosphate content of the starches which are synthesised in the plant cells and plants according to the invention. Compared with starches from corresponding non-genetically modified wild-type plant cells, starches from the plant cells according to the invention, which exhibit increased biological activity of the R1 protein, are distinguished in that they synthesise a starch with an increased phosphate content, preferably an increased phosphate content in the C6 position of the glucose monomer.
Moreover, an increased biological activity of the R1 protein can be determined by measuring the amount of R1-transcripts, e.g. by Northern blotting analysis, compared with the amount of R1-transcripts of corresponding non-genetically modified wild-type plants.
Furthermore, an increased biological activity of the R1 protein can be determined by measuring the amount of R1 protein, e.g. by Western blotting analysis, compared with the amount of R1 protein of corresponding non-genetically modified wild-type plants.

It has been found that the monocotyledon plant cells according to the invention synthesise a starch with an increased phosphate content in the C6 position of the glucose monomer and/or a modified phoshorylation pattern and/or modified viscosity properties compared with starch from corresponding non-genetically modified plant cells of wild-type-plants.

Therefore, the present invention preferably relates to plant cells according to the invention which synthesise a starch has an increased phosphate content in the

C6 position of the glucose monomer and/or a modified phoshorylation pattern and/or modified viscosity properties compared with starch from corresponding non-genetically modified plant cells of wild-type-plants.

In the sense of the present invention, the term "phosphate content" denotes the content of phosphate which is covalently bonded in the form of starch phosphate monoesters.

In the sense of the present invention, the term "C6 position" is to be understood to mean phosphate groups, which are bonded to the glucose monomer of the starch at carbon atom position "6".

In connection with the present invention, the expression "increased phosphate content in the C6 position" is to be understood to mean that glucose monomer phosphate groups can be detected in the in the C6 position by means of an optical-enzymatic test (Nielsen et al., Plant Physiol. 105, (1994), 111-117, see methods). The expression "increased phosphate content in the C6 position" is preferably understood to mean an increase in the phosphate content of the starch in the C6 position of the glucose monomer by at least 20%, preferably by at least 50% and most preferably by at least 100%, compared with the phosphate content of starch from corresponding non-genetically modified wild-type-plants.
In principle, the C2, C3 and C6 positions of the glucose units can be phosphorylated in the starch in vivo. In connection with the present invention, the phosphate content in the C6 position (= C6 P content) can be determined by a glucose-6-phosphate determination by means of an optical-enzymatic test (Nielsen et al., Plant Physiol. 105, (1994), 111-117) (see methods).

In the sense of the present invention, the term "modified phoshorylation pattern means that the total phosphate content of phosphate groups which are covalently bonded to the starch within the various layers of the starch grain is modified compared with chemically phosphorylated starches which are produced from starches from corresponding non-genetically modified plants. Chemically phosphorylated starches are distinguished by a phosphate gradient inside the

starch grains, wherein the outer layers are generally more strongly phosphorylated than are the inner layers. In contrast to this, the starches according to the invention are distinguished in that the phosphate groups are distributed differently over the various fayers of the starch grain, i.e. the starches according to the invention can be characterised in that they do not comprise the gradients from the outside to the inside which are typical of chemically phosphorylated starches.

Methods of investigatingh the phoshorylation pattern of starch are known to one skilled in the art (see Jane and Shen, Carbohydrate Research 247, (1993), 279-290; Gough and Pybus, Staerke 25, (1973), 123-130, for example). These methods are based on step-wise chemical conglutination of the various layers of the starch grains, in which the conglutinated layers of the starch grains are mechanically removed in steps. This shelling procedure is followed by a determination of the total phosphate content of the various starch grain layers by standard methods.

In the sense of the present invention, the term "chemically phosphorylated starch" is to be understood to mean a starch which is produced by the chemical phoshorylation of native starch from corresponding non-genetically modified plant cells and/or plants. In connection with the present invention, chemically phosphorylated starch is preferably distinguished by an amylose content which is comparable with the amylose content of the starches according to the invention. During the chemical phoshorylation of native starches, the phosphate content in the C6 position is adjusted by selecting suitable test conditions so that the phosphate content in the C6 position of the chemically phosphorylated starch is identical to and/or comparable with the phosphate content in the C6 position of the glucose monomer of the starches according to the invention.

In connection with the present invention, the term "modified viscosity properties" is to be understood in particular to mean an increase in the final viscosity in an RVA profile and/or a lowering of the peak temperature in DSC analysis.

In a further preferred embodiment, the plant cells according to the invention synthesise a starch which has an increased final viscosity compared with starch from corresponding non-genetically modified wild-type plants.

In connection with the present invention, the term "final viscosity" is to be understood to mean the viscosity which can be determined from a viscosity profile (see Figure 1) and which is denoted there as "final viscosity = Fin". The viscosity profile can be obtained by means of a Rapid Visco Analyzer (RVA) (Newport Scientific Pty Ltd, Investment Support Group, Warriewood, NSW 2102, Australia). In the analysis of wheat starches, the viscosity profile is determined by the following procedure: 2.5 g starch (dry substance) are taken up in 25 ml H_2O and used for analysis in a Rapid Visco Analyzer (Newport Scientific Pty Ltd., Investment Support Group, Warriewood NSW 2102, Australia). The instrument is operated according to the manufacturer's instructions. The complete temperature programme is illustrated schematically in Figure 1. Performing an RVA analysis is described in detail below (see methods).

In the sense of the present invention, the term "increased final viscosity" means that the final viscosity is increased by at least 10%, preferably by at least 30%, particularly by at least 50% and most preferably by at least 80% compared with starches from corresponding non-genetically modified wild-type plant cells, wherein the final viscosity can be increased by 1000% at most, preferably by 500% at most, particularly by 250% at most, compared with starches from corresponding non-genetically modified wild-type plant cells.

In a further preferred embodiment, the plant cells according to the invention synthesise a starch which has an identical or comparable phosphate content in the C6 position of the glucose monomer and/or a reduced peak temperature compared with starch from corresponding non-genetically modified wild-type plants and/or compared with chemically phosphorylated starches.

In the context of the present invention, the term "peak temperature" should be understood to mean the temperature Tp which can be determined by means of

differential scanning calorimetry (DSC), which is known to one skilled in the art. The peak temperature is the temperature which can generally be assigned to the first peak maximum of the DSC curve. It can be determined, for example, using an instrument supplied by Perkin Elmer (instrument designation: DSC-7) using capsules of large volume, wherein the sample to be investigated comprises a ratio of starch to total water content of about 1:4 and measurements are made over a temperature range from 10°C to 160°C at a heating rate of 10°C/min (see Example 4).

The term "reduced peak temperature" Tp means that compared with starches from corresponding non-genetically modified wild-type plant cells the peak temperature Tp is reduced by at least 1.5°C, preferably by at least 2.5°C, particularly by at least 4°C and most preferably by at least 6°C, and is reduced at most by 5°C, by 12°C or by 9°C.

In a further preferred embodiment, the present invention relates to plant cells according to the invention which synthesise a starch which after conglutination forms a gel, which has an increased gel strength compared with a gel formed from starch from corresponding non-genetically modified wild-type plant cells.

In the sense of the present invention, the term "increased gel strength" is to be understood to mean an increase gel strength by at least 20%, particularly by at least 50%, preferably by at least 80% and most preferably by at least 100%, with the maximum reduction being 500% at most or 250% at most compared with the gel strength of starch from corresponding non-genetically modified wild-type plant cells.
In connection with the present invention, the gel strength can be determined by means of a texture analyser under the conditions described below (see methods).

In a further embodiment of the invention, the increase in the phosphate content of the starches can relate to the amylopectin component of the starch.
Therefore, the present invention also relates to plant cells according to the invention which synthesise a starch, the amylopectin component of which is

phosphorylated and the amylose component of which has a reduced total phosphate content compared with the amylose component of corresponding chemically phosphorylated starches with the same starch phosphate content in the C6 position of the glucose monomer.
Therefore, in contrast to chemically phosphorylated starches with the same phosphate content in the C6 position of the glucose monomer, the starches of the plant cells according to the invention are distinguished in that that the amylose component of the starch from the plant cells according to the invention has a reduced total phosphate content and/or a reduced phosphate content in the C6 position of the glucose monomer compared with the amylose component of chemically phosphorylated starch with the same phosphate content in the C6 position of the glucose monomer, which is produced from starch, which preferably has a comparable amylose content, from corresponding non-genetically modified plants.

In connection with the present invention, the term "total phosphate content" is to be understood to mean the content of phosphate which is covalently bound in the form of starch phosphate monoesters in the C2, C3 and C6 positions of the glucose units. According to the invention, the content of phosphorylated non-glucans, such as phospholipids, is not included under the term "total phosphate content". Phosphorylated non-glucans therefore have to be quantitatively separated before the determination of the total phosphate content.
Methods of separating phosphorylated non-glucans (e.g. phospholipids) from starch are known to one skilled in the art.

In the sense of the present invention, the term "reduced total phosphate content" is to be understood to mean a reduction of the total phosphate content by at least 5%, particularly by at least 20%, preferably by at least 50% and most preferably by at least 80%, compared with the total phosphate content of the amylose component of a chemically phosphorylated starch with the same C6 phosphate content which is produced from starch from corresponding non-genetically modified plants.

Methods of determining of the total phosphate content are known to one skilled in the art and are described below (see methods).

In the sense of the present invention, the term "reduced phosphate content in the C6 position of the glucose monomer" should be understood to mean a reduction of the phosphate content in the C6 position of the glucose monomer of the amylose component by at least 10%, particularly by at least 20%, preferably by at least 50% and most preferably by at least 80% compared with the phosphate content in the C6 position of the glucose monomer of the amylose component of a chemically phosphorylated starch of the same C6 phosphate content which is produced from starch from corresponding non-genetically modified plants. Methods of determining of the phosphate content in the C6 position of the glucose monomer are known to one skilled in the art and are described below (see methods).

In the sense of the present invention the term "amylopectin component" is to be understood to mean the amylopectin of the starch.

In the sense of the present invention, term "amylose component" is to be understood to mean the amylose of the starch.

Methods of separating amylose and amylopectin are known to one skilled in the art. For example, the amylose component can be obtained by aqueous leaching of the starch, as described, for example, by Roger & Colonna (International Journal of Biological Macromolecules 19, (1996), 51-61).

In a further preferred embodiment of the present invention, the starches according to the invention are additionally characterised in that the ratio of the C6 phosphate content of the glucose monomer in the C6 position of the amylose component to the C6 phosphate content of the glucose monomer in the C6 position of the starch is less than 0.75, particularly less than 0.5, preferably less than 0.25 and most preferably less than 0.20. In contrast to chemically phosphorylated starches, the starches according to the invention are

distinguished in that the major part of the phosphate in the C6 position of the glucose monomer is detected in the amylopectin component of the starch and not in the amylose component.

The plant cells according to the invention originate from monocotyledon plants. They are preferably plant cells from plants which are used in agriculture, i.e. from plants, which are cultivated by humans for the purpose of nourishment or for technical purposes, particularly industrial purposes. Thus the present invention preferably relates to plant cells from starch-synthesising or starch-storing plants, e.g. rye, barley, oats, wheat, millet, rice or maize.

In one preferred embodiment of the present invention, the plant cells according to the invention originate from a plant of the group consisting of wheat, rice, barley, oats, rye and maize. Plant cells from wheat, rice and maize plants are preferred; plant cells from wheat plants are particularly preferred.

In one particularly preferred embodiment, the plant cells according to the invention synthesise a starch which in the C6 position of the glucose monomer has a phosphate content of at least 0.1 nmol C6 P mg^{-1} starch, particularly at least 0.5 nmol C6 P mg^{-1}, preferably at least 1 nmol C6 P mg^{-1} starch, more preferably at least 2 nmol C6 P mg^{-1} starch, most preferably at least 5 nmol C6 P mg^{-1} starch, most particularly preferably at least 10 nmol C6 P mg^{-1} starch, wherein the plant cells according to the invention synthesise a starch which in the C6 position of the glucose monomer has a phosphate content of 100 nmol C6 P mg^{-1} starch at most, particularly 50 nmol C6 P mg^{-1} starch at most, and most particularly 25 nmol C6 P mg^{-1} starch at most.

In a further embodiment of the invention, the plant cells according to the invention can synthesise a starch which in the C6 position of the glucose monomer has a phosphate content of at least 15 nmol C6 P mg^{-1} starch, wherein the plant cells according to the invention synthesise a starch which in the C6 position of the glucose monomer have a phosphate content of 100 nmol C6 P mg^{-1} starch at

most, particularly 50 nmol C6 P mg^{-1} starch at most, and most particularly 25 nmol C6 P mg^{-1} starch at most.

In a further embodiment, the present invention relates to a method of producing a plant cell according to the invention, wherein a cell of a monocotyledon plant is genetically modified, and wherein the genetic modification consists of the introduction of at least one extraneous nucleic acid molecule.

There is a multiplicity of techniques which are available for the transfer of DNA into a plant host cell. These techniques include the transformation of plant cells with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as a transformation agent, fusion of protoplasts, injection, electroporation of DNA, the introduction of DNA by means of biolistic techniques and other possible techniques.

The use of the transformation of plant cells facilitated by agrobacteria has been intensively investigated and is satisfactorily described in EP 120516; Hoekema, IN: The Binary Plant Vector System Offsetdrukkerij Kanters B.V., Alblasserdam (1985), Chapter V; Fraley et al., Crit. Rev. Plant Sci. 4, 1-46 and An et al. EMBO J. 4, (1985), 277-287. For the transformation of potatoes, see Rocha-Sosa et al., EMBO J. 8, (1989), 29-33.), for example.

The transformation of monocotyledon plants has in the meantime been routinely accomplished by means of a biolistic technique and by means of agrobacteria (Komari et al., (1998), Advances in cereal gene transfer; Current Opinion in Plant Biotechnology 1, page 161 et seq.; Bilang et al. (1999), Transformation of Cereals, Genetic Engineering, 12, pages 113-148, edited by: JK Setlow, Kluwer Academic / Plenum Publisher, New York). Vectors based on agrobacteria have been described (Chan et al., Plant Mol. Biol. 22, (1993), 491-506; Hiei et al., Plant J. 6, (1994) 271-282; Deng et al, Science in China 33, (1990), 28-34; Wilmink et al., Plant Cell Reports 11, (1992), 76-80; May et al., Bio/Technology 13, (1995), 486-492; Conner and Domisse, Int. J. Plant Sci. 153 (1992), 550-555; Ritchie et al, Transgenic Res. 2, (1993), 252-265). Alternative systems for the transformation of monocotyledon plants include transformation by means of a biolistic technique (Wan and Lemaux, Plant Physiol. 104, (1994), 37-48; Vasil et

al., Bio/Technology 11 (1993), 1553-1558; Ritala et al., Plant Mol. Biol. 24, (1994), 317-325; Spencer et al., Theor. Appl. Genet. 79, (1990), 625-631), protoplast transformation, electroporation of partially permeabilised cells, and the introduction of DNA by means of glass fibres. In particular, the transformation of maize has been described many times in the literature (see WO 95/06128, EP 0513849, EO 0465875, EP 292435; Fromm et al., Biotechnology 8, (1990), 833-844; Gordon-Kamm et al., Plant Cell 2, (1990), 603-618; Koziel et al., Biotechnology 11 (1993), 194-200; Moroc et al., Theor. Appl. Genet. 80, (1990), 721-726, for example).

The successful transformation of other types of cereals has also been described, e.g. for barley (Wan and Lemaux, see above; Ritala et al., see above; Krens et al., Nature 296, (1982), 72-74) and for wheat (Becker et al., Plant J. 5 (2), (1994), 229-307; Nehra et al., Plant J. 5, (1994), 285-297).

Various transformation methods have been described for rice, e.g. transformation facilitated by agrobacteria (Hiei et al., Plant J. 6 (1994), 271-282; Hiei et al., Plant Mol. Biol. 35 (1997), 205-218; Park et al., J. Plant Biol. 38 (1995), 365-371), protoplast transformation (Datta, In "Gene transfer to plants", Potrykus, Spangenberg (Eds.), Springer-Verlag, Berlin, Heidelberg, 1995, 66-75; Datta et al., Plant Mol. Biol. 20 (1992), 619-629; Sadasivam et al., Plant Cell Rep. 13 (1994), 394-396), a biolistic technique for plant transformation (Li et al., Plant Cell Rep. 12 (1993), 250-255; Cao et al., Plant Cell Rep. 11 (1992), 586-591; Christou, Plant Mol. Biol. (1997), 197-203), and electroporation (Xu et al., In "Gene transfer to plants", Potrykus, Spangenberg (Eds.), Springer-Verlag, Berlin, Heidelberg, 1995, 201-208).

The present invention further relates to plants containing the plant cells according to the invention and/or which can be produced by regeneration from plant cells according to the invention. These plants are preferably useful monocotyledon plants, e.g. rye, barley, oats, wheat, millet, rice and maize. Wheat, rice and maize are preferred; wheat is particularly preferred.

The plants according to the invention synthesise a modified starch, the phosphate content of which and/or the phoshorylation pattern of which and/or the viscosity

properties of which differ from those of starches from corresponding non-genetically modified wild-type-plants, as described in connection with the plant cells according to the invention.

Therefore, the present invention also relates to plants according to the invention which synthesise a starch, which has an increased phosphate content in the C6 position of the glucose monomer and/or exhibits a modified phoshorylation pattern and/or modified viscosity properties compared with starch from corresponding non-genetically modified plants.
The statements made in connection with the plant cells according to the invention are applicable with regard to the increase of the phosphate content in the C6 position of the glucose monomer of the starch, the modification of the phoshorylation pattern and the modification of the viscosity properties of the starch.
The present invention preferably relates to plants according to the invention which synthesise a starch after conglutination forms a gel, which has an increased gel strength compared with a gel formed from starch from corresponding non-genetically modified wild-type-plants.

The term "increased gel strength" was defined in connection with the plant cells according to the invention.

In a further preferred embodiment of the invention, the plants according to the invention, preferably wheat plants, synthesise a starch, preferably wheat starch, which in the C6 position of the glucose monomer has a phosphate content of at least 0.1 nmol C6 P mg^{-1}starch, particularly at least 0.5 nmol C6 P mg^{-1}, preferably at least 1 nmol C6 P mg^{-1} starch, most preferably at least 2 nmol C6 P mg^{-1} starch, particularly at least 5 nmol C6 P mg^{-1} starch, quite most preferably at least 10 nmol C6 P mg^{-1} starch, wherein the plants according to the invention synthesise a starch which in the C6 position of the glucose monomer has a phosphate content of 100 nmol C6 P mg^{-1} starch at most, particularly 50 nmol C6 P mg^{-1} starch at most, or 25 nmol C6 P mg^{-1} starch at most.

In a further embodiment of the invention, the plants according to the invention can synthesise a starch which in the in the C6 position of the glucose monomer has a phosphate content of at least 15 nmol C6 P mg^{-1} starch, wherein the plants according to the invention synthesise a starch which in the C6 position of the glucose monomer has a phosphate content of 100 nmol C6 P mg^{-1} starch at most, particularly 50 nmol C6 P mg^{-1} starch at most or 25 nmol C6 P mg^{-1} starch at most.

In a further preferred embodiment of the invention, the plants according to the invention synthesise the starch according to the invention in the starch-storing organs of the plant according to the invention.

In a most preferred embodiment, the plants according to the invention synthesises a starch in its starch-storing organs which has an increased phosphate content in the C6 position of the glucose monomer and/or a modified phosphorylation pattern and/or modified viscosity properties compared with starch from starch-storing organs of corresponding non-genetically modified wild-type plants.

In this connection, the expression "starch-storing organs" is to be understood to mean those organs, e.g. the grains of maize, rice and wheat plants, which incorporate stored starch, in contrast to those organs, such as leaves, which only synthesise starch transiently.

Expression of the extraneous nucleic acid molecule is particularly advantageous in starch-storing organs of monocotyledon plants, especially in wheat plants, and results in an increase of the phosphate content of the starches which can be isolated from the starch-storing organs compared with starches which can be isolated from the starch-storing organs of corresponding non-genetically modified wild-type plants, particularly wheat plants.

Expression of the extraneous nucleic acid molecule in starch-storing organs of monocotyledon plants can be achieved firstly by the use of constitutive promoters, e.g. the promoter of the 35S RNA of the cauliflower mosaic virus (see US-A-

5,352,605, for example) and the ubiquitin promoter of maize (see US-A-5,614,399, for example).

Promoters are preferably used which are specific for starch-storing organs, e.g. endosperm-specific promoters, such as the glutelin promoter (Leisy et al., Plant Mol. Biol. 14, (1990), 41-50; Zheng et al., Plant J. 4, (1993), 357-366; Yoshihara et al., FEBS Lett. 383, (1996), 213-218), the shrunken-1 promoter (Werr et al., EMBO J. 4, (1985), 1373-1380), the HMW promoter of wheat (Anderson, Theoretical and Applied Genetics 96, (1998), 568-576, Thomas, Plant Cell 2 (12), (1990), 1171-1180), the USP promoter, the phaseolin promoter (Sengupta-Gopalan, Proc. Natl. Acad. Sci. USA 82 (1985), 3320-3324, Bustos, Plant Cell 1 (9) (1989), 839-853) or promoters of zein genes from maize (Pedersen et al., Cell 29, (1982), 1015-1026; Quatroccio et al., Plant Mol. Biol. 15 (1990), 81-93) or caryopsis-specific promoters of GBSSI (granule bound starch synthase I) (DE10041861.9) and of SSII (soluble starch synthase II) from wheat (DE10032379.0).

In one particularly preferred embodiment, the plants according to the invention exhibit R1 gene expression in the starch-storing organs of said plants.

R1 gene expression can be determined, for example, by measuring the amount of R1 transcripts, e.g. by Northern blotting analysis, compared with the amount of R1 transcripts of wild-type plants.

Moreover, in connection with the present invention, R1 gene expression can be determined by measuring the amount of R1 protein, e.g. by Western blotting analysis. The amount of R1 protein is preferably determined by means of protein extracts which are isolated from plant cells of endosperm.

In a further embodiment of the invention, the plants according to the invention exhibit R1 gene expression in the starch-storing organs of said plants, which is increased compared with R1 gene expression in starch-storing organs of corresponding non-genetically modified wild-type-plants, preferably by at least

50%, most preferably by at least 100%, particularly by at least 250% and most particularly by at least 500%.

In one most preferred embodiment, the plants according to the invention exhibit a organ-specific expression of the extraneous nucleic acid molecule in the starch-storing organs of the plants according to the invention.

In the sense of the present invention, the term "organ-specific" is to be understood to mean that the selected promoter favours expression of the extraneous nucleic acid molecule in the starch-storing organs compared with mature leaves, and results in significantly increased degree of expression, such as a level of expression which is increased at least 2- to 5 times, preferably 5- to 10 times, most preferably 10- to 100 times.
In this embodiment of the invention, the plants according to the invention are distinguished in that due to the organ-specific expression of the extraneous nucleic acid molecule they exhibit increased R1 gene expression in the starch-storing organs compared with the gene expression of the endogeneous R1 gene in the starch-storing organs of a corresponding non-genetically modified wild-type-plant. In this embodiment of the invention, an increase in R1 gene expression in the leaves of the plants according to the invention cannot be detected to the same extent as that in the starch-storing organs. The increase in R1 gene expression is preferably related solely to the starch-storing organs.

In a preferred embodiment of the invention, the plants according to the invention are plants from the group consisting of wheat, rice, barley, millet, oats, rye and maize. Wheat, rice and maize plants are preferred; wheat plants are particularly preferred.

The invention also relates to a method of producing a plant as defined according to the invention, wherein

(a) a cell of a monocotyledon plant is genetically modified, wherein the genetic modification consists of the introduction of at least one extraneous nucleic acid molecule;

(b) a plant is regenerated from the cell according to step (a); and optionally

(c) further plants are produced from the plant produced according to step (b).

The present invention further relates to a method of producing a plant as defined according to the invention, which in the starch-storing organs of said plant synthesises a starch with an increased phosphate content and/or a modified phoshorylation pattern and/or an increased final viscosity and/or a reduced peak temperature compared with starch from starch-storing organs of wild-type-plants, wherein

(a) a cell of a monocotyledon plant is genetically modified, wherein the genetic modification consists of the introduction of at least one extraneous nucleic acid molecule as defined according to the invention;

(b) a plant is regenerated from the cell according to step (a); and optionally

(c) further plants are produced from the plant produced according to step (b).

The same applies to the genetic modification which is introduced according to step (a) as that which has already been explained above in connection with the plant cells and plants according to the invention.
The regeneration of plants according to step (b) can be accomplished by methods known to one skilled in the art.
The production of further plants according to step (c) of the method according to the invention can be accomplished, for example, by vegetative propagation) e.g. via cuttings, tubers or via callus cultivation and the regeneration of whole plants) or by sexual propagation. Sexual propagation preferably occurs in a controlled manner, i.e. selected plants with defined properties are crossed and propagated with each other.

In one preferred embodiment of the invention, the methods according to the invention are distinguished in that the extraneous nucleic acid molecule which is introduced into the plant cell according to step (a) is selected from the group consisting of:

(i) nucleic acid molecules which comprise the coding region of the nucleotide sequence represented in Seq ID No. 1;

(ii) nucleic acid molecules which encode an R1 protein from Solanum tuberosum with the amino acid sequence given in Seq ID No. 2;

(iii) nucleic acid molecules which constitute a derivative of the nucleotide sequence given in Seq ID No. 1; and

(iv) nucleic acid molecules which constitute fragments of the nucleic acid molecules cited in (i), (ii) or (iii).

In one particularly preferred embodiment, the methods according to the invention are distinguished in that the extraneous nucleic acid molecule is under the control of a promoter, preferably an endosperm-specific and/or caryopsis-specific promoter, which organ-specifically facilitates R1 gene expression in starch-storing tissue.

Promoters of this type, such as endosperm-specific and caryopsis-specific promoters, have been exemplified above in connection with the plants according to the invention.

In a further embodiment, the methods according to the invention relate to useful monocotyledon plants, e.g. rye, barley, oats, wheat, millet, rice and maize. Wheat, rice and maize are preferred; wheat is particularly preferred.

The present invention also relates to the plants which can be obtained by the method according to the invention.

The present invention also relates to propagation material for plants containing plant cells according to the invention, and to the plants produced by the method according to the invention. In the sense of the present invention, the term "propagation material" comprises any constituents of the plant which are suitable for production or propagation by a vegetative or generative route. Cuttings, callus cultures, rhizomes or tubers are suitable for vegetative propagation, for example. Other propagation material comprises fruit, seeds, seedlings, protoplasts, cell cultures etc., for example. Seeds are the preferred propagation material.

In one preferred embodiment, the present invention relates to maize grains.

In a most preferred embodiment, the present invention relates to wheat grains.

The maize and wheat grains according to the invention are particularly suitable for the production of fodder and of food products.

The present invention further relates to the use of extraneous nucleic acid molecules as defined according to the invention for the production of plants according to the invention, preferably of wheat, maize and rice plants, most preferably wheat plants, or for the production of monocotyledon plant cells according to the invention.

The plant cells and plant according to the invention synthesise, particularly in their starch-storing organs, a starch, the physicochemical properties of which, particularly the phosphate content of and/or the viscosity behaviour of which and/or the phoshorylation pattern of which is modified compared with that of starch synthesised in wild-type-plants and compared with chemically phosphorylated starches.

Therefore, the present invention also relates to starch which can be obtained from the plant cells, plants and/or propagation material according to the invention.

In one preferred embodiment of the invention, the starches according to the invention are characterised in that they have an increased phosphate content in the C6 position of the glucose monomer and/or a modified phoshorylation pattern and/or an increased final viscosity and/or a reduced peak temperature compared with starch from corresponding non-genetically modified wild-type plants.
The present invention also relates starch which is characterised in that it has an increased phosphate content in the C6 position of the glucose monomer and/or a modified phoshorylation pattern and/or an increased final viscosity and/or a reduced peak temperature compared with starch from corresponding non-genetically modified wild-type plants.

The statements made in connection with the plant cells according to the invention are applicable with regard to the increase of the phosphate content in the C6 position, the modification of the phoshorylation pattern and the modification of the viscosity properties (final viscosity, peak temperature).

In a further embodiment, the invention relates to starches, preferably wheat starches, which are characterised in that in the C6 position of the glucose monomer they have a phosphate content of at least 0.1 nmol C6 P mg^{-1} starch, particularly at least 0.5 nmol C6 P mg^{-1}starch, preferably at least 1 nmol C6 P mg^{-1} starch, most preferably at least 2 nmol C6 P mg^{-1} starch, most particularly at least 5 nmol C6 P mg^{-1} starch, quite most preferably at least 10 nmol C6 P mg^{-1} starch and/or an increased final viscosity and/or a reduced peak temperature, wherein in the C6 position of the glucose monomer the starch according to the invention has a phosphate content of 100 nmol C6 P mg^{-1} starch at most, particularly 50 nmol C6 P mg^{-1} starch at most, or 25 nmol C6 P mg^{-1} starch at most.

In a further embodiment of the invention, the starches according to the invention can be characterised in that in the C6 position of the glucose monomer they have a phosphate content of at least 15 nmol C6 P mg^{-1} starch, wherein in the C6 position of the glucose monomer the starch according to the invention has a phosphate content of 100 nmol C6 P mg^{-1} starch at most, particularly 50 nmol C6 P mg^{-1} starch at most, or 25 nmol C6 P mg^{-1} starch at most.

In a further preferred embodiment, the starches according to the invention, preferably wheat starches, are characterised in that after conglutination the starches according to the invention form gels which exhibit an increased gel strength compared with gels of corresponding chemically phosphorylated starches of the same phosphate content and/or compared with gels of starches from corresponding non-genetically modified plants.

In a further preferred embodiment of the invention, the starches according to the invention can be characterised in that the major part of the phosphate groups are bonded to the amylopectin component of the starch, whilst the amylose only has a very low content of covalently bonded starch monophosphate esters. Compared with chemically phosphorylated starches of comparable C-6 phosphate content, the starches according to the invention from the plant cells according to the invention are distinguished in that the amylose of the starches according to the invention is less strongly phosphorylated than is the amylopectin component.

In a further embodiment of the invention, the starches according to the invention can be characterised in that the amylose component of said starch has a reduced total phosphate content in the amylose component compared with the amylose component of chemically phosphorylated starch, which preferably has the same phosphate content in the C6 position of the glucose monomer.

Methods of determining of the total phosphate content are known to one skilled in the art and are described below (see methods).

In a further embodiment the invention, the starch according to the invention can be characterised in that the total phosphate content, which can be detected by 31P NMR (Kasemsuwan and Jane (Cereal Chemistry 73 (6), (1996), 702-707) of the amylose component of said starch is reduced by at least 5%, preferably by at least 20%, particularly by at least 50% and most preferably by at least 80%, compared with the amylose component of chemically phosphorylated starch with the same phosphate content in the C6 position of the glucose monomer which is produced from starch of corresponding non-genetically modified plants.
In one preferred embodiment of the invention, the starches according to the invention can be characterised in that they exhibit a modified phoshorylation pattern compared with chemically phosphorylated starches.
The term "modified phoshorylation pattern" has already been defined in connection with the plant cells according to the invention.

In one preferred embodiment of the invention, the starches are starches from useful monocotyledon plants, such as rye-, barley, oats, wheat, millet, rice and maize starch. Wheat, rice and maize starch are preferred; wheat starch is particularly preferred.

The present invention further relates to a method of producing the starch according to the invention, comprising the steps of extracting the starch from a plant or plant cell as described above and/or from starch-storing parts of plant such as this and/or from the propagation material according to the invention. A method such as this also preferably comprises the step of harvesting the cultivated plants and/or starch-storing parts of said plants before the extraction of the starch, and most preferably further comprises the step of cultivating plants according to the invention and/or of propagation materials according to the invention before harvesting. Methods of extracting starch from plants or from starch-storing parts of plants are known to one skilled in the art. Methods of extracting starch from various starch-storing plants are also described, for example, in "Starch: Chemistry and Technology (edited by: Whistler, BeMiller and Paschall (1994), 2nd Edition, Academic Press Inc. London Ltd; ISBN 0-12-746270-8; see Chapter XII, pages 412-468, for example: Maize 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; and Chapter XVI, pages 507 to 528: Rice starch: production and uses; by Rohmer and Klem; and see Maize starch: Eckhoff et al., Cereal Chem. 73 (1996) 54-57. The extraction of maize starch on an industrial scale is generally effected by what is termed "wet milling"). Apparatuses which are customarily used for methods of extracting starch from plant material include mills, separators, decanters, hydrocyclones, spray driers and fluidised-bed driers.

The present invention also relates to starch according to the invention which can be obtained by the methods according to the invention which were described above.

The starches according to the invention can be subsequently modified by methods known to one skilled in the art, and in their unmodified or modified form are suitable for various uses in the food or non-food sectors.

Therefore, the present invention also relates to starches as defined according to the invention which have been subsequently chemically and/or physically modified.

Various options which can be used for chemical and/or physical modification are described in detail below.

The production of modified starches by means of genetic engineering operations on a plant can firstly modify the properties of the starch obtained from the plant so that further modification by means of chemical or physical methods no longer appear to be necessary. Secondly, starches which have been modified by genetic engineering methods can be subjected to further chemical and/or physical modifications, which results in additional improvements in quality for some of the areas of use described above. These chemical and physical modifications are known in principle. In particular, they comprise modifications by means of:

- heat treatment,
- treatment with acids,
- production of starch ethers,
  starch alkyl ethers, O-allyl ethers, hydroxylalkyl ethers, O-carboxylmethyl ethers, N-containing starch ethers, P containing starch ethers, S-containing starch ethers,
- production of crosslinked starches,
- production of starch graft polymers,
- oxidation, and
- esterification operations which are used for the production of phosphate, nitrate, sulphate, xanthate, acetate and citrate starches. Other organic acids can also be used for esterification.

In a further embodiment, the present invention relates to the use of the starches according to the invention in industry, preferably for the production of foodstuffs.

In a further embodiment, the invention relates to wheat flour which contains the starches according to the invention. Amongst its other properties, the wheat flour according to the invention is distinguished by modified baking properties compared with conventional wheat flours. Methods of producing wheat flour from wheat grains are known to one skilled in the art. By means of these methods, the wheat flour according to the invention can be isolated from the plant cells and plants according to the invention and from the propagation material according to the invention.

Thus the present invention also relates to wheat flour which can be obtained from the plant cells and plants according to the invention and from the propagation material according to the invention.

Compared with flours from corresponding non-genetically modified wild-type plants, the flours according to the invention are distinguished in particular by their enhanced water absorption capacity. Methods of determining water absorption capacity are known to one skilled in the art and are described in detail below (see methods).

In one preferred embodiment of the invention, the wheat flours exhibit a water absorption capacity which is increased by at least 5%, particularly by at least 15%, preferably by at least 20% and most preferably by at least 25% compared with wheat flours from corresponding non-genetically modified wheat plants.

Due to their high water absorption capacity, the flours according to the invention have the advantage for the industrial production of dough that less water has to be supplied to the flour during the dough preparation stage.
In a further embodiment, the present invention relates to the use of the wheat flour according to the invention and/or of the starch according to the invention for the production of a baking mixture and/or for the production of a food product.

In a further embodiment, the present invention relates the use of the wheat flour according to the invention and/or of the starch according to the invention for the coating of packages.

In a particularly preferred embodiment, the present invention relates to a baking mixture which contains the wheat flour according to the invention and/or the starch according to the invention. In this connection, the term "baking mixture" is to be understood to mean any mixture which can be used for the production of dough (e.g. yeast dough, pasta dough, shortcrust pastry, sour dough etc.) and/or of bakery products (e.g. bread, cakes, rolls, croissants, etc.).

In a further particularly preferred embodiment, the present invention relates to a food product, preferably bakery and dough products (e.g. pasta) which is produced using the wheat flour according to the invention and/or the baking mixture according to the invention and/or the starch according to the invention.

Compared with bakery products which are produced from wheat flours from corresponding non-genetically modified wheat wild-type wheat plants, the bakery products and/or food products according to the invention are distinguished on storage by a slowing down of the ageing process which the bakery products undergo.

The ageing process of a bakery product is closely linked to the degree of retrogradation (recrystallisation) of the amylopectin component of the starch. It is assumed that the ageing process of a bakery product and/or food product has progressed further, the higher is the degree of retrogradation of the amylopectin component The enthalpies of fusion of the amylopectin which can be determined by DSC provide information on the degree of retrogradation of the amylopectin. The degree of ageing of a bakery product can thus be determined by determining the enthalpy of fusion of the amylopectin at a given point in time by means of DSC (=differential scanning calorimetry) analysis (see methods).

If the enthalpy of fusion of the amylopectin of the bakery products and/or food products according to the invention is determined after storage for three and/or seven and/or thirteen days, for example, it is found that the enthalpy of fusion of the amylopectin is reduced compared with the enthalpy of fusion of the amylopectin of bakery products and/or food products which are stored for the same length of time and which are based on wheat flours of corresponding non-genetically modified wild-type plants. This shows that the bakery products and food products according to the invention are distinguished by a slower ageing process compared with conventional products.

The Figures are as follows:

Figure 1: is a schematic illustration of an RVA profile;

Figure 2: is a plasmid map of the vector pUbi2afp

Figure 3: is a plasmid map of the vector pUbiR1

Figure 4: is a plasmid map of the vector p35SAcS (a derivative of pUC18 (Pietrzak M. et al., Nucleic Acids Res.14, (1986), 5857-5868): contains the pat gene from Streptomyces viridochromogenes (Wohlleben et al., Gene 70, (1988), 25-37) under the control of the CaMV35S promoter.

Figure 5: is a plasmid map of the vector pAct1-Fneo/Calgus (pUC19-derivative (Yannish-Perron et al., 1985, Gene 33: 103-119): formed from pAct1-Fneo (Müller et al., Plant Science 114, (1996), 71-82) and pCalgus, which contains the CaMV35S promoter (see US-A-5,352,605, for example), the AdhI intron from maize (Genes Dev 1987 Dec;1(10):1183-200) and the beta-glucuronidase (GUS) gene (The GUS Reporter System as a Tool to Study Plant Gene expression in: GUS Protocols: Using the GUS Gene as a Reporter of Gene expression, Academic Press (1992), 23-43).

Figure 6: shows the enthalpy of breadcrumbs during storage after 1, 3, 7 and 13 days.

Methods:

Determination of the phosphate content in the C6 position of the glucose monomer (= C6 P content) of the starch (Nielsen et al. Plant Physiol. 105, (1994), 111-117):

In order to determine the C6 P content of the starch, 50 mg starch were hydrolysed in 500 µl 0.7 M HCl for 4 hours at 95°C. The batches were subsequently centrifuged for 10 min at 15500 g and the supernatants were taken off. 7µl of the supernatants were mixed with 193 µl imidazole buffer (100 mM imidazole, pH 7.4; 5 mM $MgCl_2$, 1 mM EDTA and 0.4 mM NAD). Measurements were made in a photometer at 340 nm. After establishing the base absorption, the enzyme reaction was initiated by adding 2U glucose-6-phosphate dehydrogenase (from Leuconostoc mesenteroides, Boehringer Mannheim). The change in absorption was directly proportional to the concentration of the C-6 P content of the starch.

Determination of the total phosphate content of the starch:

Before determining the total phosphate content of the starch, the starch had to be completely separated from phosphorylated non-glucans such as phospholipids. The Determination of the total phosphate content was determined by the method of Ames (Methods in Enzymology VIII, (1966), 115-118), as follows:
About 50 mg starch were treated with 30 µl of 10% ethanolic magnesium nitrate solution and were ignited for three hours in a muffle furnace. The residue was treated with 500 µl 0.5 M hydrochloric acid and incubated for 30 min at 60°C. A 20 µl aliquot was then made up to 300 µl with 0,5 M hydrochloric acid, was added to a mixture of 100 µl of 10% ascorbic acid and 600 µl of 0.42% ammonium molybdate in 1 M sulphuric acid, and was incubated for 20 min at 45°C. This was followed by a photometric determination at 820nm using a phosphate calibration series as a standard.

Determination of the gel strength (texture analyser):

2.5 g starch (TS) were conglutinated in 25 ml H_2O in an RVA instrument (see determination of the viscosity properties by means of a Rapid Visco Analyzer (RVA)) and were subsequently stored for 24 hours at room temperature. The

samples were fixed under the sensor (a cylindrical plunger with a planar surface) of a TA-XT2 texture analysers supplied by Stable Micro Systems (Surrey, UK) and the gel strength was determined using the following instrument settings:

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

Determination of the viscosity properties (e.g. final viscosity) by means of a Rapid Visco Analyzer (RVA):

2.5 g starch (dry weight) were taken up in 25 ml H_2O and used for analysis in a Rapid Visco Analyzer (Newport Scientific Pty Ltd., Investment Support Group, Warriewod NSW 2102, Australia). The instrument was operated according to the manufacturer's instructions. In order to determine the viscosity of the aqueous solution of the starch, the starch suspension was first heated for one minute to a temperature of 50°C and was then heated from 50°C to 95°C at a rate of 12°C per minute. The temperature was subsequently held for 2.5 minutes at 95°C. Thereafter, the solution was cooled from 95°C to 50°C at a rate of 12°C per minute. Finally, the temperature was held for a further 6 minutes at 50°C. The viscosity was determined over the entire duration of the test.

Isolation of the amylose component of the starch by aqueous leaching:

It is known that amylose can be obtained from starch by aqueous leaching (Roger and Colonna (International Journal of Biological Macromolecules 19, (1996), 51-61).

In orde to isolate the amylose component of the starch, 25ml of an aqueous starch suspension (10% w/v) were prepared and heated in an RVA (Newport Scientific) with stirring, using the following profile:

Time [min]	Type	Value [°C] or [rpm]
0	Temp	30°
0	Speed	960 rpm

0.5	Speed	300 rpm
10	Temp	95°

Directly after the viscosity of the suspension or solution exceeded 200 cP, the programme was terminated and the starch suspension or solution was transferred into a cold, 50ml reaction vessel, mixed with 25ml of cold water and cooled for 10 min in an ice bath to about room temperature (RT). Thereafter, the undissolved glucan was formed into a pellet by centrifugation (20min at 2300g at RT) and the clear supernatant was treated with 10mg NaCl/ml. The dissolved glucan was then precipitated on to ice from 80% ethanol, was washed with ethanol again, and was dried for 3 days at 37°C. The pellet was subsequently ground (15s 30Hz with tungsten carbide balls in a in a vibrating mill supplied by Retsch, Germany), and 25mg of the pellet was suspended in 500µl of 0.7M HCl hydrolysed at 95 °C for 4 hours. Undissolved particles were then removed by centrifugation and the content of glucose-6P was determined enzymatically (see above: "Determination of the phosphate content in the C6 position of the glucose monomer").

Procedure for the small scale water absorption test on wheat flour:

Materials

Eppendorf cups (2 ml) with screw cap from Sarsted.

Eppendorf centrifuge (Sigma 202)

Vortex (Wilten)

Analytical Balance (Mettler)

Method

Measure the weight of the epp.cup to four decimal places using the analytical balance.

Fill the cup with 100 to 110 mg flour and note the exact weight.

Put the cup with flour on the vortex and add 1.0 ml water during vortexing.

Keep on vortexing for 10 seconds.

Add 0.9 ml water and close the cup with the screw cap.

Wait ten minutes.

Put the cups in the 6*10 rotor (swing out) in the centrifuge.

Run the centrifuge for 30 minutes at 10,000 rpm (about 8600*g).

Pour off the water from the cup.
Dry the inside of the cup with filter paper without disturbing the wet residue.
Measure the weight of the Eppendorf cup with the wet flour to four decimal places using the analytical balance.
Calculate the weight of the absorbed water.
Determine the waterabsorption of each flour ten times and calculate the average.

Differential Scanning Calorimetry :{tc "Differential Scanning Calorimetry " \l 2}

Differential Scanning Calorimetry (DSC) is a technique studying thermal transitions, in this case of breadcrumbs.
With this technique the heat flow into a sample is determined at a continuous (relatively low) heating rate. Either a peak (offset from the base line (endotherm)) or a step in the base line (glass transition) determines transitions.
Integration of the peak area with respect to the base line gives the enthalpy of the transition. For breadcrumbs, an amylopectin heat of fusion between 40 and 70°C (60°C peak) and an amylose-lipid decomplexation at about 120°C are generally observed.
Differences in the heat of fusion of amylopectin between the various flour samples indicate differences in retrogradation behaviour, and hence indicate that the bread is becoming stale. The enthalpy of fusion of the amylopectin crystals increases with storage time after baking. An increase in the enthalpy of fusion during storage is a measure of an increase in the amount of recrystallised amylopectin with time. The enthalpies (j/g) were calculated on a dry matter basis in order to correct for differences in moisture content.

DSC measurements were made using a TA Instruments Type 2920 calorimeter with LNCA cooling unit. This cooling unit uses liquid nitrogen for fast cooling after the experiment.
Samples (40 - 50 mg breadcrumbs) were placed in a DSC pan and compressed by hand with a nail. After sealing the DSC pan with a compression unit the sample was placed in the DSC-apparatus.

Samples (40 - 50 mg breadcrumbs) were heated from 20°C to 160°C at a rate of 7.5 C/min in stainless steel hermetically sealed pans (Perkin Elmer). A medium pressure pan (120µl volume), filled with 100 mg aluminium, was used as a reference.
The enthalpies of the crumbs of the mini-loaves were measured after 1, 3, 7 and 14 days of storage in single measurements.

The following examples serve to explain the invention without limiting it in any respect:

Example 1

Production of vector pUbiR1 for the transformation of wheat plants

In order to produce vector pUbiR1, vector pUbi.cas was first produced as follows:
Vector pUbi2afp (see Figure 2) was partially cut with restriction enzymes PstI/EcoRI. The 4.19 Kb fragment which resulted therefrom, consisting of the pUC19 vector (Yannish-Perron et al., 1985, Gene 33: 103-119), the 1.5 Kb ubiquitin promoter and of the first exon of the ubi gene and a shortened ubiquitin1 intron (ClaI deletion) (Christensen et al., Plant Mol. Biol. 18, (1992), 675-689) was used further after gel elution.
The nos terminator was isolated as a PstI/EcoRI fragment from the vector pAct.cas (Product Number Cambia TG0063, supplied by Cambia, GPO Box 3200, Canberra ACT 2601, Australia) and ligation of the two fragments was effected to give the vector pUbi.cas.
The cDNA of the potato R1 gene (SEQ ID No.1) was subsequently isolated as a partial digest (SmaI/Asp718 fragment) from the vector pRL2 (WO97/11188, Example 4, deposited at the German Collection for Microorganisms with the number DSM 10225) and was integrated in the vector pUbi.cas (restriction with SmaI/Asp718). The resulting construct was designated as pUbiR1 (see Figure 3).

This construct comprises the coding region of the R1 cDNA from potatoes, which is under the control of the ubiquitin promoter (Christensen et al., 1992, Plant Mol. Biol. 18: 675-689), and also comprises, as additional regulation units, the first exon of the ubi gene (Christensen et al., Plant Mol. Biol. 18, (1992), 675-689) and the shortened first intron (Christensen et al., Plant Mol. Biol. 18, (1992), 675-689), the internal ClaI fragment being deleted).

Example 2

Production of wheat plants which express an R1 gene from Solanum tuberosum, and analysis of the phosphate content of the starch of these plants

The biolistic transformation method was used for the transformation of wheat (Becker et al., Plant J. 5 (2), (1994), 229-307). The vector pUbiR1 and, for biolistic cotransformation, the vectors p35SAcS (see Fig. 4) or pAct1-Fneo/Calgus (Müller et al., Plant Science 114, (1996), 71-82, see Fig. 5) were used in identical molar ratios in the DNA particle precipitation batch.
Vector p35SAcS was produced as follows: The pat gene from S. *viridochromogenes* (Wohlleben et al., Gene 70, (1988), 25-37) was amplified via a polymerase chain reaction. The primers used were designed so that a *Bam*HI cleavage site was created on both sides of the amplificate. The *Bam*HI fragment was subsequently cloned into the *Bam*HI cleavage site placed between the 35S promoter and the 35S terminator of the vector pDH51 (Pietzrak et al., Nucleic Acids Res. 14, (1986), 5857-5868). The cassette containing the 35S promoter, the *pat* gene and the 35S terminator was cut as an EcoRI fragment and was cloned into in the EcoRI cleavage site of the vector pUC18 (Pietzrak et al., Nucleic Acids Res. 14, (1986), 5857-5868).
Scutelli from 14-day old, unripe embryos of wheat plants were used as target cells for transformation. Transformation was followed by *in vitro* culture on MS medium

(PCT/EP97/02793) containing 2 mg/l 2,4-D (=2,4-dichlorophenoxyacetic acid). Two weeks after transformation, subculture was effected on the same medium, to which 2 mg/l phosphinotricin or 150 mg/l kanamycin sulphate had been added. After a further two weeks, the developing calli were transplanted on to a regeneration medium (MS medium with 0.1 mg/l 2,4-D and 2 mg/l PPT or 150 mg/l kanamycin). The developing shoots were transferred to semi-concentrated MS medium without 2,4-D and phosphinotricin or kanamycin and were subsequently transferred into the ground. About 14 days after their establishment in the ground, the transgenic plants were identified by spraying twice with an aqueous solution containing 150 and 200 mg/l phosphinotricin, respectively, 0.1% Tween 20 (ICI America, corresponding to polysorbate 20) or by spraying twice with 2.5% kanamycin sulphate, 0.1% Tween 20.

Expression of the potato R1 gene in T_0 plants

Expression of the potato R1 gene in transgenic wheat T_0 plants was detected by Northern and Western blotting analyses and by the enzymatic determination of the phosphate content in the C6 position of the glucose monomer of the starch from caryopses (Nielsen et al., Plant Physiol. 105, (1994), 111-117).

R1 protein was detected in the transgenic wheat plants with the aid of an anti-potato R1 protein antibody (Ritte et al., Plant J. 21(4), (2000), 387-391).

Protein extracts from the endosperm of unripe caryopses about 20 days old was used for screening the transgenic plants.

Starch from transgenic T_0 plants was isolated from unripe and ripe wheat caryopses in order to determine C6 phosphate. The caryopses were triturated in a mortar to form a powder. After adding 15 ml of 100 mM Tris buffer, pH 8.0, the suspension was filtered through a 100 µm sieve and the starch was pelletised by centrifugation (2600 g, 5 min, 4°C). The supernatant was discarded. The starch pellet was subsequently re-suspended in 2 ml of 100 mM Tris buffer, pH 8.0 and transferred to an 8 ml Percoll gradient. The starch was palletised by centrifugation for 15 minutes at 170 g and 4°C. The starch pellet was subsequently washed three times with 10 ml of 100 Tris buffer, pH 8.0. Finally, the starch was degreased by acetone incubation and was dried.

The C6 P content was determined by glucose-6-phosphate determination by means of an optical-enzymatic test (Nielsen et al., Plant Physiol. 105, (1994), 111-117) in the manner described above.

The tests showed that of the wheat T_0 plants which gave a positive result in Southern blotting analysis about 50% of the lines in the caryopses synthesised a starch which had an increased phosphate content in the C6 position of the glucose monomer compared with starch from corresponding non-genetically modified wild-type-plants of the Florida variety. Table 1 gives the data for some selected lines.

Analysis of natural descendants

Seeds which were obtained naturally from R1-expressing parental lines was sown and the segregation ratios were determined. It was possible to detect both the integration and also the expression of the R1 gene in T_1descendants of different lines by Southern and Northern blotting analyses. The phosphate content of these lines in the C6 position of the starch was determined. Table 1 shows the data for some selected lines.

Table 1:

Line No.	C6 P in nmol/mg	Gene-ration	Standard deviation
Wild-type Florida variety	Not detectable	-	0.0
19	2.8	T0	0.2
20-25	6.7	T1	0.2
37	1.4	T0	0.3
40A-11-8	10.7	T2	0.7

Example 3

RVA analysis of the starch of plants from transgenic wheat plants which express an R1 gene from Solanum tuberosum, and investigation of the gel strength

The starches from the wheat plants described in Examples 1 and 2 were subjected to an RVA analysis.
The results of the RVA analysis (experimental: see methods) showed that the viscosity behaviour of the starches from the transgenic wheat plants which express an R1 gene from Solanum tuberosum is significantly modified compared with starches from corresponding non-genetically modified wild-type-wheat plants (Florida variety) (see Table 2).

Table 2:

Line No.	RVA Max (%)	RVA Min (%)	RVA Fin (%)	RVA Set (%)	RVA T (%)
Wild-type (Florida)	100	100	100	100	100
19 (T0)	98.7	113.8	116.8	120.7	102
20-25 (T1)	155.3	190	193.8	198.8	99
37 (T0)	143.8	156.2	148.8	139	101
40-11-8 (T2)	156.2	185.5	187.7	190.6	97

Legend:

RVA = Rapid Visco Analyzer

Max = see Figure 1

Min = see Figure 1

Fin = final viscosity, see Figure 1

Set = Setback = Difference between Min and Fin, see Figure 1

T = Conglutination temperature, see Figure 1

The percentages are given with respect to the wild-type (= 100 %).

The gel strengths of the starches (determination of the gel strength: see methods) from the transgenic wheat plants which express an R1 gene from Solanum tuberosum differed both from those of starches from corresponding non-genetically modified wild-type-wheat plants (Florida variety) and from those of wild-type-starches (Florida variety), which had subsequently been chemically phosphorylated (see Table 3).

In contrast to chemically phosphorylated starches with a comparable phosphate content in the C6 position of the glucose monomer, after conglutination the gels of the starches according to the invention exhibited an increased gel strength compared with gels of starches of wild-type-plants. In contrast to this, the

chemically phosphorylated starches which had been produced by the method described by Lim compared with gels of starches of wild-type plants.

Table 3:

Line No.	Gel strength (TA) (%)	Phosphate content in the C6 position in µmol phosphate/g starch
Wild-type (Florida)	100	Not detectable
19 (T0)	167	2.8
20-25 (T1)	192	6.7
37 (T0)	168	1.4
40-11-8 (T2)	224	10.7
Chemically phosphorylated wild-type (Florida) starch		
STMP1	84	2.1
STMP6	75	6.5
STMP8	67	11.5

TA = texture analyzer
The percentages are given with respect to the wild-type (= 100 %).

Example 4

DSC analysis of the starch of plants from transgenic wheat plants which express an R1 gene from Solanum tuberosum

The starches from the wheat plants described in Examples 1 and 2 were subjected to DSC analysis.

The peak temperature was determined using an instrument supplied by Perkin Elmer (instrument designation: DSC-7) using large volume capsules, wherein the sample to be investigated had a ratio of starch to total water content of about 1:4

and measurements were made over the temperature range from 10°C to 160°C at a heating rate of 10°C/min.

The results of the DSC analysis (Table 4) show that the peak temperature of the starches from the transgenic wheat plants which express an R1 gene from Solanum tuberosum exhibit a reduced peak temperature Tp compared with starches from corresponding non-genetically modified wild-type-wheat plants (Florida variety) and also compared with starches of comparable phosphate content in the C6 position of the glucose monomer which were subsequently chemically phosphorylated.

Table 4:

Sample	Water content of sample (%)	Starch (dry weight): water (total water content)	Peak temperature in °C (first peak maximum)	Other peak maxima in °C
Wild-type (Florida)	16.6	1 : 4.01	61	101
19 (T0)	10.5	1 : 3.99	58	100
20-25 (T1)	16.9	1 : 4.05	55	100
40-11-8 (T2)	16.9	1 : 3.98	56	100
STMP2*	10.9	1 : 4.00	60	101
STMP6	10.7	1 : 4.00	61	101

* The chemically phosphorylated starch STMP2 had a phosphate content in the C6 position of 3.0 µmol phosphate/g starch. All the other phosphate data are given in Table 3.

Example 5

Determination of the C6 P content of the amylose component of various starches

Apart from starch from transgenic wheat plants which express an R1 gene from Solanum tuberosum, two chemically phosphorylated starches (STMP3 and STPP4) were investigated. Chemical phoshorylation was effected by the method described by Lim and Seib (Cereal Chem. 70 (2), (1993), 137-144), wherein STMP3 was produced by reacting the starch for 2 hours at pH 6, and STPP4 was produced by reaction for 30 minutes at pH 8. The abbreviation STMP represents the selected phoshorylation reagent, namely sodium trimetaphosphate; STPP represents sodium tripolyphosphate.

The amylose component of various starches was isolated as described above (isolation of the amylose component of the starch by aqueous leaching).
The phosphate content in the C6 position was determined as described above (determination of the phosphate content in the C6 position of the glucose monomer).

Table 5:

Sample	C6 P content in µmol phosphate/g starch	C6 P content in µmol phosphate/g amylose	C6 P content of amylose/ C6 P content of starch
40-11-8 (T2)	10.7	1.9	0.18
STPP4	20.5	20.4	0.99
STMP3	6.0	5.1	0.85

It can be seen that the ratio of the C6 P content of the amylose to the C6 P content of the starch in the chemically phosphorylated starches is considerably higher than in the starches from the transgenic wheat plants which superexpress

an R1 Gen from potatoes. The chemically phosphorylated starches have a higher content of phosphorylated amylose than do the starches from the transgenic wheat plants.

Example 6

DSC analysis of breadcrumbs from micro-loaves produced from flour of different origins

Wheat flour from wheat wild-type plants (Florida variety) and from genetically modified wheat plants which superexpress the R1 gene from potato was used to produce micro-loaves, using the following recipe:

Wheat flour	10.00 g
Yeast	0.25 g
Salt	0.20 g
Dextrose	0.10 g
Ascorbic acid	20 ppm
Water	the amount of water as determined in the water absorption test and corrected for consistency (see method "Procedure for small scale water absorption test on wheat flour")

The dough of the genetically modified wheat plants became very sticky during the mixing process. To obtain doughs with correct handling properties a smaller amount of water was used. The dough consistency was corrected by technical sensory perception of the stiffness of the dough.

The dough was proofed in a proofing cabinet for 45 minutes (28°C), manually worked through, proofed for 15 minutes (28°C) for a second time, formed and put in a baking tin. Subsequently, the dough was given a final proof for 60 minutes (28°C). The loaves were baked in an oven for 15 minutes (200°C), with the tins embedded in a moist wooden frame. This embedding was done to shield the baking tin from high amounts of radiative heat. Finally, the loaves were baked for 5 minutes, without the wooden frame, to allow the crust to brown.

Differential scanning calorimetry (DSC) (see methods) was used to determine thermal transitions of the breadcrumbs. The enthalpies of crumbs from the loaves were measured after 1, 3, 7 and after 14 days of storage (Figure 6).

Figure 6 shows the enthalpy of the amylopectin fusion peak (40°C-70°C) for stored bread crumbs after 1, 3, 7 and 13 days of storage, giving an indication of staling effects. The breadcrumbs from loaves made from wild-type (Florida) flour have a higher enthalpy compared with breadcrumbs of the same age made from flour from two different lines (GMO1 and GMO2) of genetically modified wheat plants which superexpress the R1 gene from potato. This indicates a lower level of recrystallised starch, less retrogradation of starch and less staling of the bread.

Sequence protocol:

SEQ ID No. 1 (R1 cDNA potato, nucleotide sequence 5061 bp, coding region: bp 216 to bp 4607):

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SEQ ID No.2 (R1 protein from potato, amino acid sequence: 1464 amino acids)

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Claims

1. A genetically modified monocotyledon plant cell, wherein the genetic modification consists of the introduction of at least one extraneous nucleic acid molecule and the extraneous nucleic acid molecule is selected from the group consisting of:

a) nucleic acid molecules which comprise the coding region of the nucleotide sequence represented in Seq ID No. 1;
b) nucleic acid molecules which encode an R1 protein from Solanum tuberosum with the amino acid sequence given in Seq ID No. 2;
c) nucleic acid molecules which constitute a derivative of the nucleotide sequence given in Seq ID No. 1; and
d) nucleic acid molecules which constitute fragments of the nucleic acid molecules cited in (a), (b) or (c).

2. A plant cell according to claim 1, which exhibits an increased biological activity of R1 protein compared with a corresponding non-genetically modified wild-type plant cell.

3. A plant cell according to one or more of claims 1 or 2, which synthesises a starch which has an increased phosphate content in the C6 position of the glucose monomer compared with starch from corresponding non-genetically modified wild-type plant cells.

4. A plant cell according to one or more of claims 1 to 3, which synthesises a starch which has an increased final viscosity and/or a reduced peak temperature compared with starch from corresponding non-genetically modified wild-type plant cells.

5. A plant cell according to one or more of claims 1 to 4, which synthesises a starch which after conglutination forms a gel which has an increased gel strength compared with a gel of starch from corresponding non-genetically modified wild-type plant cells.

6. A plant cell according to one or more of claims 1 to 5, which originates from a plant of the group consisting of wheat, rice, barley, oats, millet, rye and maize.

7. A plant cell according to one or more of claims 1 to 5, which originates from a wheat plant.

8. A plant cell according to claim 7, which synthesises a starch which in the C6 position of the glucose monomer has a phosphate content of at least 0.1 nmol C6 P mg^{-1} starch.

9. A method of producing a plant cell according to one or more of claims 1 to 8, wherein a cell of a monocotyledon plant is genetically modified, and wherein the genetic modification consists of the introduction of at least one extraneous nucleic acid molecule.

10. A plant containing plant cells according to one or more of claims 1 to 8.

11. A plant according to claim 10, which in its starch-storing organs synthesises a starch which has an increased phosphate content in the C6 position of the glucose monomer compared with starch from the starch-storing organs of corresponding non-genetically modified wild-type-plants.

12. A plant according to one or more of claims 10 to 11, which is a rice, wheat or maize plant.

13. A method of producing a plant according to one or more of claims 10 to 12, wherein

(a) a cell of a monocotyledon plant is genetically modified, wherein the genetic modification consists of the introduction of at least one extraneous nucleic acid molecule;
(b) a plant is regenerated from the cell according to step (a); and optionally
(c) further plants are produced from the plant produced according to step (b).

14. A method according to claim 13, wherein the extraneous nucleic acid molecule is under the control of a promoter which organ-specifically facilitates R1 gene expression in starch-storing tissues.

15. A method according to one or more of claims 13 to 14, which relates to a wheat plant.

16. A propagation material for a plant according to one or more of claims 10 to 12 containing plant cells according to one or more of claims 1 to 8.

17. Use of nucleic acid molecules as defined in claim 1 for the production of monocotyledon plants according to one or more of claims 10 to 12 or of monocotyledon plant cells according to one or more of claims 1 to 8.

18. A starch obtainable from a plant cell according to one or more of claims 1 to 8 or a plant according to one or more of claims 10 to 12.

19. A starch according to claim 18, characterised in that it has an increased phosphate content in the C6 position of the glucose monomer and/or an increased final viscosity and/or a reduced peak temperature compared with starch from corresponding wild-type plants.

20. A starch, characterised in that it has an increased phosphate content in the C6 position of the glucose monomer and/or an increased final viscosity and/or a reduced peak temperature compared with starch from corresponding wild-type plants.

21. A starch, characterised in that in the C6 position of the glucose monomer it has a phosphate content of at least 0.1 nmol C6 P mg^{-1} starch.

22. A starch according to claim 21, characterised in that it has a final viscosity which is increased by at least 50% and/or a peak temperature which is reduced by at least 1.5°C.

23. A starch according to one or more of claims 18 to 22, characterised in that after conglutination said starch forms gels which exhibit an increased gel strength compared with gels of corresponding chemically phosphorylated starches with the same phosphate content in the C6 position of the glucose monomer and/or compared with gels of starches from corresponding non-genetically modified plants.

24. A starch according to one or more of claims 18 to 23, characterised in that the amylose component of said starch has a reduced total phosphate content in its amylose component compared with the amylose component of chemically phosphorylated starch.

25. A starch according to one or more of claims 18 to 24, characterised in that it exhibits a modified phoshorylation pattern compared with chemically phosporylated starch.

26. A starch according to one or more of claims 18 to 25, characterised in that it is a wheat starch.

27. A method of producing a starch according to one or more of claims 18 to 26 comprising the extraction of the starch from a plant according to one or more of claims 10 to 12.

28. Use of a starch according to one or more of claims 18 to 26 in the industrial field, preferably for the production of foodstuffs.

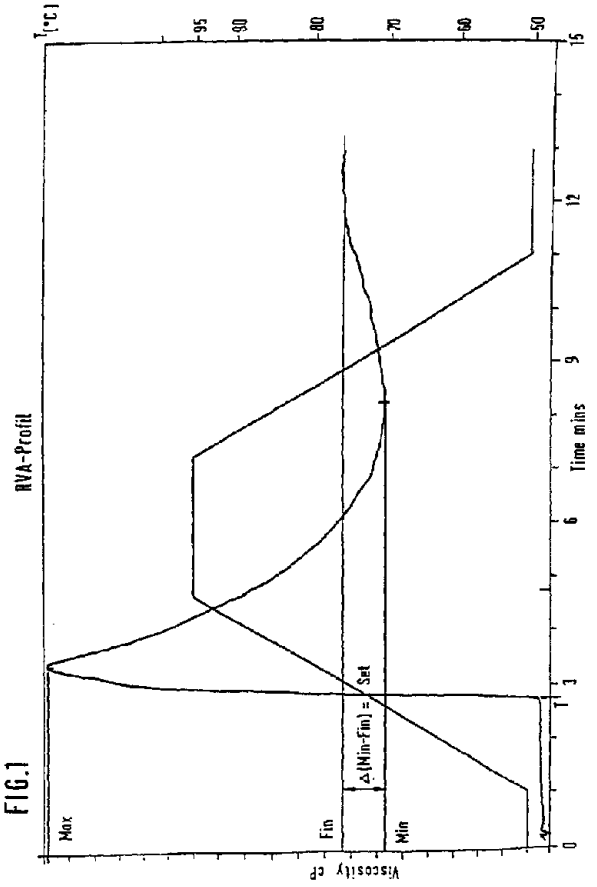
29. Wheat flour,containing a starch according to one or more of claims 18 to 26.

30. Wheat flour obtainable from a plant cell according to one or more of claims 1 to 8 or from a plant according to one or more of claims 10 to 12 or from propagation material according to claim 16.

31. Use of the wheat flour according to claim 29 or 30 or of the starch according to one or more of claims 18 to 26 for the production of a baking mixture and/or for the production of a food product.

32. A baking mixture which contains the wheat flour according to claims 29 or 30 or the starch according to one or more of claims 18 to 26.

33. A food product which has been produced using the wheat flour according to claims 29 or 30 and/or using the baking mixture according to claim 32 and/or using the starch according to one or more of claims 18 to 26.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70
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15
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Fin
Min
Set
Δ (Min-Fin) =

FIG.1

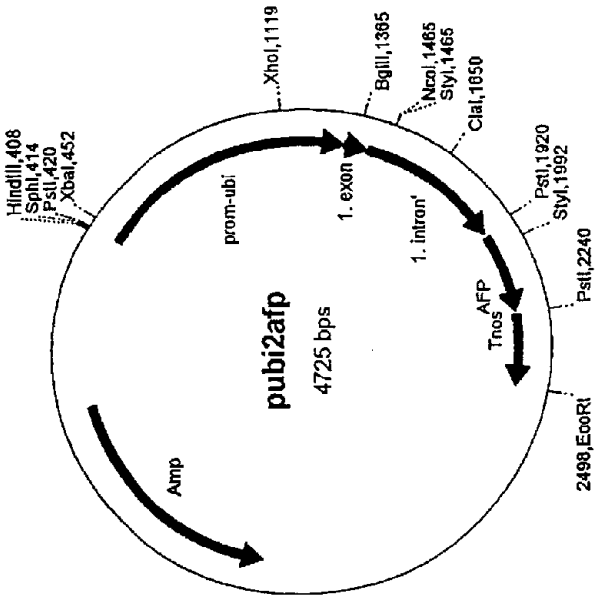
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Fig. 2

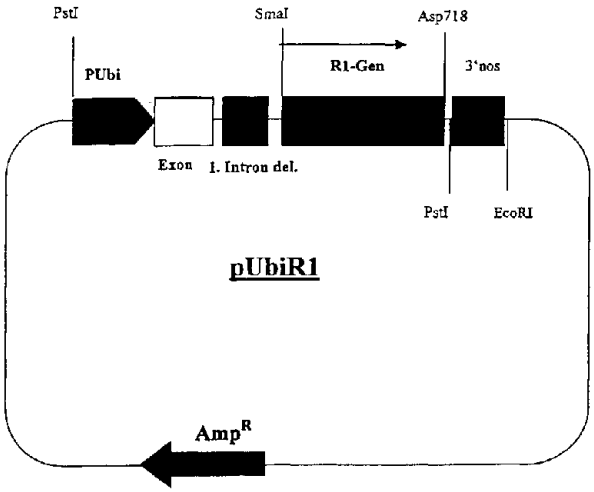
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Fig.3

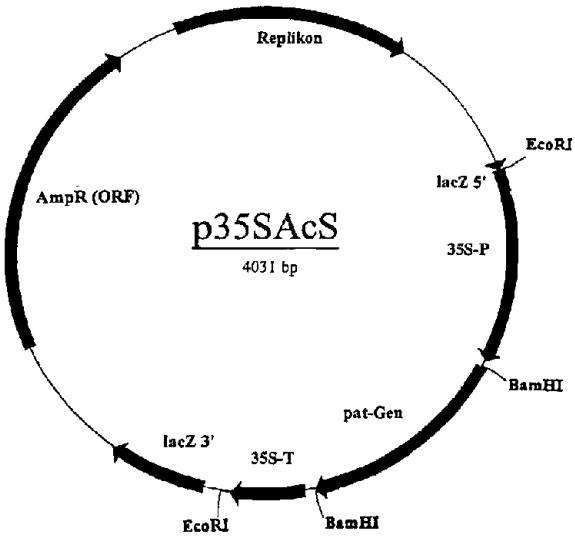
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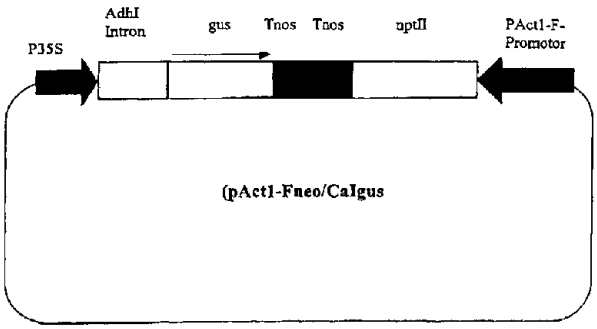
FIG. 4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Promotor
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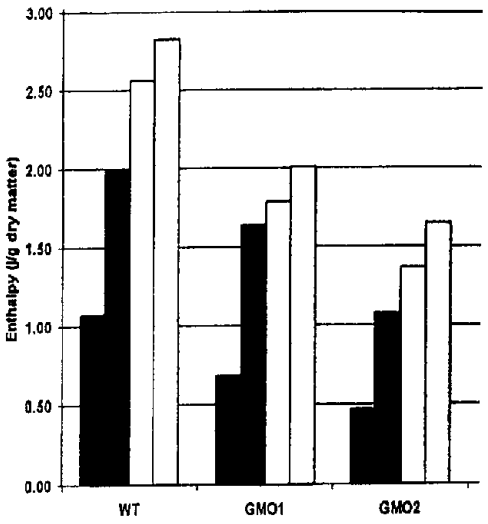
FIG.5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0.00
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GMO1
GMO2

Figure 6

SEQUENCE LISTING

<110> Aventis CropScience GmbH

<120> Monokotyledone Pflanzenzellen und Pflanzen, die eine modifizierte Stärke synthetisieren

<130> 2000/M 233

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<150> DE 10052492.3

<151> 2000-10-23

<150> DE 10064805.3

<151> 2000-12-22

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<170> PatentIn Ver. 2.1

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aat aac ttg ctg tac cag gga ttc cta acc tca aca gtg ttg gaa cat 281
Asn Asn Leu Leu Tyr Gln Gly Phe Leu Thr Ser Thr Val Leu Glu His
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Lys Ser Arg Ile Ser Pro Pro Cys Val Gly Gly Asn Ser Leu Phe Gln
25 30 35

caa caa gtg atc tcg aaa tca cct tta tca act gag ttt cga ggt aac 377
Gln Gln Val Ile Ser Lys Ser Pro Leu Ser Thr Glu Phe Arg Gly Asn
40 45 50

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Arg Leu Lys Val Gln Lys Lys Lys Ile Pro Met Gly Lys Asn Arg Ala
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Phe Ser Ser Ser Pro His Ala Val Leu Thr Thr Asp Thr Ser Ser Glu
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cta gca gaa aag ttc agt cta gaa ggg aat att gag cta cag gtt gat   521
Leu Ala Glu Lys Phe Ser Leu Glu Gly Asn Ile Glu Leu Gln Val Asp
            90                  95                 100

gtt agg cct ccc act tca ggt gat gtg tcc ttt gtg gat ttt caa gct   569
Val Arg Pro Pro Thr Ser Gly Asp Val Ser Phe Val Asp Phe Gln Ala
        105                 110                 115

aca aat ggt agt gat aaa ctg ttt ttg cac tgg ggg gca gta aag ttc   617
Thr Asn Gly Ser Asp Lys Leu Phe Leu His Trp Gly Ala Val Lys Phe
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gga aaa gaa aca tgg tct ctt cct aat gat cgt cca gat ggg acc aaa   665
Gly Lys Glu Thr Trp Ser Leu Pro Asn Asp Arg Pro Asp Gly Thr Lys
135                 140                 145                 150

gtg tac aag aac aaa gca ctt aga act cca ttt gtt aaa tct ggc tct   713
Val Tyr Lys Asn Lys Ala Leu Arg Thr Pro Phe Val Lys Ser Gly Ser
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aac tcc atc ctg aga ctg gag ata cgg gac act gct atc gaa gct att   761
Asn Ser Ile Leu Arg Leu Glu Ile Arg Asp Thr Ala Ile Glu Ala Ile
            170                 175                 180

gag ttt ctc ata tac gat gaa gcc tac gat aaa tgg ata aag aat aat   809
Glu Phe Leu Ile Tyr Asp Glu Ala Tyr Asp Lys Trp Ile Lys Asn Asn
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ggt ggc aat ttt cgt gtc aaa ttg tca aga aaa gag ata cga ggc cca   857
Gly Gly Asn Phe Arg Val Lys Leu Ser Arg Lys Glu Ile Arg Gly Pro
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gat gtt tca gtt cct gag gag ctt gta cag atc caa tca tat ttg agg   905
Asp Val Ser Val Pro Glu Glu Leu Val Gln Ile Gln Ser Tyr Leu Arg
215                 220                 225                 230

tgg gag agg aag gga aaa cag aat tac acc cct gag aaa gag aag gag   953
Trp Glu Arg Lys Gly Lys Gln Asn Tyr Thr Pro Glu Lys Glu Lys Glu
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gaa tat gag gct gct cga act gag cta cag gag gaa ata gct cgt ggt  1001
Glu Tyr Glu Ala Ala Arg Thr Glu Leu Gln Glu Glu Ile Ala Arg Gly
            250                 255                 260

gct tcc ata cag gac att cga gca agg cta aca aaa act aat gat aaa  1049
Ala Ser Ile Gln Asp Ile Arg Ala Arg Leu Thr Lys Thr Asn Asp Lys
        265                 270                 275

agt caa agc aaa gaa gag cct ctt cat gta aca aag agt gaa ata cct  1097
Ser Gln Ser Lys Glu Glu Pro Leu His Val Thr Lys Ser Glu Ile Pro
    280                 285                 290

gat gac ctt gcc caa gca caa gct tac att agg tgg gag aaa gca gga  1145
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aga aga gaa ttg caa ctt gag ctt gag aaa ggc att acc ctt gat gag   1241
Arg Arg Glu Leu Gln Leu Glu Leu Glu Lys Gly Ile Thr Leu Asp Glu
            330                 335                 340

ttg cgg aaa aag att aca aaa ggg gag ata aaa act aag gcg gaa aag   1289
Leu Arg Lys Lys Ile Thr Lys Gly Glu Ile Lys Thr Lys Ala Glu Lys
        345                 350                 355

cac gtg aaa aga agc tct ttt gcc gtt gaa aga atc caa aga aag aag   1337
His Val Lys Arg Ser Ser Phe Ala Val Glu Arg Ile Gln Arg Lys Lys
    360                 365                 370

aga gac ttt ggg cag ctt att aat aag tat cct tcc agt cct gca gta   1385
Arg Asp Phe Gly Gln Leu Ile Asn Lys Tyr Pro Ser Ser Pro Ala Val
375                 380                 385                 390

caa gta caa aag gtc ttg gaa gaa cca cca gcc tta tct aaa att aag   1433
Gln Val Gln Lys Val Leu Glu Glu Pro Pro Ala Leu Ser Lys Ile Lys
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ctg tat gcc aag gag aag gag gag cag att gat gat ccg atc ctt aat   1481
Leu Tyr Ala Lys Glu Lys Glu Glu Gln Ile Asp Asp Pro Ile Leu Asn
            410                 415                 420

aaa aag atc ttt aag gtc gat gat ggg gag cta ctg gta ctg gta gca   1529
Lys Lys Ile Phe Lys Val Asp Asp Gly Glu Leu Leu Val Leu Val Ala
        425                 430                 435

aag tcc tct ggg aag aca aaa gta cat ata gct aca gat ctg aat cag   1577
Lys Ser Ser Gly Lys Thr Lys Val His Ile Ala Thr Asp Leu Asn Gln
    440                 445                 450

cca att act ctt cac tgg gca tta tcc aaa agt cgt gga gag tgg atg   1625
Pro Ile Thr Leu His Trp Ala Leu Ser Lys Ser Arg Gly Glu Trp Met
455                 460                 465                 470

gta cca cct tca agc ata ttg cct cct gga tca att att tta gac aag   1673
Val Pro Pro Ser Ser Ile Leu Pro Pro Gly Ser Ile Ile Leu Asp Lys
                475                 480                 485

gct gcc gaa aca cct ttt tcc gcc agt tct tct gat ggt cta act tct   1721
Ala Ala Glu Thr Pro Phe Ser Ala Ser Ser Ser Asp Gly Leu Thr Ser
            490                 495                 500

aag gta caa tct ttg gat ata gta att gaa gat ggc aat ttt gtg ggg   1769
Lys Val Gln Ser Leu Asp Ile Val Ile Glu Asp Gly Asn Phe Val Gly
        505                 510                 515

atg cca ttt gtt ctt tta tct ggt gaa aaa tgg att aag aac caa ggg   1817
Met Pro Phe Val Leu Leu Ser Gly Glu Lys Trp Ile Lys Asn Gln Gly
    520                 525                 530

tcg gat ttc tat gtt gac ttc agt gct gca tcc aaa tta gca ctc aag   1865
Ser Asp Phe Tyr Val Asp Phe Ser Ala Ala Ser Lys Leu Ala Leu Lys
535                 540                 545                 550
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gct gct ggg gat ggc agt gga act gca aag tct tta ctg gat aaa ata   1913
Ala Ala Gly Asp Gly Ser Gly Thr Ala Lys Ser Leu Leu Asp Lys Ile
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gca gat atg gaa agt gag gct cag aag tca ttt atg cac cgg ttt aat   1961
Ala Asp Met Glu Ser Glu Ala Gln Lys Ser Phe Met His Arg Phe Asn
            570                 575                 580

att gct gct gac ttg ata gaa gat gcc act agt gct ggt gaa ctt ggt   2009
Ile Ala Ala Asp Leu Ile Glu Asp Ala Thr Ser Ala Gly Glu Leu Gly
        585                 590                 595

ttt act gga att ctt gta tgg atg agg ttc atg gct aca agg caa ctg   2057
Phe Thr Gly Ile Leu Val Trp Met Arg Phe Met Ala Thr Arg Gln Leu
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ata tgg aac aaa aac tat aac gta aaa cca cgt gaa ata agc aag gct   2105
Ile Trp Asn Lys Asn Tyr Asn Val Lys Pro Arg Glu Ile Ser Lys Ala
615                 620                 625                 630

cag gac aga ctt aca gac ttg ttg cag aat gct ttc acc agt cac cct   2153
Gln Asp Arg Leu Thr Asp Leu Leu Gln Asn Ala Phe Thr Ser His Pro
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caa tac cgt gaa att ttg cgg atg att atg tca act gtt gga cgt gga   2201
Gln Tyr Arg Glu Ile Leu Arg Met Ile Met Ser Thr Val Gly Arg Gly
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ggt gaa ggg gat gta gga cag cga att agg gat gaa att ttg gtc atc   2249
Gly Glu Gly Asp Val Gly Gln Arg Ile Arg Asp Glu Ile Leu Val Ile
        665                 670                 675

cag agg aaa aat gac tgc aag ggt ggt atg atg gaa gaa tgg cat cag   2297
Gln Arg Lys Asn Asp Cys Lys Gly Gly Met Met Glu Glu Trp His Gln
    680                 685                 690

aaa ttg cat aat aat act agt cct gat gat gtt gtg atc tgt cag gca   2345
Lys Leu His Asn Asn Thr Ser Pro Asp Asp Val Val Ile Cys Gln Ala
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ttg att gac tac atc aag agt gat ttt gat ctt ggt gtt tat tgg aaa   2393
Leu Ile Asp Tyr Ile Lys Ser Asp Phe Asp Leu Gly Val Tyr Trp Lys
                715                 720                 725

acc ctg aat gag aac gga ata aca aaa gag cgt ctt ttg agt tat gac   2441
Thr Leu Asn Glu Asn Gly Ile Thr Lys Glu Arg Leu Leu Ser Tyr Asp
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cgt gct atc cat tct gaa ccg aat ttt aga gga gat caa aag aat ggt   2489
Arg Ala Ile His Ser Glu Pro Asn Phe Arg Gly Asp Gln Lys Asn Gly
        745                 750                 755

ctt ttg cgt gat tta ggt cac tat atg aga aca ttg aag gct gtt cat   2537
Leu Leu Arg Asp Leu Gly His Tyr Met Arg Thr Leu Lys Ala Val His
    760                 765                 770

tca ggt gca gat ctt gag tct gct att gca aac tgc atg ggc tac aaa   2585
Ser Gly Ala Asp Leu Glu Ser Ala Ile Ala Asn Cys Met Gly Tyr Lys
775                 780                 785                 790
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act gag gga gaa ggc ttt atg gtt gga gtc cag ata aat cct gta tca   2633
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                795                 800                 805

ggc ttg cca tct ggc ttt cag ggc ctc ctc cat ttt gtc tta gac cat   2681
Gly Leu Pro Ser Gly Phe Gln Gly Leu Leu His Phe Val Leu Asp His
            810                 815                 820

gtg gaa gat aaa aat gtg gaa act ctt ctt gag gga ttg cta gag gct   2729
Val Glu Asp Lys Asn Val Glu Thr Leu Leu Glu Gly Leu Leu Glu Ala
        825                 830                 835

cgt gag gag ctt agg ccc ttg ctt ctc aaa cca aac aac cgt cta aag   2777
Arg Glu Glu Leu Arg Pro Leu Leu Leu Lys Pro Asn Asn Arg Leu Lys
    840                 845                 850

gat ctg ctg ttt ttg gac ata gca ctt gat tct aca gtt aga aca gca   2825
Asp Leu Leu Phe Leu Asp Ile Ala Leu Asp Ser Thr Val Arg Thr Ala
855                 860                 865                 870

gta gaa agg gga tat gaa gaa ttg aac aac gct aat cct gag aaa atc   2873
Val Glu Arg Gly Tyr Glu Glu Leu Asn Asn Ala Asn Pro Glu Lys Ile
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atg tac ttc atc tcc ctc gtt ctt gaa aat ctc gca ctc tct gtg gac   2921
Met Tyr Phe Ile Ser Leu Val Leu Glu Asn Leu Ala Leu Ser Val Asp
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gat aat gaa gat ctt gtt tat tgc ttg aag gga tgg aat caa gct ctt   2969
Asp Asn Glu Asp Leu Val Tyr Cys Leu Lys Gly Trp Asn Gln Ala Leu
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tca atg tcc aat ggt gga gac aac cat tgg gct tta ttt gca aaa gct   3017
Ser Met Ser Asn Gly Gly Asp Asn His Trp Ala Leu Phe Ala Lys Ala
    920                 925                 930

gta ctt gac aga atc cgt ctt gca ctt gca agc aag gca gag tgg tac   3065
Val Leu Asp Arg Ile Arg Leu Ala Leu Ala Ser Lys Ala Glu Trp Tyr
935                 940                 945                 950

cat cac tta ttg cag cca tct gcc gaa tat cta gga tca atc ctt ggg   3113
His His Leu Leu Gln Pro Ser Ala Glu Tyr Leu Gly Ser Ile Leu Gly
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gtg gac caa tgg gct ttg aac ata ttt act gaa gaa att ata cgt gct   3161
Val Asp Gln Trp Ala Leu Asn Ile Phe Thr Glu Glu Ile Ile Arg Ala
            970                 975                 980

gga tca gca gct tca tta tcc tct ctt ctt aat aga ctc gat ccc gtg   3209
Gly Ser Ala Ala Ser Leu Ser Ser Leu Leu Asn Arg Leu Asp Pro Val
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ctt cgg aaa act gca aat cta gga agt tgg cag att atc agt cca gtt   3257
Leu Arg Lys Thr Ala Asn Leu Gly Ser Trp Gln Ile Ile Ser Pro Val
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gaa gcc gtt gga tat gtt gtc gtt gtg gat gag ttg ctt tca gtt cag   3305
Glu Ala Val Gly Tyr Val Val Val Val Asp Glu Leu Leu Ser Val Gln
1015                1020                1025                1030
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gga gag gag gaa att cct gat ggt gct gtt gcc ctg ata aca cca gac   3401
Gly Glu Glu Glu Ile Pro Asp Gly Ala Val Ala Leu Ile Thr Pro Asp
            1050                1055                1060

atg cca gat gtt ctt tca cat gtt tct gtt cga gct aga aat ggg aag   3449
Met Pro Asp Val Leu Ser His Val Ser Val Arg Ala Arg Asn Gly Lys
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gtt tgc ttt gct aca tgc ttt gat ccc aat ata ttg gct gac ctc caa   3497
Val Cys Phe Ala Thr Cys Phe Asp Pro Asn Ile Leu Ala Asp Leu Gln
    1080                1085                1090

gca aag gaa gga agg att ttg ctc tta aag cct aca cct tca gac ata   3545
Ala Lys Glu Gly Arg Ile Leu Leu Leu Lys Pro Thr Pro Ser Asp Ile
1095                1100                1105                1110

atc tat agt gag gtg aat gag att gag ctc caa agt tca agt aac ttg   3593
Ile Tyr Ser Glu Val Asn Glu Ile Glu Leu Gln Ser Ser Ser Asn Leu
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gta gaa gct gaa act tca gca aca ctt aga ttg gtg aaa aag caa ttt   3641
Val Glu Ala Glu Thr Ser Ala Thr Leu Arg Leu Val Lys Lys Gln Phe
            1130                1135                1140

ggt ggt tgt tac gca ata tca gca gat gaa ttc aca agt gaa atg gtt   3689
Gly Gly Cys Tyr Ala Ile Ser Ala Asp Glu Phe Thr Ser Glu Met Val
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gga gct aaa tca cgt aat att gca tat ctg aaa gga aaa gtg cct tcc   3737
Gly Ala Lys Ser Arg Asn Ile Ala Tyr Leu Lys Gly Lys Val Pro Ser
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tcg gtg gga att cct acg tca gta gct ctt cca ttt gga gtc ttt gag   3785
Ser Val Gly Ile Pro Thr Ser Val Ala Leu Pro Phe Gly Val Phe Glu
1175                1180                1185                1190

aaa gta ctt tca gac gac ata aat cag gga gtg gca aaa gag ttg caa   3833
Lys Val Leu Ser Asp Asp Ile Asn Gln Gly Val Ala Lys Glu Leu Gln
                1195                1200                1205

att ctg acg aaa aaa cta tct gaa gga gac ttc agc gct ctt ggt gaa   3881
Ile Leu Thr Lys Lys Leu Ser Glu Gly Asp Phe Ser Ala Leu Gly Glu
            1210                1215                1220

att cgc aca acg att tta gat ctt tca gca cca gct caa ttg gtc aaa   3929
Ile Arg Thr Thr Ile Leu Asp Leu Ser Ala Pro Ala Gln Leu Val Lys
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gag ctg aag gaa aag atg cag ggt tct ggc atg cct tgg cct ggt gat   3977
Glu Leu Lys Glu Lys Met Gln Gly Ser Gly Met Pro Trp Pro Gly Asp
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gaa ggt cca aag cgg tgg gaa caa gca tgg atg gcc ata aaa aag gtg   4025
Glu Gly Pro Lys Arg Trp Glu Gln Ala Trp Met Ala Ile Lys Lys Val
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aaa ctg gat cat gac tat ctg tgc atg gct gtc ctt gtt caa gaa ata   4121
Lys Leu Asp His Asp Tyr Leu Cys Met Ala Val Leu Val Gln Glu Ile
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ata aat gct gat tat gca ttt gtc att cac aca acc aac cca tct tcc   4169
Ile Asn Ala Asp Tyr Ala Phe Val Ile His Thr Thr Asn Pro Ser Ser
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gga gac gac tca gaa ata tat gcc gag gtg gtc agg ggc ctt ggg gaa   4217
Gly Asp Asp Ser Glu Ile Tyr Ala Glu Val Val Arg Gly Leu Gly Glu
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aca ctt gtt gga gct tac cca gga cgt gct ttg agt ttt atc tgc aag   4265
Thr Leu Val Gly Ala Tyr Pro Gly Arg Ala Leu Ser Phe Ile Cys Lys
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aaa aag gat ctc aac tct cct caa gtg tta ggt tac cca agc aaa ccg   4313
Lys Lys Asp Leu Asn Ser Pro Gln Val Leu Gly Tyr Pro Ser Lys Pro
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atc ggc ctt ttc ata aaa aga tct atc atc ttc cga tct gat tcc aat   4361
Ile Gly Leu Phe Ile Lys Arg Ser Ile Ile Phe Arg Ser Asp Ser Asn
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ggg gaa gat ttg gaa ggt tat gcc ggt gct ggc ctc tac gac agt gta   4409
Gly Glu Asp Leu Glu Gly Tyr Ala Gly Ala Gly Leu Tyr Asp Ser Val
        1385                1390                1395

cca atg gat gag gag gaa aaa gtt gta att gat tac tct tcc gac cca   4457
Pro Met Asp Glu Glu Glu Lys Val Val Ile Asp Tyr Ser Ser Asp Pro
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ttg ata act gat ggt aac ttc cgc cag aca atc ctg tcc aac att gct   4505
Leu Ile Thr Asp Gly Asn Phe Arg Gln Thr Ile Leu Ser Asn Ile Ala
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cgt gct gga cat gct atc gag gag cta tat ggc tct cct aaa gac atc   4553
Arg Ala Gly His Ala Ile Glu Glu Leu Tyr Gly Ser Pro Gln Asp Ile
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gag ggt gta gtg agg gat gga aag att tat gtc gtt cag aca aga cct   4601
Glu Gly Val Val Arg Asp Gly Lys Ile Tyr Val Val Gln Thr Arg Pro
            1450                1455                1460

cag atg tgatcatatt ctcgttgtat gttgttcaga gaagaccata gatgtgatca    4657
Gln Met

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Gly Asn Ser Leu Phe Gln Gln Gln Val Ile Ser Lys Ser Pro Leu Ser
        35                  40                  45

Thr Glu Phe Arg Gly Asn Arg Leu Lys Val Gln Lys Lys Lys Ile Pro
    50                  55                  60

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

Thr Asp Thr Ser Ser Glu Leu Ala Glu Lys Phe Ser Leu Glu Gly Asn
                85                  90                  95

Ile Glu Leu Gln Val Asp Val Arg Pro Pro Thr Ser Gly Asp Val Ser
            100                 105                 110

Phe Val Asp Phe Gln Ala Thr Asn Gly Ser Asp Lys Leu Phe Leu His
        115                 120                 125

Trp Gly Ala Val Lys Phe Gly Lys Glu Thr Trp Ser Leu Pro Asn Asp
    130                 135                 140

Arg Pro Asp Gly Thr Lys Val Tyr Lys Asn Lys Ala Leu Arg Thr Pro
145                 150                 155                 160

Phe Val Lys Ser Gly Ser Asn Ser Ile Leu Arg Leu Glu Ile Arg Asp
                165                 170                 175

Thr Ala Ile Glu Ala Ile Glu Phe Leu Ile Tyr Asp Glu Ala Tyr Asp
            180                 185                 190

Lys Trp Ile Lys Asn Asn Gly Gly Asn Phe Arg Val Lys Leu Ser Arg
        195                 200                 205

Lys Glu Ile Arg Gly Pro Asp Val Ser Val Pro Glu Glu Leu Val Gln
    210                 215                 220

Ile Gln Ser Tyr Leu Arg Trp Glu Arg Lys Gly Lys Gln Asn Tyr Thr
225                 230                 235                 240

Pro Glu Lys Glu Lys Glu Glu Tyr Glu Ala Ala Arg Thr Glu Leu Gln
                245                 250                 255
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Glu Glu Ile Ala Arg Gly Ala Ser Ile Gln Asp Ile Arg Ala Arg Leu
            260                 265                 270

Thr Lys Thr Asn Asp Lys Ser Gln Ser Lys Glu Glu Pro Leu His Val
        275                 280                 285

Thr Lys Ser Glu Ile Pro Asp Asp Leu Ala Gln Ala Gln Ala Tyr Ile
    290                 295                 300

Arg Trp Glu Lys Ala Gly Lys Pro Asn Tyr Pro Pro Glu Lys Gln Ile
305                 310                 315                 320

Glu Glu Leu Glu Glu Ala Arg Arg Glu Leu Gln Leu Glu Leu Glu Lys
                325                 330                 335

Gly Ile Thr Leu Asp Glu Leu Arg Lys Lys Ile Thr Lys Gly Glu Ile
            340                 345                 350

Lys Thr Lys Ala Glu Lys His Val Lys Arg Ser Ser Phe Ala Val Glu
        355                 360                 365

Arg Ile Gln Arg Lys Lys Arg Asp Phe Gly Gln Leu Ile Asn Lys Tyr
    370                 375                 380

Pro Ser Ser Pro Ala Val Gln Val Gln Lys Val Leu Glu Glu Pro Pro
385                 390                 395                 400

Ala Leu Ser Lys Ile Lys Leu Tyr Ala Lys Glu Lys Glu Glu Gln Ile
                405                 410                 415

Asp Asp Pro Ile Leu Asn Lys Lys Ile Phe Lys Val Asp Asp Gly Glu
            420                 425                 430

Leu Leu Val Leu Val Ala Lys Ser Ser Gly Lys Thr Lys Val His Ile
        435                 440                 445

Ala Thr Asp Leu Asn Gln Pro Ile Thr Leu His Trp Ala Leu Ser Lys
    450                 455                 460

Ser Arg Gly Glu Trp Met Val Pro Pro Ser Ser Ile Leu Pro Pro Gly
465                 470                 475                 480

Ser Ile Ile Leu Asp Lys Ala Ala Glu Thr Pro Phe Ser Ala Ser Ser
                485                 490                 495

Ser Asp Gly Leu Thr Ser Lys Val Gln Ser Leu Asp Ile Val Ile Glu
            500                 505                 510

Asp Gly Asn Phe Val Gly Met Pro Phe Val Leu Leu Ser Gly Glu Lys
        515                 520                 525

Trp Ile Lys Asn Gln Gly Ser Asp Phe Tyr Val Asp Phe Ser Ala Ala
    530                 535                 540

Ser Lys Leu Ala Leu Lys Ala Ala Gly Asp Gly Ser Gly Thr Ala Lys
545                 550                 555                 560

Ser Leu Leu Asp Lys Ile Ala Asp Met Glu Ser Glu Ala Gln Lys Ser
                565                 570                 575
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Phe Met His Arg Phe Asn Ile Ala Ala Asp Leu Ile Glu Asp Ala Thr
            580                 585                 590

Ser Ala Gly Glu Leu Gly Phe Thr Gly Ile Leu Val Trp Met Arg Phe
        595                 600                 605

Met Ala Thr Arg Gln Leu Ile Trp Asn Lys Asn Tyr Asn Val Lys Pro
    610                 615                 620

Arg Glu Ile Ser Lys Ala Gln Asp Arg Leu Thr Asp Leu Leu Gln Asn
625                 630                 635                 640

Ala Phe Thr Ser His Pro Gln Tyr Arg Glu Ile Leu Arg Met Ile Met
                645                 650                 655

Ser Thr Val Gly Arg Gly Gly Glu Gly Asp Val Gly Gln Arg Ile Arg
            660                 665                 670

Asp Glu Ile Leu Val Ile Gln Arg Lys Asn Asp Cys Lys Gly Gly Met
        675                 680                 685

Met Glu Glu Trp His Gln Lys Leu His Asn Asn Thr Ser Pro Asp Asp
    690                 695                 700

Val Val Ile Cys Gln Ala Leu Ile Asp Tyr Ile Lys Ser Asp Phe Asp
705                 710                 715                 720

Leu Gly Val Tyr Trp Lys Thr Leu Asn Glu Asn Gly Ile Thr Lys Glu
                725                 730                 735

Arg Leu Leu Ser Tyr Asp Arg Ala Ile His Ser Glu Pro Asn Phe Arg
            740                 745                 750

Gly Asp Gln Lys Asn Gly Leu Leu Arg Asp Leu Gly His Tyr Met Arg
        755                 760                 765

Thr Leu Lys Ala Val His Ser Gly Ala Asp Leu Glu Ser Ala Ile Ala
    770                 775                 780

Asn Cys Met Gly Tyr Lys Thr Glu Gly Glu Gly Phe Met Val Gly Val
785                 790                 795                 800

Gln Ile Asn Pro Val Ser Gly Leu Pro Ser Gly Phe Gln Gly Leu Leu
                805                 810                 815

His Phe Val Leu Asp His Val Glu Asp Lys Asn Val Glu Thr Leu Leu
            820                 825                 830

Glu Gly Leu Leu Glu Ala Arg Glu Glu Leu Arg Pro Leu Leu Leu Lys
        835                 840                 845

Pro Asn Asn Arg Leu Lys Asp Leu Leu Phe Leu Asp Ile Ala Leu Asp
    850                 855                 860

Ser Thr Val Arg Thr Ala Val Glu Arg Gly Tyr Glu Glu Leu Asn Asn
865                 870                 875                 880

Ala Asn Pro Glu Lys Ile Met Tyr Phe Ile Ser Leu Val Leu Glu Asn
                885                 890                 895
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Leu Ala Leu Ser Val Asp Asp Asn Glu Asp Leu Val Tyr Cys Leu Lys
            900                 905                 910

Gly Trp Asn Gln Ala Leu Ser Met Ser Asn Gly Gly Asp Asn His Trp
        915                 920                 925

Ala Leu Phe Ala Lys Ala Val Leu Asp Arg Ile Arg Leu Ala Leu Ala
    930                 935                 940

Ser Lys Ala Glu Trp Tyr His His Leu Leu Gln Pro Ser Ala Glu Tyr
945                 950                 955                 960

Leu Gly Ser Ile Leu Gly Val Asp Gln Trp Ala Leu Asn Ile Phe Thr
                965                 970                 975

Glu Glu Ile Ile Arg Ala Gly Ser Ala Ala Ser Leu Ser Ser Leu Leu
            980                 985                 990

Asn Arg Leu Asp Pro Val Leu Arg Lys Thr Ala Asn Leu Gly Ser Trp
        995                1000                1005

Gln Ile Ile Ser Pro Val Glu Ala Val Gly Tyr Val Val Val Val Asp
   1010                1015                1020

Glu Leu Leu Ser Val Gln Asn Glu Ile Tyr Lys Lys Pro Thr Ile Leu
1025                1030                1035                1040

Val Ala Asn Ser Val Lys Gly Glu Glu Glu Ile Pro Asp Gly Ala Val
                1045                1050                1055

Ala Leu Ile Thr Pro Asp Met Pro Asp Val Leu Ser His Val Ser Val
            1060                1065                1070

Arg Ala Arg Asn Gly Lys Val Cys Phe Ala Thr Cys Phe Asp Pro Asn
        1075                1080                1085

Ile Leu Ala Asp Leu Gln Ala Lys Glu Gly Arg Ile Leu Leu Leu Lys
   1090                1095                1100

Pro Thr Pro Ser Asp Ile Ile Tyr Ser Glu Val Asn Glu Ile Glu Leu
1105                1110                1115                1120

Gln Ser Ser Ser Asn Leu Val Glu Ala Glu Thr Ser Ala Thr Leu Arg
                1125                1130                1135

Leu Val Lys Lys Gln Phe Gly Gly Cys Tyr Ala Ile Ser Ala Asp Glu
            1140                1145                1150

Phe Thr Ser Glu Met Val Gly Ala Lys Ser Arg Asn Ile Ala Tyr Leu
        1155                1160                1165

Lys Gly Lys Val Pro Ser Ser Val Gly Ile Pro Thr Ser Val Ala Leu
   1170                1175                1180

Pro Phe Gly Val Phe Glu Lys Val Leu Ser Asp Asp Ile Asn Gln Gly
1185                1190                1195                1200

Val Ala Lys Glu Leu Gln Ile Leu Thr Lys Lys Leu Ser Glu Gly Asp
                1205                1210                1215
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Phe Ser Ala Leu Gly Glu Ile Arg Thr Thr Ile Leu Asp Leu Ser Ala
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Pro Ala Gln Leu Val Lys Glu Leu Lys Glu Lys Met Gln Gly Ser Gly
        1235                1240                1245

Met Pro Trp Pro Gly Asp Glu Gly Pro Lys Arg Trp Glu Gln Ala Trp
    1250                1255                1260

Met Ala Ile Lys Lys Val Trp Ala Ser Lys Trp Asn Glu Arg Ala Tyr
1265                1270                1275                1280

Phe Ser Thr Arg Lys Val Lys Leu Asp His Asp Tyr Leu Cys Met Ala
                1285                1290                1295

Val Leu Val Gln Glu Ile Ile Asn Ala Asp Tyr Ala Phe Val Ile His
            1300                1305                1310

Thr Thr Asn Pro Ser Ser Gly Asp Asp Ser Glu Ile Tyr Ala Glu Val
        1315                1320                1325

Val Arg Gly Leu Gly Glu Thr Leu Val Gly Ala Tyr Pro Gly Arg Ala
    1330                1335                1340

Leu Ser Phe Ile Cys Lys Lys Lys Asp Leu Asn Ser Pro Gln Val Leu
1345                1350                1355                1360

Gly Tyr Pro Ser Lys Pro Ile Gly Leu Phe Ile Lys Arg Ser Ile Ile
                1365                1370                1375

Phe Arg Ser Asp Ser Asn Gly Glu Asp Leu Glu Gly Tyr Ala Gly Ala
            1380                1385                1390

Gly Leu Tyr Asp Ser Val Pro Met Asp Glu Glu Glu Lys Val Val Ile
        1395                1400                1405

Asp Tyr Ser Ser Asp Pro Leu Ile Thr Asp Gly Asn Phe Arg Gln Thr
    1410                1415                1420

Ile Leu Ser Asn Ile Ala Arg Ala Gly His Ala Ile Glu Glu Leu Tyr
1425                1430                1435                1440

Gly Ser Pro Gln Asp Ile Glu Gly Val Val Arg Asp Gly Lys Ile Tyr
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Val Val Gln Thr Arg Pro Gln Met
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【国際調査報告】

INTERNATIONAL SEARCH REPORT

National Application No
PCT/EP 01/12179

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C08L3/02 A01H5/00 C08B30/00 C12N5/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, SEQUENCE SEARCH, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 195 34 759 A (INST GENBIOLOGISCHE FORSCHUNG) 20 March 1997 (1997-03-20) examples SEQ ID NOs:1 and 2	1-33
X	DE 196 53 176 A (PLANTTEC BIOTECHNOLOGIE GMBH F) 25 June 1998 (1998-06-25) examples SEQ ID NOS:1 and 2	1-33

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 May 2002	13/06/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Burkhardt, P

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INTERNATIONAL SEARCH REPORT

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PCT/EP 01/12179

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LORBERTH ET AL: "inhibiton of a starch-granule-bound protein leads to modified starch and repression of cold sweetening" NATURE BIOTECHNOLOGY, NATURE PUBLISHING, US, vol. 16, May 1998 (1998-05), pages 473-477, XP002111459 ISSN: 1087-0156 cited in the application page 473, last paragraph -page 474, last paragraph; figure 1 ---	1-33
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P,X	WO 00 77229 A (AVENTIS CROPSCIENCE GMBH) 21 December 2000 (2000-12-21) the whole document SEQ ID NOs:7 and 8 ---	1-33
T	RITTE GERHARD ET AL: "The starch-related R1 protein is an alpha -glucan, water dikinase." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (2002 MAY 14) 99 (10) 7166-71. , XP002200501 -----	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 01/12179

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(81)指定国 AP(GH,GM,KE,LS,MW,MZ,SD,SL,SZ,TZ,UG,ZW),EA(AM,AZ,BY,KG,KZ,MD,RU,TJ,TM),EP(AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE,TR),OA(BF,BJ,CF,CG,CI,CM,GA,GN,GQ,GW,ML,MR,NE,SN,TD,TG),AE,AG,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BY,BZ,CA,CH,CN,CO,CR,CU,CZ,DE,DK,DM,DZ,EC,EE,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KP,KR,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,MZ,NO,NZ,PH,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZW

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Ｆターム(参考) 2B030 AA02 AB03 AD09 CB02 CD17
4B024 AA08 BA80 CA04 CA05 CA06 DA01 EA04 FA02 FA07 FA10
GA11 GA17 HA08 HA14
4B032 DG02 DK15
4B065 AA88X AA88Y AB01 AC14 BA02 BA25 CA19 CA24 CA41