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(54) **Title**: METHOD FOR MODULATING THE LEVEL OF PHOSPHORYLATION OF STARCH IN A PLANT LINE, METHOD FOR SELECTING A PLANT OR PART THEREOF, INCLUDING A SEED AND TUBER, AND USE THEREOF

(57) **Abstract**: The invention relates to method for modulating the degree of phosphorylation of starch in plants by changing the allele composition of the alpha-glucan water dikinase (GWD) gene. A decrease in the amount (dosage) of the A allele and/or an increase in the amount (dosage) of the C and/or H allele infer an increase of phosphate content of the starch. The shifts in allele composition can be established by means of breeding, such as introgression, or by transgenic means, such as homologous recombination.

TITLE: Method for modulating the level of phosphorylation of starch in a plant line, method for selecting a plant or part thereof, including a seed and tuber, and use thereof.

FIELD OF THE INVENTION

The present invention relates to genetic engineering of plants, more especially plants that can produce starch, more particularly potato plants. Especially the invention relates to methods for modulating, especially increasing the level of phosphorylation of starch in a plant line. Further, the invention relates to breeding and selection of plants in a breeding program, more particularly to select plants with a modified phosphorylation of starch.

BACKGROUND OF THE INVENTION

Potato is a healthy and nutritious part of the average Western human diet, contributing carbohydrates and important amino acids and vitamins. It is, next to corn and wheat, one of the main sources for starch. Starch and its derivatives are already widely employed in the manufacture of paper, textiles and adhesives, and due to their biodegradable and renewable nature they are increasingly being considered as an environmentally-friendly alternative to the use of synthetic additives in many other products, including plastics, detergents, pharmaceutical tablets, pesticides, cosmetics and even oil-drilling fluids. The thermal and rheological properties of potato starch, as well as properties in processing are related to the degree of starch phosphorylation. The presence of phosphate groups in starch increases the hydration capacity of starch pastes after gelatinization.

Natural starch from many plant species does contain small amounts of covalently-bound phosphate (which may help to stabilise the physical structure of starch grains and play a role in starch breakdown). A crucial enzyme responsible for phosphorylating starch, an alpha-glucan water dikinase (GWD, EC 2.7.9.4) has been identified in potatoes (Lorberth et al. 1998; Ritte et al. 2002) (potato starch is particularly rich in phosphate), and it has been proven that genetic modification of plants to overexpress said gene may permit the production of high-phosphate starch. This has been demonstrated in wheat (WO 02/034923) that normally does not produce phosphorylated starch and in corn (WO 2005/002359). Increasing the phosphate content in starch is highly desirable because a high natural degree of

phosphorylation avoids or reduces expensive and environmentally unfriendly industrial chemical processes.

GWD catalyzes the *de novo* phosphorylation of unphosphorylated starch by introducing phosphate groups exclusively into the C6 position of the glucose molecules of starch (Ritte et al. 2006).

Although genetic engineering with the GWD enzyme would be possible to influence starch phosphorylation in potato, it still has the drawback that consumer acceptation is relatively low and that the production of genetically engineered crops is subject to governmental regulation. In general, therefore, an increased interest in breeding technologies combined with genetic knowledge for breeding and selection, has been observed in the recent decades.

In the past decade of potato research, identification of genes that control the genetic variation of complex traits has mainly been done by linkage analysis in biparental segregating populations. These mapping populations are often developed from diploid parents that originate partly or completely from wild species. In contrast to this linkage mapping, association mapping is a linkage disequilibrium (LD) based technique that exploits biodiversity observed in a wider germplasm pool, e.g. existing cultivars and breeding lines. In potato, association mapping has been conducted by resequencing specific candidate genes and more recently by a genome-wide marker coverage approach (D'hoop 2008).

The most valuable diagnostic markers for identifying the genetic variation of complex traits using either linkage or association mapping are markers derived from polymorphisms in the genes causal for a trait of interest, as such markers are in complete LD with the quantitative trait alleles. SNPs are not independent of one another and only a small subset of all possible arrangements of segregating sites will be seen in a population sample. The DNA sequence variation that is found in a population is the result of the past transmission of that variation through the population. This historical past produces a block-wise structure in which a number of SNPs are in LD with one another. These LD regions are structured into haplotypes blocks and are likely to be transmitted as a unit from generation to generation (e.g. identical by descent). Information about any SNP in the block applies, in a statistical correlation sense, to the whole block. In this context, SNPs can be as informative as multi-allelic molecular markers when used as 'haplotype tags', i.e. several SNPs that tag all the detected haplotypes in a given locus. For direct haplotype identification, homozygous and monoploid (2n=1x=12) potato accessions can be used. Alternatively, potato accessions of lower ploidy might help with haplotype phase identification. The goal of haplotype

interference is to find a set of haplotypes explaining every genotype present in a given population. This genetic composition can then be used to identify alleles and genotypes associated with a quantitative trait of interest.

One of the unresolved questions is how the trait of phosphorylation of starch is affected by the molecular and genetic diversity of the GWD gene. It remains one of the goals in research to identify the genetic variation in the GWD gene across the population of potato accessions to detect markers that would assist breeding attempts for improving the starch phosphorylation trait in a commercial variety of potato.

SUMMARY OF THE INVENTION

The inventors now have developed a method for increasing the degree of phosphorylation in starch in a plant, comprising enriching a plant for the presence of the alleles C or H of glucan water dikinase (GWD) (increasing the dosage of GWD alleles C and/or H) by

a) selecting at least one parental genotype wherein at least one of alleles, C or H of GWD is present,
b) crossing said plant(s),
c) selecting progeny from crossing step b) for a genotype wherein at least one of alleles C or H of GWD is present,
d) selfing and/or further crossing said progeny selected in step c),
e) selecting progeny resulting from step d) for a genotype wherein at least one of alleles C or H of GWD is present, and
f) optionally repeating steps d) and e), whereby homozygosy for allele C or H may be obtained,

wherein said method optionally comprises at least one selection as performed in steps a), c) or e) is performed by marker-assisted selection and wherein said method optionally comprises increasing the ploidy or the use of unreduced gametes of the parental genotype selected at step a) or the progeny of step c) to tetraploid level before conducting step e).
Preferably in this method the genotype of the plant in step a), d) or f) comprises at most two copies of the GWD allele A, more preferably at most one copy, and most preferably allele A is absent..

In an alternative embodiment, the invention comprises a method for increasing the degree of phosphorylation in starch in a plant, comprising:

a) selecting at least one parental genotype wherein allele A of glucan water dikinase (GWD) is absent

b) crossing said plant(s),

c) selecting progeny from crossing step b) for a genotype wherein allele A of GWD is absent,

d) selfing and /or further crossing said progeny selected in step c),

e) selecting progeny resulting from the crossing in step d) for a genotype wherein the allele A of GWD is absent,

f) optionally repeating said steps of selfing and/or crossing and selection of steps d) and e) to provide a plant having a genotype wherein allele A of GWD is completely absent,

wherein said method optionallycomprises at least one selection as performed in steps a), c) or e) is performed by marker-assisted selection and wherein said method optionally comprises increasing the ploidy or the use of unreduced gametes of the parental genotype selected at step a) or the progeny of step c) to tetraploid level before conducting step e) and wherein said method preferably is a method wherein the genotype of the plant in step a), d) or f) comprises at least one copy of the GWD alleles C and/or H, preferably at least two alleles.

Preferably in a method according to the invention the increase in the degree of phosphorylation is caused by an increase in the dosage of alleles C and/or H of GWD and a simultaneous decrease in the dosage of alleles A of GWD.

Further part of the invention is a method for increasing the degree of phosphorylation in starch in a plant, comprising:

a) introgressing the GWD allele C and/or H in the genome of a starch producing plant

b) optionally selecting a plant wherein allele A of GWD is not present

c) optionally selecting for a plant wherein multiple GWD alleles are present selected from the group consisting of C and H,

wherein said method optionally comprises a marker assisted selection step.

In another embodiment the invention comprises a method for selecting a plant or part thereof, including a seed and tuber, said method comprising:

a) testing a plant or part thereof for the absence of at least one marker that is indicative for allele A of GWD, and/or testing a plant or part thereof for the presence of at least one marker that is indicative for at least one of GWD alleles selected from the group C, and H; and

b) selecting said plant or part thereof based on the information derived from said testing.

Preferably in one or more of the above described methods the presence of said marker is determined at DNA level, more preferably said marker is selected from the group consisting of the single nucleotide polymorphisms (SNP) as indicated in Table 1.

Also preferable is a method according to the invention, wherein said plant is tetraploid, preferably wherein said plant is a potato plant, more preferably wherein said potato plant is a *S. tuberosum* Group Tuberosum cultivar.

In a further embodiment, the present invention comprises a method for obtaining a transgenic plant with a higher degree of phosphorylation in starch than the corresponding wild-type plant, comprising introducing a GWD gene, more preferably a GWD gene comprising one or more of the alleles C and/or H of GWD into said plant, preferably wherein an allele C or H of GWD is introduced by site-directed mutagenesis, preferably by homologous recombination, more preferably by homologous recombination with an allele A of GWD.

Also comprised in the invention is the use of a marker for selecting a plant or part thereof, including a seed and tuber, with a genotype wherein the allele A of GWD is absent or wherein at least one of the alleles C and H of GWD are present, wherein said marker is a SNP selected from the SNPs listed in Table 1, or a marker in linkage disequilibrium therewith. Further part of the invention is the use of the above described method for selection of plants for marker-assisted breeding of a plant.

Preferably, in the above described applications, said plant is tetraploid, preferably said plant is a potato plant, more preferably said potato plant is a *S. tuberosum* Group Tuberosum cultivar.

A further part of the invention is a potato plant having tubers with an increased phosphorylation degree of starch, wherein the starch-bound phosphate content [nmol/ng starch] is more than 38.0. Said potato plant preferably is a *S. tuberosum* Group Tuberosum cultivar, more preferably said potato plant is a tetraploid *S. tuberosum* Group Tuberosum cultivar.

Also part of the invention is starch with a phosphorus content of at least 38 nmol/mg, more preferably at least 40 nmol/mg, more preferably at least 45 nmol/mg, more preferably at least 50 nmol/mg, more preferably at least 55 nmol/mg, more preferably at least 60 nmol/mg, more preferably at least 65 nmol/mg, more preferably at least 70 nmol/mg, more preferably at least 80 nmol/mg, produced by a plant according to claim 13 or 17.

Said starch is suitable for food and thus the invention also pertains to a food product comprising such a high phosphate containing starch.

A further part of the invention is the use of a high phosphate containing starch as described above for the production of paper. Accordingly, the invention also pertains to a paper product comprising such a starch.

LEGENDS TO THE FIGURES

Fig. 1. Gene model of the GWD gene, showing the location of the regions GWDex7 en GWD56.

Fig. 2. Dendrogram with allele frequencies of the ten GWD alleles. Letters represent the different alleles.

Fig. 3. Timetable of occurrences of haplotypes G and H.

Fig. 4. Association of genotypes and starch phosphate content. Letters representing the four alleles of a genotypic class. Horizontal lines are representing the overall mean and the SEM (dotted lines). Vertically indicated are the median (thick line) for each variety and quartiles (bar).

Fig. 5. Association of genotype and starch phosphate content for haplotypes A, C and H

Fig. 6. QTL analysis of phosphorous content in starch of the diploid CxE potato mapping population in two subsequent years (2002/2003) showing QTLs on chromosome 2, 5 and 9

Fig. 7. Bar chart showing means of starch phosphate content (Pi) of the four genotypic GWD classes of CxE progeny . Error bars show 95% Confidence Limit of Mean.

Fig. 8. Bar chart showing means of starch phosphate content (Pi) of the four genotypic StPh02 classes of CxE progeny in two subsequent years 2002 (A) and 2003 (B). Error bars show 95% Confidence Limit of Mean.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, the term “plant or part thereof” means any complete or partial plant, single cells and cell tissues such as plant cells that are intact in plants, cell clumps and tissue cultures from which potato plants can be regenerated. Examples of plant parts include, but are not limited to, single cells and tissues from pollen, ovules, leaves, embryos, roots, root tips, anthers, flowers, fruits, stems shoots, tubers, including potato tubers for consumption or ‘seed tubers’ for cultivation or clonal propagation, and seeds; as well as pollen, ovules, leaves,

embryos, roots, root tips, anthers, flowers, fruits, stems, shoots, scions, rootstocks, seeds, protoplasts, calli, and the like.

As used herein, the term "population" means a genetically heterogeneous collection of plants sharing a common genetic derivation.

As used herein, the term "variety" is as defined in the UPOV treaty and refers to any plant grouping within a single botanical taxon of the lowest known rank, which grouping can be: (a) defined by the expression of the characteristics that results from a given genotype or combination of genotypes, (b) distinguished from any other plant grouping by the expression of at least one of the said characteristics, and (c) considered as a unit with regard to its suitability for being propagated unchanged.

The term “cultivar” (for cultivated variety) as used herein is defined as a variety that is not normally found in nature but that has been cultivated by humans, i.e. having a biological status other than a "wild" status, which "wild" status indicates the original non-cultivated, or natural state of a plant or accession. The term “cultivar” specifically relates to a potato plant having a ploidy level that is tetraploid. The term "cultivar" further includes, but is not limited to, semi-natural, semi-wild, weedy, traditional cultivar, landrace, breeding material, research material, breeder's line, synthetic population, hybrid, founder stock/base population, inbred line (parent of hybrid cultivar), segregating population, mutant/genetic stock, and advanced/improved cultivar.

As used herein, “crossing” means the fertilization of female plants (or gametes) by male plants (or gametes). The term "gamete" refers to the haploid or diploid reproductive cell (egg or sperm) produced in plants by meiosis, or by first or second restitution, or double reduction from a gametophyte and involved in sexual reproduction, during which two gametes of opposite sex fuse to form a diploid or polyploid zygote. The term generally includes reference to a pollen (including the sperm cell) and an ovule (including the ovum). "Crossing" therefore generally refers to the fertilization of ovules of one individual with pollen from another individual, whereas "selfing" refers to the fertilization of ovules of an individual with pollen from genetically the same individual.

The term "backcrossing" as used herein means the process wherein the plant resulting from a cross between two parental lines is crossed with one of its parental lines, wherein the parental line used in the backcross is referred to as the recurrent parent. Repeated backcrossing results in the genome becoming more and more similar to the recurrent parent, as far as this can be achieved given the level of homo- or heterozygosity of said parent.

As used herein, "selfing" is defined as refers to the process of self-fertilization wherein an individual is pollinated or fertilized with its own pollen.

The term "marker" as used herein means any indicator that is used in methods for inferring differences in characteristics of genomic sequences. Examples of such indicators are restriction fragment length polymorphism (RFLP) markers, amplified fragment length polymorphism (AFLP) markers, single nucleotide polymorphisms (SNPs), insertion mutations, microsatellite markers (SSRs), sequence-characterized amplified regions (SCARs), cleaved amplified polymorphic sequence (CAPS) markers or isozyme markers or combinations of the markers described herein which defines a specific genetic and chromosomal location.

As used herein, "gene" means a hereditary unit (often indicated by a sequence of DNA) that occupies a specific location on a chromosome and that contains the genetic instruction for the presence or absence of a particular phenotypic characteristics or trait in a plant.

As used herein, "locus" is defined as the genetic or physical position that a given gene occupies on a chromosome of a plant.

The term "allele(s)" as used herein means any of one or more alternative forms of a gene, all of which alleles relate to the presence or absence of a particular phenotypic trait or characteristic in a plant. In a diploid cell or organism, the two alleles of a given gene occupy corresponding loci on a pair of homologous chromosomes. It is in some instance more accurate to refer to "haplotypes" (i.e. an allele of a chromosomal segment) in stead of "allele", however, in these instances, the term "allele" should be understood to comprise the term "haplotype".

The term "heterozygous" as used herein, and confined to diploids, means a genetic condition existing when different alleles reside at corresponding loci on homologous chromosomes.

As used herein, and confined to diploids, "homozygous" is defined as a genetic condition existing when identical alleles reside at corresponding loci on homologous chromosomes.

As used herein, and confined to tetraploids, the term "nulliplex", "simplex", "duplex", "triplex" and "quadruplex", is defined as a genetic condition existing when a specific allele at a corresponding locus on corresponding homologous chromosomes is present 0, 1, 2, 3 or 4 times, respectively. At the tetraploid level the phenotypic effect associated with a recessive allele is only observed when the allele is present in quadruplex condition, whereas the phenotypic effect associated with a dominant allele is already observed when the allele is present in a simplex or higher condition.

The term "dosage" when used in relation to an allele means the amount of alleles present in the genotype of the plant.

The term "recessive allele" as used herein is defined as an allele whose phenotypic effect is not expressed in an individual organism which genotype is heterozygous for the allele, but is only expressed in an individual organism which genotype is homozygous or quadruplex for the recessive allele.

As used herein, the term "dominant allele" is defined as an allele whose phenotypic effect is expressed in an individual organism due to the presence of at least one copy, irrespective of the zygosity of the plant.

The terms "haploid", "diploid" and "tetraploid" as used herein are defined as having respectively one, two and four pairs of each chromosome in each cell (excluding reproductive cells).

The term "polymorphism" is defined as an allelic variant that occurs in a population. The polymorphism can be a single nucleotide difference present at a locus, or can be an insertion or deletion of one or a few nucleotides such as an indel, or the insertion of a large

stretch of nucleotides such as a transposons insertion. As such, a single nucleotide polymorphism ("SNP") is characterized by the presence in a population of at least two, of the four nucleotides (i. e., adenosine, cytosine, guanosine or thymidine) at a particular homologous position or locus in a genome such as the potato genome. Accordingly, it will be recognized that, while the methods of the invention are exemplified primarily by the detection of SNPs, the disclosed methods or others known in the art similarly can be used to identify other polymorphisms in the exemplified genetic region or haplotype.

As used herein, the term "indel" is defined as a mutation in the DNA whereby one or more nucleotides are either inserted (Insertion) or deleted (DELetion), resulting in a net gain or loss of nucleotides. This includes any combination of insertions and deletions.

The term "haplotype" as used herein means a combination of alleles at multiple loci that are transmitted together on the same chromosome. This includes haplotypes referring to as few as two loci, and haplotypes referring to an entire chromosome depending on the number of recombination events that have occurred between a given set of loci. It also includes a set of polymorphisms on a single chromatid that are statistically associated with each other.

As used herein, the term "infer" or "inferring", when used in reference to assessing the presence of any allele as mentioned herein, means drawing a conclusion about the presence of an allele in a plant or part thereof using a process of analyzing individually or in combination nucleotide occurrence(s) of one or more polymorphism(s) of the invention in a nucleic acid sample of the plant or part thereof, and comparing the individual or combination of nucleotide occurrence(s) of the polymorphism(s) to known relationships of nucleotide occurrence(s) of one or more of the alleles as disclosed in the present invention. As disclosed herein, the nucleotide occurrence(s) can be identified directly by examining the qualitative differences or quantitative differences in expression levels of nucleic acid molecules, or indirectly by examining (the expression level of) a polypeptide encoded by a particular gene, for example, the GWD gene, when the polymorphism is associated with an amino acid change in the encoded polypeptide or has regulatory effects.

The term "primer" as used herein refers to an oligonucleotide which is capable of annealing to the amplification target allowing a DNA polymerase to attach thereby serving as

a point of initiation of DNA synthesis when placed under conditions in which synthesis of primer extension product which is complementary to a nucleic acid strand is induced, i.e., in the presence of nucleotides and an agent for polymerization such as DNA polymerase and at a suitable temperature and pH. The (amplification) primer is preferably single stranded for maximum efficiency in amplification. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the agent for polymerization. The exact lengths of the primers will depend on many factors, including temperature and source of primer. A "pair of bi-directional primers" as used herein refers to one forward and one reverse primer as commonly used in the art of DNA amplification such as in PCR amplification.

As used herein, the term "probe" means a single-stranded oligonucleotide sequence that will recognize and form a hydrogen-bonded duplex with a complementary sequence in a target nucleic acid sequence analyte or its cDNA derivative.

The terms "stringency" or "stringent hybridization conditions" refer to hybridization conditions that affect the stability of hybrids, e.g., temperature, salt concentration, pH, formamide concentration and the like. These conditions are empirically optimised to maximize specific binding and minimize non-specific binding of primer or probe to its target nucleic acid sequence. The terms as used include reference to conditions under which a probe or primer will hybridise to its target sequence, to a detectably greater degree than other sequences (e.g. at least 2-fold over background). Stringent conditions are sequence dependent and will be different in different circumstances. Longer sequences hybridise specifically at higher temperatures. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridises to a perfectly matched probe or primer.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M Na+ ion, typically about 0.01 to 1.0 M Na+ ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes or primers (e.g. 10 to 50 nucleotides) and at least about 60°C for long probes or primers (e.g. greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringent conditions or "conditions of reduced stringency" include hybridization with a buffer solution of 30% formamide, 1 M NaCl, 1%

SDS at 37°C and a wash in 2x SSC at 40°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1x SSC at 60°C. Hybridization procedures are well known in the art and are described in e.g. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D.,Seidman, J.G., Smith, J.A., Struhl, K. eds. (1998) Current protocols in molecular biology. V.B. Chanda, series ed. New York: John Wiley & Sons.

Description

The present inventors have found that there are (at least) ten distinct and highly diverse alleles for the GWD locus. Nucleotide diversity between these haplotypes translates into an average nucleotide diversity of about 1 SNP per 45 basepairs. By assessing this variation with the corresponding orthologous GWD sequence of tomato, it could be derived that the average within-potato sequence diversity was larger than the between-species diversity at the locus. From this, it can be concluded that most observed haplotype groups within the sampled population seem to have diverged long time ago, at least predating domestication in potato.

Further, and more importantly, it appears that the presence of some of the alleles (or the absence thereof) is highly related with the degree of phosphorylation of the starch. As can be seen in the examples, the allele A, which is the most abundant allele (frequency of 32.5% in the test population) generally is associated with a low degree of phosphorylation, while alleles C and H were associated with a high degree of phosphorylation.

It will be understood that breeding plants for a higher phosphorylation degree of starch based on selection of those GWD alleles that are associated with high phosphorylation degree and by crossing out those alleles that are associated with a low phosphorylation degree would be possible now the correlation of the GWD alleles with the degree of starch phosphorylation has been given.

Thus, the present invention now enables a method for increasing the degree of phosphorylation of starch in a plant comprising:

a) selecting at least one parental genotype wherein allele A of glucan water dikinase (GWD) is absent,
b) crossing said plant(s),

c) selecting progeny from crossing step b) for a genotype wherein allele A of GWD is absent,
d) selfing and/or further crossing said progeny selected in step c),
e) selecting progeny resulting from step d) for a genotype wherein allele A of GWD is absent, and
f) optionally repeating steps d) and e).

The selection of the plants in steps c) and e) may be done in any desired and possible way, but preferably by marker-assisted selection.

In a similar way, but then breeding towards the inclusion of alleles that are positively correlated with the degree of phosphorylation in starch, the invention provides a method for increasing the degree of phosphorylation in starch in a plant, comprising enriching a plant for the presence of the alleles C or H of GWD by

a) selecting at least one parental genotype wherein at least one of alleles, C or H of GWD is present,
b) crossing said plant(s),
c) selecting progeny from crossing step b) for a genotype wherein at least one of alleles C or H of GWD is present,
d) selfing and/or further crossing said progeny selected in step c),
e) selecting progeny resulting from step d) for a genotype wherein at least one of alleles C or H of GWD is present, and
f) optionally repeating steps d) and e), whereby homozygosy for allele C or H may be obtained.

It is of course to be understood that it would be advantageous to combine these two methods, i.e. both striving for a decrease of the number of allele A alleles and for an increase in the number of C or H alleles in a plant.

It has also been found that a next variation in alleles of another gene, the starch phosphorylase 2 (StPho2) gene accounts for a further variation in starch phosphate content. There, it was found that allele 2 of StPho2 was correlated with a high amount of starch phosphate, while allele 1 was correlated with a low amount of starch phosphate. StPho2 allele 2 can be distinguished from other StPho2 alleles by sequencing the PCR product obtained with primers Pho2-1F (5'-GAAGATGGAAAGGGTTCTCA-3') and Pho2-1R (5'-TTAGCCATATGCACAACAGG-3'). The nucleotide at position 278 (counting from the first

nucleotide of primer Pho2-1F) is A for allele 2, while it is G for all other alleles (SNP G278A). Furthermore, in PCR product Pho2-7F (5'-ACTTGGGTGGGATGAATCTT-3') + Pho2-7R (5'-TAGCAAGCAGTTCGAGGTTC-3') allele 2 contains a sequence of $(T)_9$ starting from position 660 counting from the Pho2-7F primer, whereas all other alleles have a sequence of $(T)_8$, or TATTTTTT.

Therefore, the invention provides a method for increasing the degree of phosphorylation in starch in a plant, comprising enriching a plant for the presence of the allele 2 of starch phosphorylase 2 (StPho2) (increasing the dosage of allele 2 of StPho2) by

a) selecting at least one parental genotype wherein at least allele 2 of starch phosphorylase 2 (StPho2) is present,
b) crossing said plant(s),
c) selecting progeny from crossing step b) for a genotype wherein at least allele 2 of starch phosphorylase 2 (StPho2) is present,
d) selfing and/or further crossing said progeny selected in step c),
e) selecting progeny resulting from step d) for a genotype wherein at least allele 2 of starch phosphorylase 2 (StPho2) is present, and
f) optionally repeating steps d) and e), whereby homozygosy for allele 2 of starch phosphorylase 2 (StPho2) may be obtained.

As will be understood by the skilled person, a simultaneous enrichment for allele 2 of starch phosphorylase 2 (StPho2) and for any of alleles C or H of the GWD gene may be used for obtaining an additional enrichment effect. Also a positive selection for allele 2 of starch phosphorylase 2 (StPho2) may be combined with a negative selection for allele A of GWD. In the same sense, the application provides for a method of producing the plants, by involving them in a breeding programme, either the programme for enriching for the C and/or H alleles, and/or the programme for decreasing the amount of A alleles, as mentioned above, select the plants of interest and grow these plants.

The above-mentioned methods result in an increase of the degree of phosphorylation in starch. It will be obvious for a person of skill that if the goal is to decrease the degree of phosphorylation in starch, the opposite effects should be desired, i.e. an enrichment for allele A of GWD and a decrease of the number of C and H alleles of GWD, and an enrichment for allele 1 of StPho2.

Any suitable method known in the art for crossing selected plants may be applied in the methods according to the invention. This includes both *in vivo* and *in vitro* methods. A person skilled in the art will appreciate that *in vitro* techniques such as protoplast fusion or embryo rescue may be applied when deemed suitable.

Selected plants that are used for crossing purposes in the methods according to the invention may have any type of ploidy. For example, selected plants may be haploid, diploid or tetraploid. However, crossing diploid plants will only provide diploid offspring. Crossing a diploid plant with a tetraploid plant will result in triploid offspring that is sterile.

Thus, when plants are selected that are diploid, their ploidy must be increased to tetraploid level before they can be crossed with another tetraploid plant in the methods according to the invention. Methods for increasing the ploidy of a plant are well known in the art and can be readily applied by a person skilled in the art. For example, ploidy of a diploid plant for crossing purposes can be increased by using 2N gametes of said diploid plant. Ploidy can also be increased by inhibiting chromosome segregation during meiosis, for example by treating a diploid plant with colchicine. By applying such methods on a diploid plant, embryos or gametes are obtained that comprise double the usual number of chromosomes. Such embryos or gametes can then be used for crossing purposes.

Preferably, selected plants are crossed with each other using classical *in vivo* crossing methods that comprise one or more crossing steps including selfing. By applying such classical crossing steps characteristics of both the parents can be combined in the progeny. For example, a plant that provides a high yield can be crossed with a plant that contains large amounts of a certain nutrient. Such a crossing would provide progeny comprising both characteristics, i.e. plants that not only comprise large amounts of the nutrient but also provide high yields.

When applying backcrossing, F1 progeny is crossed with one of its high-yielding parents P to ensure that the characteristics of the F2 progeny resemble those of the high-yielding parent. For example, a selected diploid potato with a high degree of phosphorylation in starch (e.g. a plant containing one or more of the GWD alleles C or H) is made tetraploid by using colchicine and then crossed with a selected high-yielding tetraploid potato cultivar, with the purpose of ultimately providing a high-yielding tetraploid progeny having a high degree of phosphorylation. Also selfing may be applied. Selected plants, either parent or progeny, are then crossed with themselves to produce inbred varieties for breeding. For example, selected specimens from the above mentioned F1 progeny are crossed with

themselves to provide an F2 progeny from which specimens can be selected that have an increased level of phosphorylation. However, potato is know to display severe inbreeding depression upon selfing, and selfing requires both male and female fertility which may not be the case, and therefore this strategy is cumbersome.

Preferred, therefore is a strategy of increasing the number of desired alleles in one parent plant by marker-assisted breeding, then cross said parent plant with a high yielding variety in order to produce plants that have retained both characteristics from their parents.

When selecting and crossing a parental genotype in a method according to the invention, a marker is used to assist selection in at least one selection step. It is known in the art that markers, indicative for a certain trait or condition, can be found *in vivo* and *in vitro* at different biological levels. For example, markers can be found at peptide level or at gene level. At gene level, a marker can be detected at RNA level or DNA level. Preferably, in the present invention the presence of such a marker is detected at DNA level. Markers at DNA level are known in the art. For example, an SNP or haplotype that is unique for a certain gene or allele is indicative for a specific trait or condition associated with that gene or allele and is thus a suitable marker for that gene or allele.

The inventors now have identified two regions in the genomic sequence of the glucan water dikinase (GWD) gene containing SNPs that enable discrimination between 10 different alleles for the gene. These regions are a large part (627 basepairs) of the region exon 8 to exon 9 of the gene (hereinafter identified as GWDex7) and a region (606 basepairs) covering exon 15 to exon 17 (hereinafter identified as GWD56).

The sequences of GWDex7 and GWD56 for allele A^1 are given below.

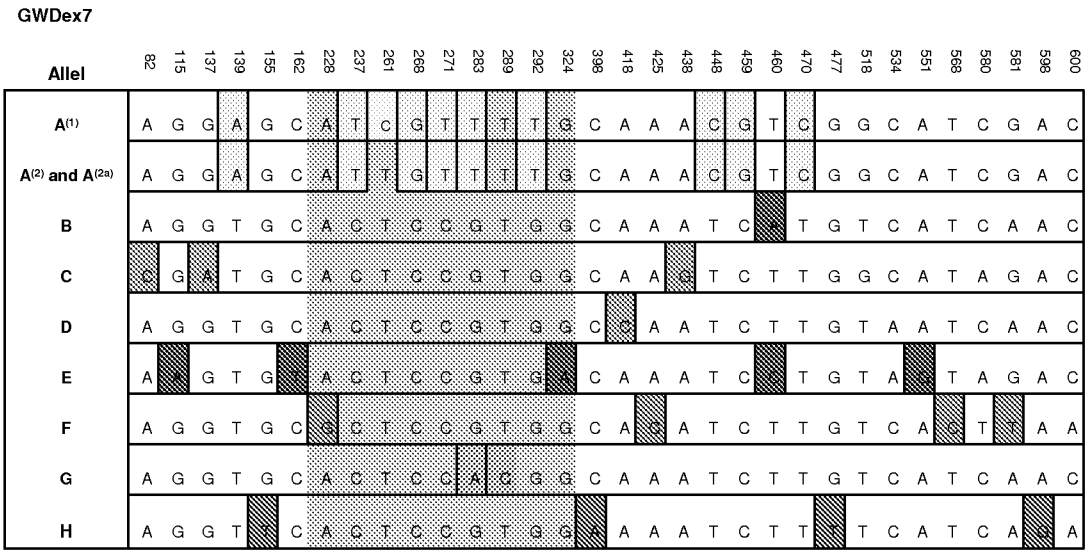
>REF_GWDex7_7322
GGAATATGAGGCTGCTCGAACTGAGCTACAGGAGGAAATAGCTCGTGGTGCTTCC
ATACAGGACATTCGAGCAAGGCTAACAAAAACTAATGATAAAAGTCAAAGCAAA
GAAGAGCCTCTTCATGTAACAAAGAGTGAAATACCTGATGACCTTGCCCAAGCAC
AAGCTTACATTAGGTGGGAGAAAGCAGGAAAGCCGAACTATCCTCCAGAAAAGC
AAATTGTAAATGCTGAACTTTTCTTACAGTTTATGTAGTGTGCGTATATGTCTAGG
CGCATAAATCTGGGTATTCTGTCTTCTATAGATGTGAGACTTTGATCAAGCATTGT
TTTATTAACAGGAAGAACTCGAAGAAGCAAGAAGAGAATTGCAACTTGAGCTTG
AGAAAGGCATTACCCTTGATGAGTTGCGGAAAAAGATTACAAAAGGGGAGATAA
AAACTAAGGCGGAAAAGCACGTGAAAAGAAGCTCTTTTGCCGTTGAAAGAATCC
AAAGAAAGAAGAGAGACTTTGGGCAGCTTATTAATAAGTATCCTTCCAGTCCTGC
AGTACAAGTACAAAAGGTCTTGGAAGAACCACCGGCCTTATCTAAAATTAAGCTG
TATGCCAAGGAGAAGGAGGAGCAGA

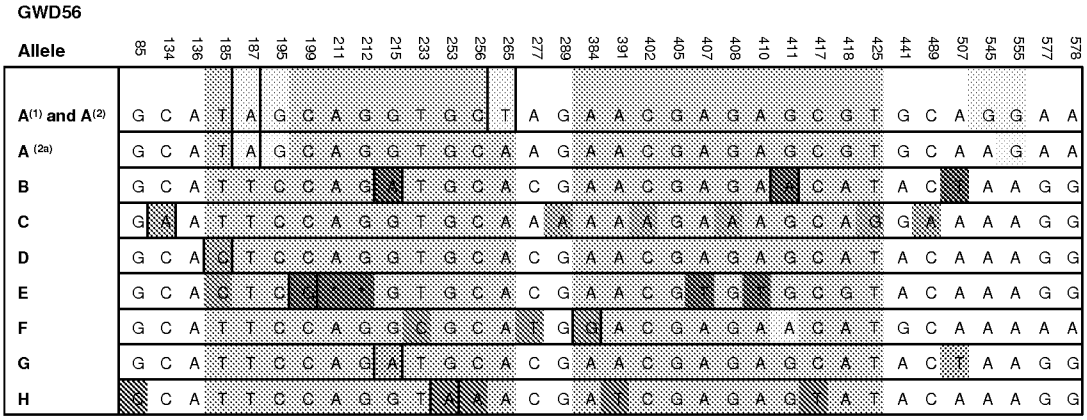
>REF_GWD56_7322
TGAAATAAGCAAGGCTCAGGACAGACTTACAGACTTGTTGCAGAATGCTTTCACC
AGTCACCCTCAATACCGTGAAATTTTGCGGATGATTATGTCAACTGTTGGACGTG

GAGGTGAAGGGGATGTAGGACAGCGAATTAGGGATGAAATTTTGGTCATCCAGG
TTCATATATATCTTTTATCTTCACACCGGTGTTTCTATTGATAGACAGGGGTATCT
AGTTTCCTTAAGTGAATAGCATGTTGATAAGAGTTCTTTTCCTATTTTCAGAGGAA
AAATGACTGCAAGGGTGGTATGATGGAAGAATGGCATCAGAAATTGCATAATAA
TACTAGTCCTGATGATGTTGTGATCTGTCAGGTAGTGAGAATGCAGAGGGAATAT
TGATGAAACTTCTTTCCCAGAAGTAGTTTAACGATGTACTTCTTTTGCAGGCATTG
ATTGACTACATCAAGAGTGATTTTGATCTTGGTGTTTATTGGAAAACCCTGAATG
AGAACGGAATAACAAAAGAGCGTCTTTTGAGTTATGACCGTGCTATCCGTTCTGA
ACCGAATTTTAGAGGAGATCAAAAGAATGGTCTTTTGCGTGATTTAGGTCACTAT

In the below table the differences for each of the ten alleles are indicated. From the Table the complete sequences of the areas GWDex7 and GWD56 for the alleles A^2, A^{2a}, B, C, D, E, F, G and H can be reconstructed. For instance, the allele A^2 as compared to A^1 has a C261T mutation in the GWDex7 area and is identical to A^1 in the GWD56 area. Allele B as compared to allele A1 differs in many places in the GWDex7 area: positions 139 (A→T), 237 (T→C), 261 (C→T), 268 (G→C), 271 (T→C), 283 (T→G), 292 (T→G), 448 (C→T), 459 (G→C), 460 (T→A) and 470 (C→T). In the case of allele B the GWDex7 area is unique with respect to all other alleles by the T460A mutation. Further, as can be seen, allele B differs from allele A1 in 8 positions in the GWD56 area. In the present application, the alleles will be indicated by their letter as used in the below Table.

Table 1: Single nucleotide polymorphisms in the areas GWDex7 and GWD56:





It can be seen from the above Table that each allele can uniquely be identified by SNPs in one or both of the sequenced regions. It will be clear for the skilled person that many more SNPs or other polymorphisms may exist in the genomic GWD sequences that are not contained within the tested regions GWDex7 and GWD56. As long as these SNPs can be used to discriminate between the ten alleles as identified in the Table above, and more particularly the alleles A, C and H of GWD, such SNPs are regarded to be useful in the methods according to the invention. Also SNPs in sequences outside the GWD gene, but which SNPs are in LD with the above-mentioned SNPs would be useful in the present invention. The same applies for SNPs in the StPho2 gene that lie outside the region amplified with primers Pho2-7F and Pho2-7R.

It will be clear to the skilled person that any method suitable for detecting a nucleotide change may be applied when selecting a plant or plant line in the method according to the invention. Methods for detecting a nucleotide change can utilize one or more oligonucleotide probes or primers that selectively hybridize to a target polynucleotide which contains one or more SNP positions or other markers. Such probes or primers include, for example, an amplification primer pair. Probes useful in practicing a method of the invention can include, for example, an oligonucleotide that is complementary to and spans a portion of the target polynucleotide, including the position of the marker, wherein the presence or absence of a specific nucleotide at the position (e.g, an SNP, an indel or a transposon insertion) is detected by the presence or absence of selective hybridization of the probe. Such a method can further include contacting the target polynucleotide and hybridized oligonucleotide with an endonuclease, and detecting the presence or absence of a cleavage product of the probe,

depending on whether the nucleotide occurrence at the marker site is complementary to the corresponding nucleotide of the probe. A pair of probes that specifically hybridize upstream and adjacent and downstream and adjacent to the site of the marker, wherein one of the probes includes a nucleotide complementary to a nucleotide occurrence of the marker, also can be used in an oligonucleotide ligation assay, wherein the presence or absence of a ligation product is indicative of a specific nucleotide occurrence at the marker site. An oligonucleotide also can be useful as a primer, for example, for a primer extension reaction, wherein the product (or absence of a product) of the extension reaction is indicative of the nucleotide occurrence. In addition, a primer pair useful for amplifying a portion of the target polynucleotide including the marker site can be useful, wherein the amplification product is examined to determine the nucleotide occurrence at the marker site.

Where the particular nucleotide occurrence of a marker, or nucleotide occurrences of a haplotype, is such that the nucleotide occurrence results in an amino acid change in an encoded polypeptide, the nucleotide occurrence can be identified indirectly by detecting the particular amino acid in the polypeptide. The method for determining the amino acid will depend, for example, on the structure of the polypeptide or on the position of the amino acid in the polypeptide. Where the polypeptide contains only a single occurrence of an amino acid encoded by the particular polymorphism, the polypeptide can be examined for the presence or absence of the amino acid. For example, where the amino acid is at or near the amino terminus or the carboxy terminus of the polypeptide, simple sequencing of the terminal amino acids can be performed. Alternatively, the polypeptide can be treated with one or more enzymes and a peptide fragment containing the amino acid position of interest can be examined, for example, by sequencing the peptide, or by detecting a particular migration of the peptide following electrophoresis. Where the particular amino acid comprises an epitope of the polypeptide, the specific binding, or absence thereof, of an antibody specific for the epitope can be detected. Other methods for detecting a particular amino acid in a polypeptide or peptide fragment thereof are well known and can be selected based, for example, on convenience or availability of equipment such as a mass-spectrometer, capillary electrophoresis system, magnetic resonance imaging equipment, and the like.

The marker-assisted selection steps in the methods of the invention can in principle be performed by applying any nucleic acid amplification method, such as the Polymerase Chain Reaction (PCR; Mullis 1987, U.S. Pat. No. 4,683,195, 4,683,202, en 4,800,159) or by using

amplification reactions such as Ligase Chain Reaction (LCR; Barany 1991, Proc. Natl. Acad. Sci. USA 88:189-193; EP Appl. No., 320,308), Self-Sustained Sequence Replication (3SR; Guatelli et al., 1990, Proc. Natl. Acad. Sci. USA 87:1874-1878), Strand Displacement Amplification (SDA; U.S. Pat. Nos. 5,270,184, en 5,455,166), Transcriptional Amplification System (TAS; Kwoh et al., Proc. Natl. Acad. Sci. USA 86:1173-1177), Q-Beta Replicase (Lizardi et al., 1988, Bio/Technology 6:1197), Rolling Circle Amplification (RCA; U.S. Pat. No. 5,871,921), Nucleic Acid Sequence Based Amplification (NASBA), Cleavage Fragment Length Polymorphism (U.S. Pat. No. 5,719,028), Isothermal and Chimeric Primer-initiated Amplification of Nucleic Acid (ICAN), Ramification-extension Amplification Method (RAM; U.S. Pat. Nos. 5,719,028 and 5,942,391) or other suitable methods for amplification of DNA.

In order to amplify DNA with a small number of mismatches to one or more of the amplification primers, an amplification reaction may be performed under conditions of reduced stringency (e.g. a PCR amplification using an annealing temperature of 38°C, or the presence of 3.5 mM $MgCl_2$). The person skilled in the art will be able to select conditions of suitable stringency.

The detection of the amplification products can in principle be accomplished by any suitable method known in the art. The detection fragments may be directly stained or labeled with radioactive labels, antibodies, luminescent dyes, fluorescent dyes, or enzyme reagents. Direct DNA stains include for example intercalating dyes such as acridine orange, ethidium bromide, ethidium monoazide or Hoechst dyes.

Alternatively, the DNA fragments may be detected by incorporation of labeled dNTP bases into the synthesized DNA fragments. Detection labels which may be associated with nucleotide bases include e.g. fluorescein, cyanine dye or BrdU.

When using a probe-based detection system, a suitable detection procedure for use in the present invention may for example comprise an enzyme immunoassay (EIA) format.

Probes useful for the detection of the target DNA as disclosed herein preferably bind only to at least a part of the DNA sequence region as amplified by the DNA amplification procedure. Those of skill in the art can prepare suitable probes for detection based on the nucleotide sequence of the target DNA without undue experimentation. Also the complementary sequences of the target DNA may suitably be used as detection probes in a

method of the invention, provided that such a complementary strand is amplified in the amplification reaction employed.

Any suitable method for screening the nucleic acids of a plant or part thereof for the presence or absence of polymorphisms is considered to be part of the methods according to the invention. Such screening methods include, but are not limited to: DNA sequencing, restriction fragment length polymorphism (RFLP) analysis, amplified fragment length polymorphism (AFLP) analysis; heteroduplex analysis, single strand conformational polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis (DGGE), real time PCR analysis (e.g. Taqman®), temperature gradient gel electrophoresis (TGGE), primer extension, allele-specific hybridization, and INVADER® genetic analysis assays, cleavage fragment length polymorphism (CFLP) analysis, sequence-characterized amplified region (SCAR) analysis, cleaved amplified polymorphic sequence (CAPS) analysis

The development of primers and probes useful for the detection of polymorphic positions in a nucleic acid is within the realm of ordinary skill (see for instance Sambrook, J. et al., 2001).

By using standard DNA technology it is possible to produce probes and primers that directly or indirectly hybridize to the DNA samples to be tested or cDNA produced from RNA by reverse transcription, and which can be used in assays for the detection of the markers. Nucleic acid amplification techniques allow the amplification of fragments of nucleic acids, which may be present in very low amounts.

In order to develop nucleic acid-based detection methods, the SNP-specific sequences must be determined for which primers or probes may then be developed. To detect the SNPs by nucleic acid amplification and/or probe hybridization, the nucleic acid may be isolated from any raw sample material, optionally reverse transcribed into cDNA and directly cloned and/or sequenced. DNA and RNA isolation kits are commercially available from for instance QIAGEN GmbH, Hilden, Germany, or Roche Diagnostics, a division of F. Hoffmann-La Roche Ltd, Basel, Switzerland. Nucleic acid-based detection of insertions or deletions can be accomplished accordingly.

A sample useful for practicing a method of the invention can be any biological sample from a plant or a part thereof that contains nucleic acid molecules, including portions of the allele sequences to be examined, or corresponding encoded polypeptides, depending on the

particular method. As such, the sample can be a cell or tissue sample. As some of the markers are located in a non-coding region, the nucleic acid sample generally is a deoxyribonucleic acid (DNA) sample, particularly genomic DNA or an amplification product thereof. However, where hetero-nuclear ribonucleic acid (RNA), which includes unspliced mRNA precursor RNA molecules, is available, a cDNA or amplification product thereof can be used. The nucleic acid sample can thus be DNA or RNA, or products derived therefrom such as, for example, amplification products.

Using either the cloned nucleic acid as a hybridization probe, using sequence information derived from the clone, or by designing degenerative primers based on the sequence of the SNP and its flanking sequences, nucleic acid hybridization probes and/or nucleic acid amplification primers may be designed an used in a detection assay for detecting the above identified SNPs in a sample as defined herein.

The DNA, or alternatively, the cDNA may be PCR amplified by using for instance Pfu and Taq DNA polymerases and amplification primers specific for the SNP DNA sequences. Also complete commercially available systems may be used for PCR (e.g. available form various suppliers such as Roche Diagnostics). A suitable method may for instance include mixing into a suitable aqueous buffering system (e.g. a commercially available PCR buffer) a suitable amount of total DNA as a template (e.g. 1 to 5 μg), a suitable amount (e.g. 10 pmol) of a pair of bi-directional amplification primers, a suitable amount of dNTPs and the DNA polymerase, denaturing the nucleic acids by boiling for 1 min, and performing a cycling reaction of around 10-50 alternating cycles of stringent primer hybridization, strand elongation and denaturing, at suitable temperatures to obtain DNA copies of the DNA template as amplification product. The amount of copies produced upon a certain number of cycles correlates directly to the amount of target DNA in the DNA template.

The skilled person is well aware of the available quantitative PCR methods presently available from commercial suppliers to quantify the amount of target DNA in the template. The term “hybridization signal” as used herein inter alia refers to the amount of amplification product produced upon a certain number of cycles and thus to the amount of target DNA available as template in the reaction.

In order to amplify a nucleic acid with a small number of mismatches to one or more of the amplification primers, an amplification reaction may be performed under conditions of reduced stringency (e.g. a PCR amplification using an annealing temperature of 38°C, or the

presence of 3.5 mM $MgCl_2$). The person skilled in the art will be able to select conditions of suitable stringency.

The primers herein are selected to be "substantially" complementary (i.e. at least 65%, more preferably at least 80% perfectly complementary) to their target regions present on the different strands of each specific sequence to be amplified. It is possible to use primer sequences containing e.g. inositol residues or ambiguous bases or even primers that contain one or more mismatches when compared to the target sequence. In general, sequences that exhibit at least 65%, more preferably at least 80% homology with the target DNA or RNA oligonucleotide sequences are considered suitable for use in a method of the present invention. Sequence mismatches are also not critical when using low stringency hybridization conditions.

The detection of the amplification products can in principle be accomplished by any suitable method known in the art. The amplified fragments may be directly stained or labeled with radioactive labels, antibodies, luminescent dyes, fluorescent dyes, or enzyme reagents. Direct DNA stains include for example intercalating dyes such as acridine orange, ethidium bromide, ethidium monoazide or Hoechst dyes.

Alternatively, the DNA or RNA fragments may be detected by incorporation of labeled dNTP bases into the synthesized fragments. Detection labels which may be associated with nucleotide bases include e.g. fluorescein, cyanine dye, digoxigenin (DIG) or bromodeoxyuridine (BrdU).

Other methods of analysing the nucleic acid suitably comprise the use of a primer extension assay; a Taqman® PCR; a differential hybridization assay; an assay which detects allele-specific enzyme cleavage; and/or allele-specific PCR.

When using a probe-based detection system, a suitable detection procedure for use in the present invention may for example comprise an enzyme immunoassay (EIA) format (Jacobs *et al.*, 1997, J Clin Microbiol 35:791–795). For performing a detection by manner of the EIA procedure, either the forward or the reverse primer used in the amplification reaction may comprise a capturing group, such as a biotin group for immobilization of target DNA PCR amplicons on e.g. a streptavidin coated microtiter plate wells or streptavidin coated Dynabeads® (Dynal Biotech, Oslo, Norway) for subsequent EIA detection of target DNA amplicons. The skilled person will understand that other groups for immobilization of target DNA PCR amplicons in an EIA format may be employed.

Probes useful for the detection of the target nucleic acid sequences preferably bind only to at least a part of the nucleic acid sequence region as amplified by the nucleic acid amplification procedure. Those of skill in the art can prepare suitable probes for detection based on the nucleotide sequence of the target nucleic acid without undue experimentation as set out herein. Also the complementary nucleotide sequences, whether DNA or RNA or chemically synthesized analogues, of the target nucleic acid may suitably be used as type-specific detection probes in a method of the invention, provided that such a complementary strand is amplified in the amplification reaction employed.

Suitable detection procedures for use herein may for example comprise immobilization of the amplicons and probing the nucleic acid sequences thereof by e.g. Northern and Southern blotting. Other formats may comprise an EIA format as described above. To facilitate the detection of binding, the specific amplicon detection probes may comprise a label moiety such as a fluorophore, a chromophore, an enzyme or a radio-label, so as to facilitate monitoring of binding of the probes to the reaction product of the amplification reaction. Such labels are well known to those skilled in the art and include, for example, fluorescein isothiocyanate (FITC), β-galactosidase, horseradish peroxidase, streptavidin, biotin, digoxigenin, ^{35}S, ^{14}C, ^{32}P or ^{125}I. Other examples will be apparent to those skilled in the art.

Detection may also be performed by a so-called reverse line blot (RLB) assay, such as for instance described by Van den Brule *et al.* (2002). For this purpose RLB probes are preferably synthesized with a 5' amino group for subsequent immobilization on e.g. carboxyl coated nylon membranes. The advantage of an RLB format is the ease of the system and its speed, thus allowing for high throughput sample processing.

The use of nucleic acid probes for the detection of RNA or DNA fragments is well known in the art. Mostly these procedures comprise the hybridization of the target nucleic acid with the probe followed by post-hybridization washings. Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For nucleic acid hybrids, the Tm can be approximated from the equation of Meinkoth and Wahl (1984): Tm = 81.5 °C + 16.6 (log M) + 0.41 (% GC)-0.61 (% form)-500/L; where M is the molarity of monovalent cations, % GC is the percentage of guanosine and cytosine nucleotides in the nucleic acid, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The Tm is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. Tm is reduced by

about 1 °C for each 1 % of mismatching; thus, the hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with > 90% identity are sought, the Tm can be decreased 10°C. Generally, stringent conditions are selected to be about 5 °C lower than the thermal melting point (Tm) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4 °C lower than the thermal melting point (Tm); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10 °C lower than the thermal melting point (Tm); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20 °C lower than the thermal melting point (Tm). Using the equation, hybridization and wash compositions, and desired Tm, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a Tm of less than 45 °C (aqueous solution) or 32 °C (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, 1993 supra; Ausubel *et al.*, 1998 supra. Alternatively, a modulation in the degree of phosphorylation may also achieved by transgenic methods. Again in such a case, for an increase of the degree of phosphorylation in starch an enrichment for the GWD alles C and H and a decrease in the GWD allele A should be sought. Also enrichment for allele 1 of StPho2 is an alternative or additional approach to reach the intended goal.

Transgenic approaches can consist of introducing the above-mentioned alleles in a plant, which is a common procedure and which is well known to the skilled person. More appropriate, however, is site directed mutagenesis, in which one or more of the above-mentioned alleles is used to replace the corresponding endogenously present alleles. Methods for site directed mutagenesis of plants are known such as, for instance, using homologous recombination as been described in WO 02/052026 and in a reviw of May, G.D. and Kmiec, E.B., 2000, AgBiotechNet, 2:1-3. Preferably an allele C or H is introduced by exchange with an allele A of GWD by homologous recombination. Additionally or alternatively, also introduction of allele 2 of StPho2 by exchange with allele 1 of StPho2 is performed.

EXAMPLES

Example 1 Identification of GWD alleles

Plant material and DNA isolation

To aid haplotype identification, five monoploid potato reference genotypes were used: 7322 (H7322, or AM79.7322 originally from G. Wenzel, Institut für Genetik, Grünbach, Germany), M47 and M133 (1022M-47 and 1022M-133) (Hoogkamp et al 2000) and M5 and M38 (851-5 and 851-38) (Uijtewaal 1987). Furthermore, DNA from nine diploid reference genotypes was used: C en E, 1024-2 and 1029-31 (87.1024/2 and 87.1029/31) (Jacobsen et al. 1989), RH and SH (RH89-039-16 and SH82-93-488) (Rouppe van der Voort et al. 1997; Van Os et al. 2006), RH90 and RH88 (RH90-038-21 and RH88-025-50) (Park et al. 2005) and G254 (Olsder and Hermsen 1976).

The cultivar collection used consisted of 221 tetraploid potato cultivars and progenitor lines chosen to represent the widest possible range of diversity (D'Hoop et al. 2008).

Genomic DNA was extracted from leaf material according to Van der Beek et al. (1992).

PCR amplification and sequencing

Primers for amplification of genomic DNA and for sequencing of the amplified products are shown in Table 2

Table 2. Primers used for (re) sequencing of the potato GWD genomic sequence

Name	Sequence (5' → 3')	Position Y09533 (cDNA)	Forward/ Reverse	Exon/Intron
AWGWD1	CAGTTGAAGCCGTTGGATATGT	3142	F	exon 23
AWGWD2	ATATTGCGTAACAACCACCAAA	3528	R	exon 25
AWGWDex7F	GGAATATGAGGCTGCTCGAACT	842	F	exon 7A
AWGWDex7R	TCTGCTCCTCCTTCTCCTTGGC	1329	R	exon 7B
AWGWD3	GGATATAGTAATTGAAGATGGC	1625	F	exon 10
AWGWD4	TGGTTTTACGTTATAGTTTTTG	1955	R	exon 12
AWGWD5	TGAAATAAGCAAGGCTCAGGAC	1979	F	exon 13
AWGWD6	ATAGTGACCTAAATCACGCAAA	2381	R	exon 15
AWGWD7	CTTAGGCCCTTGCTTCTCAAAC	2628	F	exon 19
AWGWD8	CCTAGATATTCGGCAGATGGCT	2968	R	exon 21
AWGWD9	GTGTGGGCTTCAAAATGGAATG	3912	F	exon 29
AWGWD10	CCCTCACTACACCCTCAATGTC	4437	R	exon 32
AWGWD11	GAAGAAATTATACGTGCTGGAT	3033	F	exon 22
AWGWD12	CATCTGGCATGTCTGGTGTTAT	3279	R	exon 23
AWGWD13	TCTTCTTAATAGACTCGATCCC	3074	F	exon 22
AWGWD14	TCCTTCAATAATACATACAGCA	—	R	intron 23
AWGWD15	AGCTGAAGGAGAAGATGCAGGG	3820	F	exon 28
AWGWD16	TCGGCATATATTTCTGAGTCGT	4063	R	exon 29
AWGWD17	ATGGTAGTGATAAACTGTTTTTG	463	F	exon 3
AWGWD18	TTATCGTGGGCTTCATCGTAT	662	R	exon 5
AWGWD19	AAGGTAAGAAAGGCAAGACTAA	—	F	intron 6
AWGWD20	CTTTGTTACATGAAGAGGCTC	954	R	exon 7A
AWGWD19A	ACTTTATTTAATCCCATTCTTA	—	F	intron 6
AWGWD20A	ATACCCTACTTTTCTTGTAGAT	—	R	intron 6
AWGWD21	GTATGCCAAGGAGAAGGAGGAG	1325	F	exon 7b

AWGWD22	AGTCAACATAGAAATCCGACCC	1704	R	exon 10
AWGWD23	GCAAACTGCATGGGCTACAAAA	2454	F	exon 16
AWGWD24	AAGGGCCTAAGCTCCTCACGAG	2617	R	exon 19
AWGWD25	GTGCTTGACAGAACCCGTCTTG	2907	F	exon 21
AWGWD26	CTAGATTTGCAGTTTTCCGAAG	3099	R	exon 22
AWGWD27	GGGAATATTGAGCTACAGGTAC	387	F	exon 2

Amplicons for sequencing were generated from 10-50 ng genomic DNA template. PCR amplifications were performed in 20 µl reactions using 1 u of Taq Polymerase, 1x reaction buffer, 200 nM dNTP and 250 nM of each primer. Standard cycling conditions were: 4 min initial denaturation at 94°C, followed by 35 cycles of 1 min denaturation at 94°C, 30 sec annealing at 57°C and 40 sec extension at 72°C. Reactions were finished by 7 min incubation at 72°C. PCR products were examined for quality on ethidium bromide stained agarose gels. Following DNA amplification, PCR products were directly sequenced on ABI377 or ABI3700 sequencers (Greenomics, Wageningen) using the dideoxy chain-termination method and ABI PRISM Reaction Kit. The forward amplification primers were used as sequencing primers.

Identification of full genomic GWD sequence

The orthologous GWD gene in *Arabidopsis* (At1g10760, SEX1), consisting of 32 exons and 31 introns, was used to compare the *Arabidopsis* genomic sequence with potato GWD mRNA sequences (AY027522 and Y09533). Primers were designed based on the sequence of Y09533 (**Table 2**). To avoid mis-priming of alleles, most of the primer pairs were designed on exon regions. The primers amplified both coding and non-coding sequence intervals of the GWD gene.

Fragment GWDex7, obtained with primers AWGWDex7F and AWGWDex7R, was used for identification of genomic BACs covering the *GWD* locus in the *HindIII* RH89-039-16 BAC library (RHPOTKEY, http://potatogenome.net/). A positive BAC clone was selected and shotgun sequenced. Gaps between contigs containing the GWD gene were closed by a combinatorial PCR approach. The assembled contig contained 3 kb of promoter sequence and the reconstructed 16.5 kb gene. The encoding region (4395 bp) differed at 3 bases with the published GWD cDNA AY027522. The deduced amino acid sequence differed at 1 position (1256G->V). Published cDNA sequence Y09533 differed at 45 bases and the deduced amino acid sequence differed at 25 positions.

SNP detection and analysis

Alignment and quality scoring was done using the Staden software package (Staden 1996). Sequence variations (SNPs and short INDELs) were detected using NovoSNP (Weckx et al. 2005). Allele dosage of SNPs were scored using both the Data Acquisition & Data Analysis software DAx7.1 (Van Mierlo Software Consultancy) and manual scoring. The dosage of bi- and tri-allelic SNPs in homozygous and heterozygous tetraploid genotypes was estimated from the area-ratio of overlapping base calling peaks. Amplicons containing heterozygous indels were subsequently sequenced form the opposite end with the second amplification primer.

Haplotype identification and diversity analysis

New haplotypes present in the germplasm collection were identified by cloning a selection of genotypes in which new base variations were detected. For this, PCR products of eight genotypes of the GWDex7 fragment and six of the GWD56 fragment were cloned in the pGEM-Teasy vector (Promega, Madison, WI) following the supplier's instructions. The DNA sequences were determined on a ABI3700 sequencer using BigDye terminator chemistry. On average, twelve cloned PCR products were sequenced for each GWD allele to obtain a consensus sequences.

For nucleotide diversity and phylogenetic analysis, the consensus haplotype sequences were compared with one another and with *S.lycopersicum*-derived sequences using MEGA 4 software (Tamura et al. 2007). Similarity between each pair of sequences was calculated on the basis of percentage identity, and tree construction was performed using the Neighbour Joining (NJ) method.

Allelic composition and genotypic analysis

The primary tag SNPs used to estimate allele dosage and composition of the genotypes are given in table 1.

To estimate allele frequencies for the GWD locus, a program for the analysis of autotetraploid genotypic data, AUTOTET (Thrall and Young 2000) was used. The following statistics were calculated to describe the levels of genetic diversity: G, genotypic richness-the number of four allele genotypes at a locus; Ai, the average number of alleles per individual at a locus; H_o, the observed heterozygosity; and H_e, the expected heterozygosity. We also calculated the mean fixation index (F) for all variable loci in each population, in order to compare the genotype proportions with those expected under Hardy–Weinberg equilibrium. The chi-squared (χ^2) test was used to evaluate deviations of F from zero.

Results

A panel of five monoploid and nine diploid potato accessions was assembled to gain a first insight into GWD nucleotide polymorphism among different *S. tuberosum* clones. A number of amplicons were directly sequenced and assessed for single locus amplification, SNPs and InDels. All amplicons derived from the monoploid accessions gave chromatogram peaks representing a single haplotype. Re-sequenced amplicons of diploid accessions displayed multiple chromatogram peaks at discrete nucleotide positions as expected for heterozygous accessions. For **3** out of **7** amplicons, no InDels were observed in the initial panel. Two of those amplicons were selected to identify SNPs in a broader gene pool of 239 tetraploid potato cultivar and progenitor lines. The GWDex7 amplicon (627 bp) includes a large part of the region exon 8 to exon 9; the GWD56 amplicon (606 bp) covers exon 15 to exon 17 (FIG 1).

High quality trace files with an average read length of 523 bp were generated for 226 (95%) tetraploid accessions. The irretrievable accessions showed low quality sequencing chromatograms in repetitive runs. In the ~1kb DNA sequence of the two amplicons, 64 polymorphic sites were detected and quantitatively scored. Four of these sites were of tri-allelic nature and 60 of bi-allelic nature .The raw overall SNP frequency – which ignores the fact that SNPs co-segregate in haplotype blocks – was one SNP every 16 bp. Nucleotide diversity among the haplotypes translate into an average nucleotide diversity of about 1 SNP per 45 basepairs

Short range linkage disequilibrium and identification of haplotypes

The two analyzed amplicons – approximately 2.7 kb apart – present a sample range of 3.25 kb. We assessed short-range LD at this range by deploying the squared correlation coefficient (r^2) between the dosage of the polymorphisms. For the current sample size of 226 potato accessions and a significance level P of 0.01, 70% of the markers showed significant associations. Significant association between polymorphisms in the two amplicon segments of the data support the strong linkage between the two discontinuous amplicons; no decay of LD was observed within this sample range

Using the sequence information of the monoploid and diploid accessions, a number of haplotype models were deduced. Three haplotypes could be directly identified in the monoploid sequences and another three could be inferred from the diploid sequences. The haplotypes of the tetraploid potato genepool could not be directly inferred from the unphased

sequence data due to the highly heterozygous state and high SNP frequency. Haplotype models for these accessions were deduced by identifying sets of co-segregating SNPs in the LD matrix. Sequences of the haplotypes were then confirmed by cloning amplicons from a limited number of corresponding potato accessions. All SNPs were assigned to haplotypes. At least ten different haplotypes could be distinguished (Table 1). In one of the amplicons (GWD56), although no indels were found in the six haplotypes present in the panel of mono- and diploid accessions, a 2 bp InDel was found in haplotype E. Analysis of the genotypes harbouring this haplotype was still possible for both amplicons. We found copy numbers for this and other haplotypes to be fully interchangeable between the two independent amplicons. Selected haplotype tag SNPs therefore were in almost complete LD.

At the protein level, the analyzed haplotypes contained 302 codons in five exons. Of those codons 36 segregated, causing 15 synonymous changes and 19 replacement changes. No well-defined dysfunctional mutations such as stop codon, splicing site or frame shift mutations were found in the haplotypes. To investigate the similarity of the detected haplotypes further, a homozygous diploid *S.lycopersicum* accession was included in the amplicon analysis and used as an out-group in phenetic analysis. Over the 1 kb of sequence, the tomato haplotype was to a high degree similar (95.4% - 96.5%) to the haplotypes observed in the *S.tuberosum* genepool. Similarity within the ten potato haplotypes ranged from 96.6% to 99.9% (FIG 3). Dissimilarity between the two most distant potato haplotypes (haplotypes $A^{(1)}$ and H) approached the potato/tomato distance. Nucleotide diversity (π) between the ten potato haplotypes was $9.6x10^{-3}$.

Haplotype frequencies and genetic diversity

For both amplicons, haplotype-specific tag SNPs were identified to assign an allelic composition to each accession. Given potential biases associated with recombination of tag SNPs and ascertainment of SNP dosage, haplotypes were pairwise-defined (e.g. based on a single tag SNP) whenever possible, using the best quantifiable tag SNP for haplotype dosage estimation (see Table 3). For the GWDex7 amplicon, we were able to pairwise-define eight haplotypes, while one haplotype was multi-marker defined (e.g. based on multiple tag SNPs). Seven of these haplotypes were reproducibly identified in the GWD56 amplicon using either pairwise- or multimarker-defined haplotypes (see Table 1). Dosage estimates of these haplotypes matched in both amplicons. One haplotype (haplotype $A^{(1)}$) was discernible only in the GWDex7 amplicon. In the GWD56 amplicon it was identical-in-state to haplotype $A^{(2)}$. Also in the GWD56 amplicon, a haplotype (haplotype $A^{(2a)}$) identical-in-state to haplotype $A^{(2)}$ was identified. Haplotype $A^{(2)}$ was the most dominant among all ten haplotypes with a

frequency of 32.5%, while frequencies of the very similar haplotypes $A^{(1)}$ and $A^{(2a)}$ were relatively low (3,7% and 4,4% respectively). To aid further analysis, these three haplotypes were grouped into a single haplotype group (haplotype A). Frequencies of the haplotypes and the used tag SNPs are presented in Table 3.

TABLE 3 haplotype frequencies and tag snps.

Haplotype	tagSNPs for GWD haplotype dosage	
	Fragment 1	**Fragment 2**
$A^{(1)}$	SNP261C	SNP265T
$A^{(2)}$	SNP283T - *SNP261C*	SNP265T
$A^{(2A)}$	SNP283T - *SNP261C*	*SNP187A* - SNP265T
B	SNP460A	*SNP411A - SNP384G*
C	SNP137A	SNP134A
D	SNP418	*SNP185 - SNP199G*
E	SNP162T	SNP199G
F	SNP228G	SNP384G
G	SNP283A	*SNP215A - SNP411A - SNP384G*
H	SNP398A	SNP253A

A genetic model based on the eight haplotype groups showed that it was possible to assign a complete four allele genotype to 220 (98%) of the potato accessions (see Table 4). Combinations of different haplotypes in the individual accessions resulted in many different genotypes. The distribution of haplotype $A^{(2)}$ among cultivars was clearly dominant, occurring in 87.6% of the accessions. A few alleles were found with frequencies less than 5%. Haplotype G and H are rare with frequencies below 5%. Homozygosity was found in only four accessions of the analyzed pool. These accessions were homozygous for haplotype A. Three of these accessions contained combinations of the grouped haplotypes $A^{(1)}$, $A^{(2)}$ and $A^{(2a)}$ and thus were not truly homozygous; one accession contained only the major haplotype $A^{(2)}$. All other accessions were heterozygous with two, three or four different haplotypes. The average number of haplotypes per individual (Ai) was 2.86. Full heterozygotes were frequently detected; 74 accessions contained four different haplotypes. We observed 70 different genotypic classes, with the number of accessions per class ranging from 1 to 13 (2.73 on average). The most abundant genotypic class was AAAB occurring in 13 accessions,

followed by AABD, AABF and AABB. Observed (Ho=0.772) and expected (He=0.759) heterozygosity were in close agreement when assuming random chromosomal segregation. A $\chi 2$ test showed that the mean fixation index (F) was in accordance with Hardy-Weinberg expectations (P>0.05).

Table 4. Haplotypes of tested potato cultivars.and the corresponding phosphate content.

P8Code	Cultivar name	Genotype	Starch-bound phosphate content [nmol/ng starch]
P80001	1256A(23) = Black 1256	ADEE	28.46
P80002	Abundance	ABBD	.
P80003	Ackersegen	AABB	26.31
P80004	Adirondack	ACEF	28.67
P80005	Adora	ABBF	18.32
P80006	Adretta	ABBB	24.45
P80007	Agata	ABEF	20.32
P80008	Agria	ABEE	24.98
P80009	Ajiba	AAAB	20.21
P80010	Albion	AABB	26.54
P80011	Alcmaria	AABF	14.9
P80012	Allure	ABFF	16.71
P80013	Alma (1978)	ABFF	24.22
P80014	Almera	BBBC	26.6
P80015	Alpha	AAAC	21.87
P80016	Am 66-42	AADF	17.84
P80018	Am 78-3704	BBBC	27.47
P80019	Amorosa	AAAA	17.75
P80020	Ampera	ABEF	25.06
P80021	Amyla	ACCF	23.52
P80022	Anosta	AACE	19.8
P80023	Aquila	AAAC	.
P80024	Arcade	AABE	23.68
P80025	Arinda	AABD	17.18
P80026	Arnova	ACDD	27.98
P80027	Arran Banner	AADF	24.12
P80028	Arran Chief	AABD	28.31
P80029	Arran Pilot	BEFF	23.96
P80030	Arran Victory	AABB	32.24
P80031	Arrow	AABC	19.59
P80032	Astarte	AACC	18.65
P80033	Asterix	AAAF	19.63
P80034	Atlantic	AACH	30.38
P80035	Aurora	AAAF	17.9
P80036	Ausonia	ABCF	19.86

P80037	Avenance	AABD	28.23
P80038	Ballydoon	AABB	23.41
P80039	Bartina	AAAB	24.28
P80040	Belle De Fontenay	BFFG	25.13
P80041	Bellini	AABC	22.89
P80042	Berber	AACE	17.77
P80043	Bildtstar	ABEH	27.41
P80044	Bintje	ABEF	25.35
P80045	Biogold	BFHH	27.71
P80046	British Queen	ABDF	25.41
P80047	Caesar	AABB	21.61
P80048	Champion	AABF	23.52
P80049	Charlotte	ACCF	15.51
P80050	Cherie	AABC	20.53
P80051	Cilena	BBDF	25.37
P80052	Civa	AABD	19.22
P80053	Clivia	AABD	29.39
P80054	Craigs Alliance	ABBB	23.01
P80055	Craigs Bounty	AADH	27.92
P80056	Craigs Defiance	AABE	15.88
P80057	Daisy	ABBH	20.59
P80059	Desiree	AAAD	16.19
P80061	Diamant	AAAA	14.43
P80062	Ditta	AACE	24.89
P80063	Donald	AABB	22.24
P80064	Doon Star	AAAE	19.59
P80065	Dorado	ABCC	28.1
P80066	Dore	AAFF	21.42
P80067	Dr Mcintosh	AAAB	16.97
P80068	Draga	AAAB	17.54
P80069	Eersteling (Duke Of York)	ABFF	18.01
P80070	Early Rose	BDEF	24.74
P80071	Edeltraut	AABF	.
P80072	Eden (2000)	AABE	17.39
P80073	Ehud	AACE	21.51
P80074	Eigenheimer	ABCF	25.87
P80075	Elisabeth	BBBD	21.01
P80076	Eos	AAAD	19.54
P80077	Epicure	ADEF	24.62
P80078	Escort	AADF	13.96
P80079	Estima	AABD	18.84
P80080	Exquisa	BBFH	27.95
P80081	Fabula	ACDE	21.01
P80082	Felsina	AABF	19.01
P80083	Festien	AADD	26.05
P80084	Fianna	AABF	18.75

P80085	Fichtelgold	ABCD	31.03
P80086	Flava	AABF	18.45
P80087	Sutton'S Flourball	AABF	24.47
P80088	Fontane	ABCE	22.25
P80089	Fresco	AAAC	14.67
P80090	Frieslander	ABBE	16.26
P80091	Fruhmolle	ABBD	.
P80092	Furore	AAAC	20.87
P80093	Gladstone	AABD	25.94
P80094	Gloria	AABF	18.36
P80095	Golden Wonder	AAAD	33.67
P80096	Goya (2000)	AAAD	24.11
P80098	Hansa	AACD	31.57
P80099	Herald	ABBD	17.94
P80100	Hermes	ACDE	22.35
P80103	Hydra	ABBF	.
P80104	Impala	AABE	17.13
P80105	Industrie	BBCD	24.05
P80106	Innovator	AABD	23.81
P80107	Inova	AAAB	18.18
P80108	Irish Cobbler	AADD	22.95
P80109	Irish Queen	AABD	22.93
P80110	Jaerla	ABFF	19.53
P80111	Fransen (Jaune D'Or)	ABEF	25.46
P80112	Jubel	ABCD	17.37
P80115	Katahdin	ABBC	30.85
P80116	Kennebec	BCFH	30.89
P80117	Kepplestone Kidney	AABE	25.98
P80118	Kerpondy	ABDF	24.13
P80119	Kerr'S Pink	AABB	.
P80120	King George	ABDF	19.17
P80121	Kondor	AABB	19.62
P80122	Kuras	ABCH	26.19
P80123	Kuroda	ABDH	26.29
P80124	Lady Christl	ABDE	25.51
P80125	Lady Claire	BEEF	26.44
P80127	Lady Rosetta	AAAA	12.61
P80128	Laura	AAAH	20.94
P80129	Lenape	AACH	.
P80130	Leyla	ABCF	21.38
P80131	Libertas	ACEE	25.06
P80132	Liseta	AACD	17.65
P80133	Magnum Bonum	ABCD	.
P80134	Majestic	ADEF	20.43
P80135	Marfona	BBCD	22.56
P80136	Maris Piper	AABF	16.6

P80137	Maritiema	ADDF	19.98
P80138	Maritta	BBCH	.
P80139	Markies	AABF	25.46
P80140	May Queen	ABFF	25.42
P80141	Mercator	AADD	26.27
P80142	Monalisa	AAAB	15.02
P80143	Mondial	AACD	20.06
P80145	Mpi 19268	AAAF	18.13
P80146	Myatt'S Ashleaf	ABFG	24.08
P80147	Nicola	AACD	23.16
P80148	Ninetyfold	ABDE	.
P80149	Niska	BBCD	.
P80150	Noisette	AABB	16.3
P80151	Nomade	AACD	29.37
P80152	Noordeling	ACEE	25.94
P80153	Obelix	AACF	26.62
P80154	Ontario	ABCD	.
P80155	Pallas	AAAF	19.34
P80157	Patersons Victoria	ADFG	23.26
P80158	Peerless	AAAB	25.55
P80160	Pepo	AABE	17.33
P80161	Picasso	BBCC	25.36
P80162	Premiere	BCDF	18.33
P80163	Prevalent	CCEE	23.75
P80164	Primura	BBDF	19.82
P80165	Profijt	AACE	20.76
P80166	Ramos	BBCE	29.15
P80167	Record	AAEE	24.34
P80168	Rode Eersteling (Red Duke Of York)	ABFF	.
P80169	Red Scarlett	AAAB	20.96
P80170	Redstar	AAAB	22.16
P80171	Reichskanzler	AAAC	.
P80172	Remarka	AABF	18.89
P80173	Romano	AAAB	18.72
P80174	Rural New Yorker No.2	AACD	25.42
P80175	Russet Burbank	ABEF	18.64
P80176	Samba	ABBB	26.35
P80177	Santana	AABC	23.54
P80178	Sante	ADDF	18.47
P80179	Saskia	ABDF	14.01
P80180	Saturna	CEEH	26.65
P80181	Seresta	AABC	22.3
P80183	Shepody	AACD	30.52
P80185	Spunta	AACH	24.96
P80186	Sunrise	BBHH	23.98

P80187	Tahi	ABEH	.
P80188	Tasso	ABCE	18.99
P80189	The Alness	ABBE	22.75
P80190	Timate	AABF	19.21
P80191	Tinwald'S Perfection	AAAB	21.77
P80193	Triplo	ABCE	22.14
P80194	Triumf	AABD	.
P80195	Triumph	DEEF	.
P80196	Ulster Glade	AAAB	19.81
P80197	Ulster Knight	AAAB	22.37
P80198	Ulster Sceptre	ABBF	14.9
P80199	Ultimus	AABC	19.76
P80200	Umatilla Russet	BCDF	25.14
P80201	Up To Date	AABE	20
P80202	Urgenta	AAAC	25.55
P80204	Ve 66-295	AAAD	18.2
P80205	Ve 70-9	AADF	20.67
P80207	Ve 74-45	AAAD	24.7
P80208	Victoria	ABEF	23.93
P80209	Virgo	ABDF	24.64
P80210	Vivaldi	ABBF	19.33
P80211	Vk 69-491	ABCD	29.23
P80211bi	Vk 69-491	AABC	.
P80212	Voran	AACC	26.82
P80213	Voyager	ACDF	21.21
P80214	Vtn 62-33-3	AAAD	20.9
P80215	W 72-22-496	AABD	19.69
P80216	Wauseon	BBCH	37.65
P80217	Wilja	ABCF	.
P80218	Winston	AAAB	.
P80219	Wisent	AADD	24.04
P80220	Y 66-13-636	AABD	22.13
P80221	Yam	AADF	24.59
P80222	Yukon Gold	ACEF	24.15
P80223	Sh83-92-488	AD[1]	23.58
P80224	Rh89-039-16	BC [1]	27.53
P80225	Merano	AADD	.
P80226	Pink Fir Apple	AABF	.
P80227	Folva	AAAB	.
P80228	Nadine	AABB	.
P80229	Panda	BBCH	.
P80230	Arielle	ABDD	18.27
P80231	Marabel	ABBF	22.77
P80232	Vitelotte Noir	AAAB	.
P80233	Quarta	AADE	.
P80234	Princess	ABFF	.

P80235	Ratte	BBGG	.
P80236	Alma (1904)	AABD	.

[1] Diploid cultivars, thus only two alleles

Pedigree analysis of haplotypes

Examination of the pedigrees of rare haplotypes showed that haplotype G was found only in ancient cultivar accessions, introduced between 1804 to 1872, and that haplotype H is relatively new in the analyzed pool (figure 3).

All analyzed accessions harboring haplotype H have a lineage descending from the USDA 96-56 accession, which has been used for the introgression of the *Phytophthora* resistance gene R1 from *S. demissum* (Leonards-Schippers et al. 1992; Ballvora et al. 2002). Also the rare haplotype $A^{(1)}$ is relatively new in the cultivated potato genepool. The first cultivar in our dataset harbouring this haplotype is Maris Piper, introduced in 1963. This is also the first cultivar harbouring the nematode resistance allele H1, providing resistance to Gro1 (Ellenby 1952; Toxopeus and Huijsman 1953; Ross 1979). The H1 resistance was introduced by introgression from *S. tuberosum ssp. andigena* clone CPC 1673. All accessions in which we could identify haplotype $A^{(1)}$ had this CPC clone as parent in their pedigree and are, according to the European potato database, resistant to Gro1.

Next, we performed pedigree analysis to see how the identified haplotype groups compared to relatedness and to verify the accuracy of the haplotype dosage estimates. In the analyzed accessions, 196 offspring-parents pairs were identified. Haplotypes of gametes were in good congruence with those predicted from parental haplotype configurations.

Association between haplotypes and starch phosphate content

Phosphorus content of starches was determined for 191 of the fully genotyped accessions (Table 4). Phosphorus content ranged from 12.7 to 37.7 nmol/mg, with an average level of 22.5±4.3. Variation in phosphorus content within the genotypic classes was large (figure 4).

Two genotypic classes differed significantly from other classes. The average phosphorus content of class AAAA (14.9 nmol/mg, n=4) was significantly ($P<0.01$) lower than other classes while the phosphorus content in the single accession representing class BBCH was significantly higher (37.7 nmol/mg, $P<0.05$). Individual haplotypes significantly ($P<0.05$) associated with phosphorus content, when assuming no population structure, were

haplotypes A (negative association), C, and H.(positive association) Correction for population structure confirmed the significant associations for these haplotypes (figure 5).

Linear regression models with the dosages of these haplotypes showed variance in phosphorus content, which is best explained by haplotype A (12% explained variance).

The phosphorus content of starches of 94 offspring of the diploid CxE potato mapping population has been determined for two successive years previously (Werij et al., unpublished). The content ranged from 9.7 to 37.6 nmol/mg, with an average level of 21.4±4.8SD. Pearson's correlation (R=0.889) between the measurements of the two successive years was high. For both years, QTL analysis showed three major additive QTLs regions on chromosome 2, 5 and 9 respectively (figure 7). The QTL on chromosome 2, with a LOD score of 6.41, explained 33.8% of the observed variance. The chromosome 5 QTL (LOD 6.06) explained 28.5% of the variance and the chromosome 9 QTL (LOD4.47) explained 23.7% of the variance.

GWD haplotypes of the diploid potato CxE mapping population were resolved using a Lightcycler amplicon designed to discriminate the maximum of GWD haplotypes. Three distinct GWD haplotypes were observed in the parental genotypes. Haplotype $A^{(2)}$ (of the haplotype A group) is shared between both parents, haplotypes F is unique to the C-parent and haplotype B unique to the E-parent. By applying Joinmap linkage analysis (Stam 1993), we mapped the GWD locus 12 cM distal to the chromosome 5 specific marker SPUD237 (De Jong et al. 1997). The QTL on chromosome 5 co-localizes with the GWD locus. In the CxE mapping population the offspring homozygous for haplotype A showed significantly lower levels of starch phosphate, similar to the results found in the association analysis for haplotype group A (figure 7).

Example 2. Allelic variation for StPho2 associated with phosphate content

QTL analysis for phosphate content of tuber starch in the CxE population (a diploid potato mapping population; Jacobs et al. 1995) resulted, besides a QTL at the position of GWD on chromosome 5, in a second QTL on chromosome 9. A candidate gene on this position is starch phosphorylase 2 (StPho2). Allelic variation for this gene in the CxE population was assessed. It was shown that the E-specific allele (allele 2) was associated with a higher level of phosphate content in tuber starch, see Figure 8.

Next, a set of 115 tetraploid cultivars was analysed for presence of this allele of StPho2. Allele 2 was found to be present in 17 cultivars, mostly in simplex. These are Agria, Arcade, Arnova, Belle de Fontenay, Bintje, Clivia, Donald, Draga (1970), Duke of York, Eigenheimer, Escort, Flava, Hansa, Jaune d'Or, Marlen, Triplo and Quarta. These cultivars can be used in crosses, to obtain progeny homozygous for StPho2 allele 2. Such progeny is expected to have an increased starch phosphate content compared with siblings lacking StPho2 allele 2, also depending on their GWD allele composition.

StPho2 allele 2 can be distinguished from other StPho2 alleles by sequencing the PCR product obtained with primers Pho2-1F (5'-GAAGATGGAAAGGGTTCTCA-3') and Pho2-1R (5'-TTAGCCATATGCACAACAGG-3'). The nucleotide at position 278 (counting from the first nucleotide of primer Pho2-1F) is A for allele 2, while it is G for all other alleles (SNP G278A). Furthermore, in PCR product Pho2-7F (5'-ACTTGGGTGGGATGAATCTT-3') + Pho2-7R (5'-TAGCAAGCAGTTCGAGGTTC-3') allele 2 contains a sequence of $(T)_9$ starting from position 660 counting from the Pho2-7F primer, whereas all other alleles have a sequence of $(T)_8$, or TATTTTTT.

Example 3. Marker assisted selection for GWD alleles

Plant material

Crosses were made between Astarte (GWD alleles AACC) and Voran (GWD alleles AACC. Using High Resolution Melting analysis with the LightScanner (Idaho Technologies, US) a selection of five classes of genotypes was made being the genotypes: AAAA, AAAC, AACC, ACCCC and CCCC. Of each class of genotypes, 10 different clones were selected and used to perform field trials. Starch of the clones of the five diferent GWD genotypes (amylose-freestarch) were analysed for starch phosphate content.

Similarly, the variety Sunrise (GWD alleles BBHH) was selfed. Using High Resolution Melting analysis with the LightScanner (Idaho Technologies, US) a selection of five classes of genotypes was made being the genotypes BBBB, BBBH, BBHH, BHHH and HHHH. Of each class of genotypes, 10 different clones were selected and used to perform field trials. Starch of the clones of the five different GWD genotypes (amylose-freestarch) were analysed for starch phosphate content.

A cross is made between Astarte (AACC) and Voran (AACC). The obtained offspring is selected for its GWD allele dosage by molecular SNP detection tools. Genotypes with

nulliplex, simplex, duplex, triplex or quadruplex of the GWD allele A or C are used to perform a field trial.

Table 6.

Plant genotype	chance
AAAA	1/36
AAAC	8/36
AACC	18/36
ACCC	8/36
CCCC	1/36

Starch of the genotypes with the GWD alllele in different dosages is analysed for starch phosphate content. It shows that with decreasing dosage of allele A and with increasing dosage of allele C starch phosphate content increases.

Example 4 High phoshate amylopectin starch potato

For various reasons, starch producers prefer high phosphate starch potatoes with different ratios of amylopectin and amylose. An earlier induced gene mutation in potatoes that affects the synthesis of the enzyme granule bound starch synthase (GBSS), and the subsequent molecular cloning of this gene (Hovenkamp-Hermelink et al., 1987, Theor. Appl. Genet. 75:217-221; Visser et al., 1989, Plant Science 64:185-192) has opened possibilities for altering the starch composition of potatoes – either through established breeding methods or through modern techniques of genetic manipulation.

The GBSS mutation in potato is similar to the so-called waxy (*wx)* mutation in maize and prevents the production of amylose, when expression or specific function of the GBSS protein is absent. Therefore, this mutation has been designated as amylose-free (*amf*) mutant of potato. Herein, the *amf*-gene mutation (allele a) stands for a modification of the GBSS-gene that leads to a complete functional loss of GBSS-activity, notwithstanding that GBSS-like gene products, without the specific activity, may still be expressed from the gene's transcripts in question, whereby the *Amf*-gene (allele A) stands for a gene from which gene products with GBSS-activity can still be obtained. The *amf*-gene character is determined by a monogenic

mendelian recessive gene, the phenotype of which can be detected in various plant parts such as columella cells of root tips, tubers, plastids in the stomatal guard cells and in microspores (Jacobsen et al., 1989, Euphytica 44:43-48). When these parts are stained with a potassium iodine solution (Lugol), starch is stained red in mutants and dark blue in the wild type.

Unlike many other phenotypic genetic markers, the mutated or functionally deleted GBBS- or *amf*-gene offers certain special advantages for genetic analysis as well as for breeding. For example, the progeny can be classified at a very early seedling stage as well as in adult plants, through pollen staining, homo- and heterozygotes can be unambiguously classified: the dosages 2-4 of the mutant allele in a polyploid can be easily detected through the ratios 5:1, 1:1 and 0:1 in stained pollen samples; different types of 2n-gametes in diploid clones can be detected and their influence on the phenotype and genotype of polyploid from 4x*2x crosses can be predicted. Besides, through molecular tools such as Minor Groove Binding MGB-Taqman assay, the SNP resulting in the one basepair deletion in the open reading frame of the GBSS amf allele can be detected in a qualitative and quantitative manner (van der Leij et al., Molec. Gen. Genetics (1991) 228: 240-248).

Prospects of using the material in conventional as well as in analytic breeding of potato have since the generation of the *amf*-gene potato mutant of Hovenkamp-Hermelink been opened. A disadvantage for breeding is the recessive nature of *amf*, which complicates the combination of this character with other agronomic traits at the tetraploid level.

Therefore, the analytic breeding method advocated by Chase (1963, J. Genet. Cytol. 5:359-364), which involves breeding of potato at the diploid level and returning to the tetraploid condition through the use of 2n-gametes, could be of considerable value for breeding *amf*-varieties. The aim of such investigations are at least two fold: a. to combine *amfamf* and *Amfamf* genotypes with that of 2n-gamete formation, and b. to create fertile, nulliplex clones as basic material for breeding amylose-free potatoes.

Using an amylose-free (amf)mutant of diploid potato (*Solanum tuberosum)*, diploid and tetraploid clones with different genotypes at the *amf*-locus and with different genotypes at the GWD and STPho2 locus were produced. In order to make use of the diploid material in analytic breeding of *amf*-potatoes, clones were selected that produced a considerable frequency of 2n-pollen and 2n-eggs. Successful attempts were made to select normal synaptic as well as desynaptic clones producing 2n-gametes. When for example microspores are stained with a potassium iodide solution (Lugol), starch is stained red in mutants (comprising only the *amf*-gene) and dark blue in the wild type (comprising only the A*mf*-gene). Based on

the phenotype of starch in the microspores or by molecular SNP analysis, tetraploid clones with nulliplex, simplex, duplex, triplex and quadriplex genotypes at the *Amf*-locus were selected. Similarly, by molecular SNP analysis, tetraploid clones with nulliplex, simplex, duplex, triplex and quadriplex genotypes at the GWD or StPho2 locus were selected in the nulliplex, simplex, duplex, triplex and quadriplex genotypes at the *Amf*-locus. We investigated amylose and starch-bound phosphate content in various parts of the mutant potato plant.

Plant material. Monoploid amylose-free (amf) clone 86.040 and the parent clone AM79.7322 are described in Hovenkamp-Hermelink et al. (1987, *ibid*). Doubled amf-plants were obtained by adventitious shoot regeneration on leaf explants, which were taken from *in vitro* propagated shoots of monoploid 86.040. After root induction in MS_{30} (Murashige & Skoog, 1962, Phyisol. Plant 15:473-497) (MS) medium supplemented with 30 g/l sucrose) a number of these diploid amf-plants were transferred into a glasshouse, at 19°C at day: 17°C at night and 16 h day length, in sterilized leaf containing soil. For better flowering, part of the doubled plants was grafted onto tomato rootstock. Pollen fertility was estimated, after aceto-carmine staining. For the crosses, a variety of wild-type potato pollen was used. The crosses were made on open flowers of diploid (2x) clones of 86.040.

Starch analysis. Starch granules in micropores and tubers were stained with I_2-KI solution according to Hovenkamp-Hermelink et al. (1987), in stomatal guard cells and other leaf cells according to the treatment described for, microspores and in root cap cells by treatment of root tips with a mixture of Lugols-solution and choralhydrate (1:1, v/v). Four gram of choralhydrate is dissolved in 2 ml of water. The amylose percentage in starch solutions of tubers was measured according to Hovenkamp-Hermelink et al., 1988, Potato Res. 31:241-246). Roottips were fixed and stained according to Pijnacker and Ferwerda (1985, Can. J. Genet. Cytol. 26:415-419) for chromosome counts and karyotypic investigations. When for example microspores are stained with a potassium iodide solution (Lugol), starch is stained red in mutants (comprising only the *amf*-gene) and dark blue in the wild type (comprising only the A*mf*-gene) (Jacobsen et al., 1989, Euphytica 44:43-48).

Identification of GWD ***allele C and H*** *in combination with the amf-gene mutants*

Based on iodine staining of microspores or molecular SNP assays based on the amf mutation, genotypes corresponding tot nulliplex (no wild-type GBSS-allele), simplex, duplex and triplex/quadruplex for the wild-type GBSS allele were selected. On the other hand, genotypes corresponding tot nulliplex (no wild-type GBSS-allele), simplex, duplex and triplex/quadruplex for **GWD allele C, or H** were selectedThis selection was based on High Resolution Melting analysis using the LightScanner (Idaho Technologies, US).

A cross is made between amfamfamfaamfgggg and AmfAmfAmfAmfGGGG, whereby G =GWD allel C or H, and g=GWD allel a, b, d, e, f, g. The offspring are duplex for AmfAmfamfamfGGgg. The obtained offspring when duplex plants (AmfAmfamfamfGGgg x AmfAmfamfamfGGgg) are crossed is shown in Table 7. The allele dosage of the amf/Amf genotypes can be distinguished after iodine staining by their segregation of blue and red microspores or by molecular SNP detection tools. The allele dosage of the GWD genotypes can be distinguished by molecular SNP detection tools. Amylose-free genotypes (amfamfamfamf) with nulliplex, simplex, duplex, triplex or quadruplex of the GWD allele G)allele C or H) are used to perform a field trial.

Table 7.

Plant genotype	chance	plant genotype	chance
Amfamfamfamf	1/36	amfamfamfamfgggg	1/1296
Amfamfamfamf	8/36	amfamfamfamfGggg	8/1286
AmfAmfamfamf	18/36	amfamfamfamfGGgg	18/1296
AmfAmfAmfamf	8/36	amfamfamfamfGGGg	8/1296
AmfAmfAmfAmf	1/36	amfamfamfamfGGGG	1/1296

Starch of the aaaa genotype (amylose-freestarch) with the GWD alllele in different dosages are analysed for starch phosphate content. It appears that with increasing dosage of GWD allele C or H starch content increases.

A cross was made between amfamfamfamfSSSS (amf = mutant Amf allele – defective GBSS allele and S = StPho2 allele 1) and AmfAmfAmfAmfssss (Amf = Amf allele – functional

GBSS allele and s = StPho2 allele 2). The offspring are duplex for AmfAmfamfamfSSss. The obtained offspring when duplex plants (AmfAmfamfamfSSss x AmfAmfamfamfSSss) are crossed is shown in Table 8. The allele dosage of the amf/Amf genotypes can be distinguished after iodine staining by their segregation of blue and red microspores or by molecular SNP detection tools. The allele dosage of the StPho2 allele 2 genotypes can be distinguished by molecular SNP detection tools. Amylose-free genotypes (aaaa) with nulliplex, simplex, duplex, triplex or quadruplex of the StPho2 allele 1 are used to perform a field trial.

Table 8

Plant genotype	chance	plant genotype	chance
amfamfamfamf	1/36	amfamfamfamfssss	1/1296
Amfamfamfamf	8/36	amfamfamfamfSsss	8/1296
AmfAmfamfamf	18/36	amfamfamfamfSSss	18/1296
AmfAmfAmfamf	8/36	amfamfamfamfSSSs	8/1296
AmfAmfAmfAmf	1/36	amfamfamfamfSSSS	1/1296

Starch of the aaaa genotype (amylose-freestarch) with the StPho2 allele 1 in different dosages is analysed for starch phosphate content. It appears that with increasing dosage of StPho2 allele 1 starch content increases.

Example 5 Examples of high phosphate starch applications

Example 5a – As anionic starch in wet-end application

A testpulp is prepared from birchsulphate (freeness 33°SR) of which the water hardness is adjusted to 40°GH with $CaCl_2.2H_20$ and the conductivity to 3000 µS/cm with Na_2SO_4. The consistency is 1%. To the pulp is added a solution of the starch (dosage 3%). As fixatives PaperPac N (PPN) (dosage 1%) and Luredur VI (poly-vinylamine MP1256) (varying dosage) are used. The contacttimes in the adsorptiontests are: starch 60 sec; PPN 40 sec and the Luredur VI 20 sec.

The pulp is subsequently filtered, and the starch content in the filtrate is determined. The adsorption is calculated as ((starch added – starch in filtrate) / starch added) * 100%

Starches used are unmodified waxy potato starch with normal and elevated phosphate contents as indicated in the Table 9.

starch	P content (mmol/mol AHG)	Luredur VI (%)	adsorption (%)
Waxy potato starch Normal P content	4.3	0	61.7
		0.5	68.2
		1.0	68.8
		2.0	69.2
Waxy potato starch Elevated P content	11	0	66.4
		0.5	75.3
		1.0	81.2
		2.0	86.9
Waxy potato starch Elevated P content	13	0	61.0
		0.5	71.5
		1.0	82.0
		2.0	90.3

It can be seen from Table 9 that the starches with elevated phosphate content show stronger starch adsorption onto the pulp.

Example 5b – As cationic starch in wet-end application

Cationic, high phosphate, waxy potato starch (or amphoteric starches with net positive charge) were prepared according to the following procedure.

High P waxy potato starch (1 mol AHG, 162 g dry, 196.6 g as is) was suspended in 244.8 g tapwater to obtain a suspension of 36.7% w/w solids. A solution of 1-chloro-2-hydroxypropyltrimethylammonium chloride (65% w/w, 11.86 g, 0.041 mol) was added, and subsequently 82.7 g of 4.4% w/w NaOH solution. The suspension was heated to 45 °C and the reaction continued for 6 h. The mixture was neutralized to pH 5 with 8.2 ml 6 N sulphuric acid, filtered on a Büchner funnel and washed with 1 liter tap water.

Waxy potato starch with normal phosphate content was obtained in a similar manner, using 0.03 mol of cationic reagent in order to obtain a similar charge density as the high phosphate starch.

Cationic regular potato starch used was Amylofax PW from AVEBE.

The test pulp consisted of 80 % birch sulphate cellulose fibres (as in Example 1) and of 20 % Hydrocarb 90. The specific conductivity and the water hardness is artificially increased to a level of 3000 µS/cm (sodium sulphate) and 40°GH (calcium chloride)

The cationic starch dosage is 0.8 %, with and without 0.3 % as is Paper Pac N (PPN).

The addition order is:

time (sec)	0	10	60
addition	Paper Pac N	Cationic starch	stop

After 60 seconds the pulp is filtered using a Büchner funnel.

The zeta-potential is measured and the starch content of the filtrate is determined.

Addition	P content (mmol/mol AHG)	Charge density (µeq/mg)	PPN (%)	Starch adsorption (%)	Zp (mV)
0.8 % cationic regular potato starch (Amylofax™ PW ex AVEBE)	4.2	+ 0.35	0	78,5	-1,4
			0.3	86,7	-0,9
0.8 % cationic waxy potato starch (Product A)	3.8	+ 0.20	0	96,6	-2,7
			0.3	98,3	-3,5
0.8 % cationic waxy potato starch, high P (Product B)	12	+ 0.18	0	98,3	-0,9
			0.3	100,0	-2,1

It is evident from Table 10 that both the amylopectin content and phosphate content contribute to the adsorption efficiency of the cationic starches.

The hand sheet tests are performed with the dynamic hand sheet former (FRET).
The machine settings are:
- stirring speed 1600 rpm
- air: 20 % of 1 Bar during 5 seconds
- vacuum: 420 mm Hg:
- for each hand sheet an amount of test pulp is used equal to a sheet weight of 100 g/m^2
The addition order and contact time is:

time (sec)	0	10	60	70
addition/event	0.3 % Paper Pac N	0.8 % Cationic starch	dilution	drainage

- at 60 seconds the pulp is diluted to 0.25% with water with a similar specific conductivity and water hardness as the test pulp
- at 70 seconds the diluted pulp is drained

Starch		Amylofax PW	Product A	Product B
Zeta-potential ± 0.9	mV	+0.8	-1.2	+1.4
Drainage ± 0.02	sec	0.96	0.96	0.98
Grammage ± 0.8	g/m^2	94.1	96.5	96.3
Porosity	sec/100 ml	45.3 ± 0.4	46.2 ± 0.4	46.2 ± 1.0
Thickness ± 2	µm	146	150	147
Tensile	kN/m	4.6 ± 0.3	4.6 ± 0.2	4.7 ± 0.2
Elongation ± 0.1	%	2.1	2.0	2.1
Scott Bond	J/m^2	426 ± 13	436 ± 6	499 ± 13
Starch content ± 0.03	%	0.50	0.68	0.69
Filler content ± 0.1	%	12.8	14.6	14.0

From Table 11 with hand sheet results it is evident that the waxy potato products show a better adsorption, resulting in a higher starch content. Remarkably, the Scott bond (internal strength) of the paper sheet is much higher for the high phosphate product B, even though the filler content is higher than the tradition caiotnic starch Amylofax PW, and only slightly lower than product A.

Example 5c – As surface sizing starch

Waxy potato starch of normal and high phosphate content were hydrolyzed to viscosity levels normally used for surface sizing applications as follows.

500 g dry starch was charged in a Hobart mixer. In an amount of demiwater, which is sufficient to bring the starch up to 30% moisture, the desired amount of 1 N HCl was dissolved. This solution was added drop wise to the starch under continuous mixing. After addition, mixing was continued for 30 minutes. The mixture was put in a plastic bag and stored in the refrigerator for one night. The mixture was dried using a Retsch drier, operated in the following mode: 2x15 min at 60°C and 1x15 min at 90°C. In between heating runs, the starch was mixed by hand. Finally the pH (1:2) and the moisture content were assessed. Moisture content should be lower than 5%.

The total amount of dried starch mixture (500 g) was charged in a pre-heated dextrination drum. In order to operate the drum at 100°C, it was heated with a heating coil. Fine tuning of the temperature was achieved using a heating lamp. After 30 minutes the product was removed by vacuum and cooled down by spreading onto a sheet of filter paper. After cooling the moisture content and the pH (1:4) were measured. With 0.5 – 1.5 g dry Na_2CO_3 the products were neutralised till pH 6-7 assessed in a 1:4 suspension.

Table 12

Product	P content [mmol/mol]	1 N HCl per 1 kg dry starch [ml]	pH before dextrinization [1:2]	Brookfield LV, 60 rpm 35%, 50° C	Charge density
Perfectamyl A4692	4.0	n.a.		435	-0.32
Product C	3.7	31.5	2.53 (1:3)	275	-0.05
Product D	11	65	2.23	300	-0.14
Product E	13	59	2.46	580	-0.15
Perfectamyl LV	4.0	n.a.		185	-0.32

The products obtained were dissolved in 35% solids using a bath with boiling water and subsequently diluted with tap water to obtain a 9% solution. The solution obtained was applied onto non-sized woodfree base paper using a Dixon coater model 160-B at 50 m/min. The base paper contained 0.8% starch. After sizing, the starch content increased to 3 – 3.5%.

The paper obtained was repulped in tap water at 20 °C using ISO method 5263 (2004) at 30000 revolutions to obtain a pulp suspension of 2% consistency.

The retainability (starch adsorption) tests were performed as in Examples 1 and 2. In each test 150 gram of pulp suspension was used. Paper Pac N (PPN) was added in varying amounts. The contact time of Paper Pac N was 60 seconds, then the pulp was filtered and the starch content of the filtrate was determined.

Table 13

Paper	Starch type	Starch content sized paper	Paper Pac N (% as is)	Starch content of filtrate (mg/kg)
492-03	Perfectamyl™ LV	3.2%	0	430
			2	373
			4	269
			6	204
			8	184
			10	171
492-05	Perfectamyl™ A4692	3.4%	0	445
			2	388
			4	299
			6	139
			8	111
			10	98
492-09	Product C	3.3%	0	459
			2	364
			4	297
			6	274
			8	258
			10	251
492-20	Product D	3.5%	0	480
			2	377
			4	296
			6	209
			8	137
			10	107

It is clear from Table 13 that the high phosphate waxy potato dextrin is just as retainable as the commercial carboxylated benchmarks, even though the charge density is considerably lower. The normal phosphate containing waxy dextrin is not very retainable.

Example 5d – As co-binder in coating colours

A formulation for a single coat was chosen as model coating colour:

- 70 parts fine carbonate (Carbital 90 ex Imerys)
- 30 parts fine clay (Capim DG ex Imerys)
- 5 parts starch
- 8 parts latex (Styronal D517)

The formulation was prepared with final dry solid contents of 64-69% by mixing pigments, starch (as 35% solution as described in Example 5c) and latex under continuous stirring. The pH of the coating formulation was finally adjusted to ca. 9 with 15% NaOH solution. The viscosity at low and high shear was determined by Brookfield (HAT, 100 rpm, 25 °C) and Hi-Shear. High shear viscosity was also determined with the ACAV at shear rates 250.000 – 1.000.000 s^{-1}.

AAGWR water retention was determined as loss of water in g/m^2 at 1,5 bar and in 15 seconds.

From Table 14 it is evident that the waxy potato derivative with elevated phophate level exhibits good thickening power at low shear, good shear thinning behaviour at high shear. Most remarkable is the unexpectedly good water retention. The value in the table is actually the amount of water lost from the formulation under pressure. Product D shows the smallest loss of water, even though the dry solids content is not the highest.

Table 14

Recipe nr.	Perfectamyl™ A4692			Nylgum™ A 55			Product C[1]			Product E[1]		
Temperature °C	25			25			25			25		
pH	8.8			8.8			8.8			8.8		
starch viscosity in 35%	435			580			275			580		
Dry solids %	69.2	67.1	64.9	69.2	66.9	64.9	68.9	67.0	65.2	69.3	66.9	64.9
Brookfield viscosity 100 rpm, mPa.s	2720	1590	1110	3600	2440	1700	1320	900	600	3120	1640	1170
Hi-shear viscosity												
100 rpm, mPa.s	422	257	191	538	363	255	245	183	131	463	290	230
1100 rpm, mPa.s	180	111	83	266	158	111	120	88	67	176	117	90
CAV viscosity, mPa.s												
shear rate [1/s]												
$0.25 * 10^6$		62				52	84				67	
$0.50 * 10^6$		66				51	82				68	
$1.00 * 10^6$		57				45	73				58	
ÅAGWR waterretention 15 sec., 1.5 bar, gr/m^2		33				40	34				24	

1. Products C and E are from Example 5c

Example 6. Effects crossing of GWD alleles on the phosphate content

From the cross of Astarte (AACC) and Voran (AACC) as described in Example 3, the offspring was tested for the presence of the GWD alleles and assayed for the amount of starch bound phosphate; for each of the genotypes the mean phosphate content was calculated. The results are indicated in Table 15.

Similarly, as described in Example 3, the variety Sunrise (BBHH) was selfed. Starch of the clones of the three different genotypes found (amylase-free starch) were analysed for starch phosphate content. The results are indicated in Table 15.

From these results it appears that the presence of an extra allele A or H increrases the phosphate content of the starch, as predicted.

Table 15

	mg P per g starch		mmol P per mol AHG	
	average	stdev	average	stdev
AAAA	625.7	32.6	3.65	0.19
AAAC	780.7	70.0	4.55	0.41
AACC	782.7	59.0	4.56	0.34
ACCC	902.7	2.1	5.26	0.01
BBBB	895.0	159.4	5.22	0.93
BBBH	1047.7	114.5	6.11	0.67
BBHH	1109.0	109.8	6.47	0.64

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Claims

1. A method for producing plants with an increased degree of phosphorylation in starch in a plant, comprising
   a. selecting a plant obtained from an enrichment programme for the presence of the alleles C or H of glucan water dikinase (GWD) , wherein said enrichment programme comprises
      i. selecting at least one parental genotype wherein at least one of alleles, C or H of GWD is present,
      ii. crossing said plant(s),
      iii. selecting progeny from crossing step ii) for a genotype wherein at least one of alleles C or H of GWD is present,
      iv. selfing and/or further crossing said progeny selected in step iii),
      v. selecting progeny resulting from step iv) for a genotype wherein at least one of alleles C or H of GWD is present, and
      vi. optionally repeating steps iv) and v), whereby homozygosy for allele C or H may be obtained,

   wherein said method optionally comprises at least one selection as performed in steps i), iii) or v) performed by marker-assisted selection and wherein said method optionally comprises increasing the ploidy or the use of unreduced gametes of the parental genotype selected at step i) or the progeny of step iii) to tetraploid level before conducting step v); and
   b. growing said plant selected in step a).

2. A method for producing plants with an increased degree of phosphorylation in starch in a plant, comprising:

a) selecting a plant obtained from a programme in which the presence of the allele A of glucan water dikinase (GWD) is decreased, wherein said programme comprises

i) selecting at least one parental genotype wherein allele A of glucan water dikinase (GWD) is absent

ii) crossing said plant(s) with another parental genotype comprising at least one allele A,

iii) selecting progeny from crossing step ii) for a genotype wherein allele A of GWD is absent,

iv) selfing and /or further crossing said progeny selected in step iii),

v) selecting progeny resulting from the crossing in step iv) for a genotype wherein the allele A of GWD is absent,

vi) optionally repeating said steps of selfing and/or crossing and selection of steps iv) and v) to provide a plant having a genotype wherein allele A of GWD is completely absent,

wherein said method optionally comprises at least one selection as performed in steps i), iii) or v) is performed by marker-assisted selection and wherein said method optionally comprises increasing the ploidy or the use of unreduced gametes of the parental genotype selected at step i) or the progeny of step iii) to tetraploid level before conducting step v) and wherein said method preferably is a method wherein the genotype of the plant in step i), ii), iv) or vi) comprises at least one copy of the GWD alleles C and/or H, preferably at least two alleles, and

b) growing said plant selected in step a).

3. The method according to claim 1, wherein the genotype of the plant in step i), iv) or vi) comprises at most two copies of the GWD allele A, preferably at most one copy, more preferably, wherein allele A is absent..

4. A method according to claim 1 or claim 2, wherein the increase in the degree of phosphorylation is caused by an increase in the number of alleles C and/or H of GWD and a simultaneous decrease in the number of alleles A of GWD.

5. Method for producing plants with an increased degree of phosphorylation in starch in a plant, comprising:

a) selecting a plant obtained from an introgression of the GWD allele C and/or H in the genome of a starch producing plant by selecting for a plant wherein allele A of GWD is not present and/or selecting for a plant wherein multiple GWD alleles are present selected from the group consisting of C and H, wherein said method optionally comprises a marker assisted selection step; and

b) growing said plant selected in step a)

6. A method for selecting a plant or part thereof, including a seed and tuber, said method comprising:

a) testing a plant or part thereof for the absence of at least one marker that is indicative for allele A of GWD, and/or testing a plant or part thereof for the presence of at least one marker that is indicative for at least one of GWD alleles selected from the group C, and H; and
b) selecting said plant or part thereof based on the information derived from said testing.

7. The method according to any one of claims 1 to 6, wherein the presence of said marker is determined at DNA level, preferably wherein said marker is selected from the group consisting of the single nucleotide polymorphisms (SNP) as indicated in Table 1.

8. The method according to any one of claims 1 to 7, wherein said plant is tetraploid, preferably wherein said plant is a potato plant, more preferably wherein said potato plant is a *S. tuberosum* Group Tuberosum cultivar.

9. A method for obtaining a transgenic plant with a higher degree of phosphorylation in starch than the corresponding wild-type plant, comprising introducing a GWD gene, more preferably a GWD gene comprising one or more of the alleles C and/or H of GWD into said plant, preferably wherein an allele C or H of GWD is introduced by site-directed mutagenesis, preferably by homologous recombination, more preferably by homologous recombination with an allele A of GWD.

10. Use of a marker for selecting a plant or part thereof, including a seed and tuber, with a genotype wherein the allele A of GWD is absent or wherein at least one of the alleles C and H of GWD are present, wherein said marker is a SNP selected from the SNPs listed in Table 1, or a marker in linkage disequilibrium therewith.

11. Use of a method according to claim 6 for marker-assisted breeding of a plant.

12. The use according to any one of claims 10 and 11, wherein said plant is tetraploid, preferably wherein said plant is a potato plant, more preferably wherein said potato plant is a *S. tuberosum* Group Tuberosum cultivar.

Fig. 1

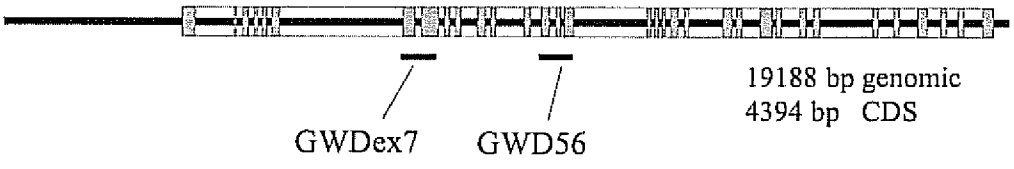


Fig. 2

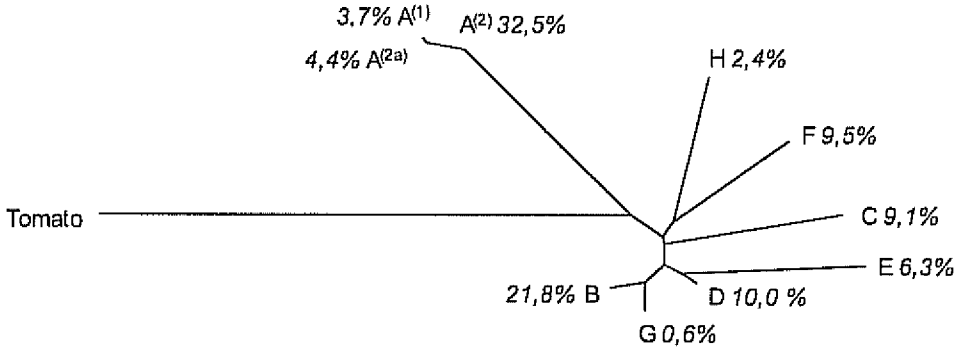


Fig. 3

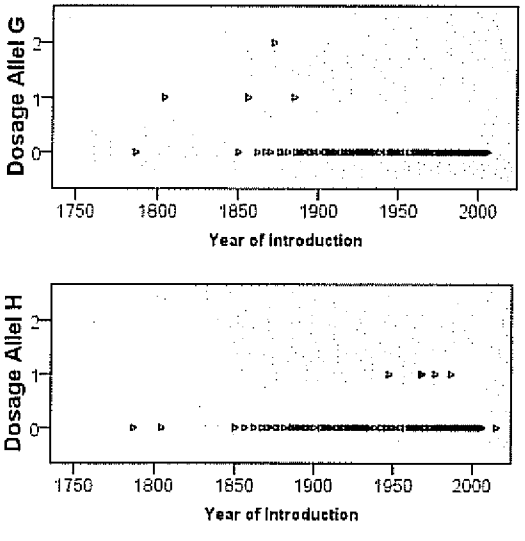


Fig. 4

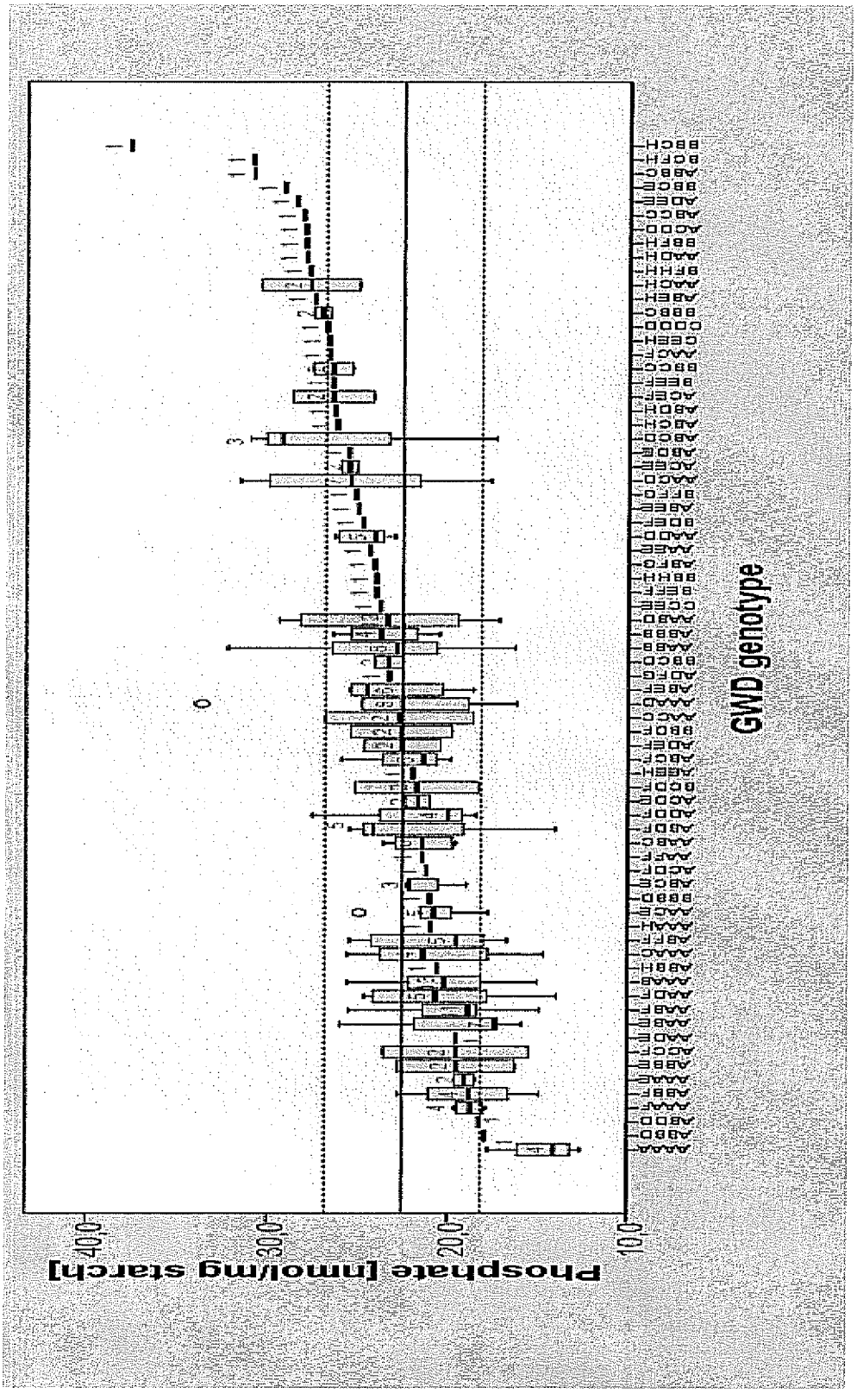
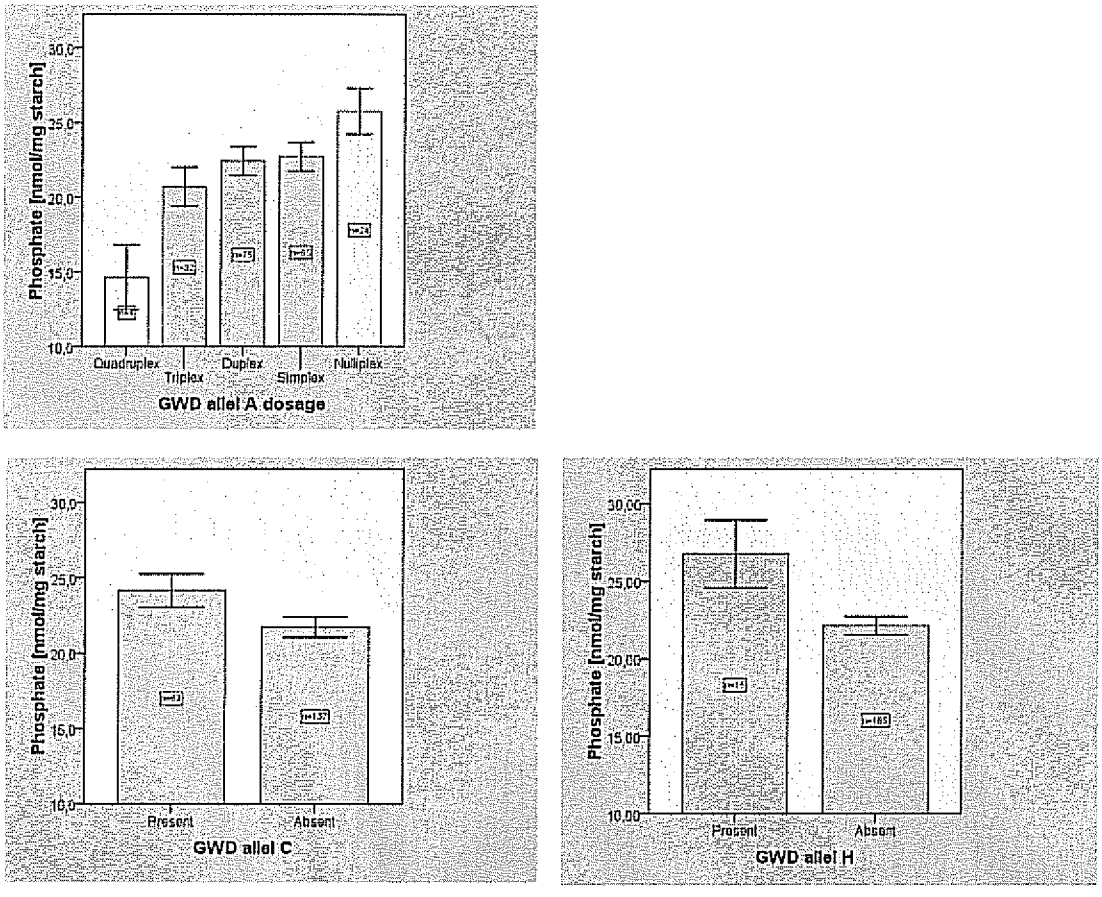


Fig. 5


Phosphate [nmol/mg starch]
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25,0
20,0
15,0
10,0
Quadruplex
Triplex
Duplex
Simplex
Nulliplex
GWD allel A dosage
Present
Absent
GWD allel C
GWD allel H

Fig. 6

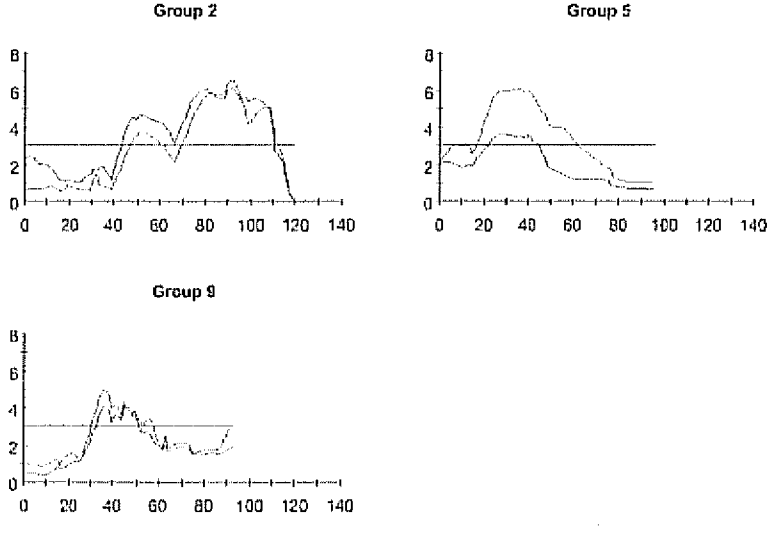
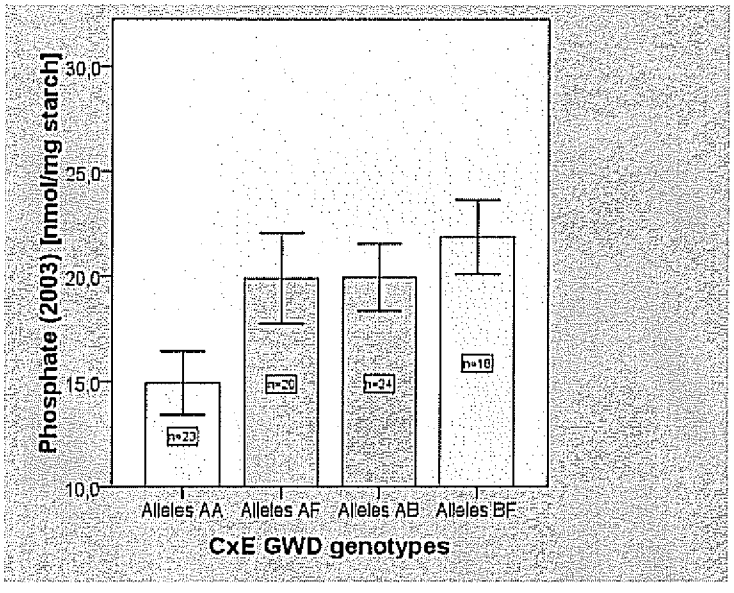

Group 2
Group 5
Group 9
0
2
4
6
8
0
20
40
60
80
100
120
140

Fig. 7


Phosphate (2003) [nmol/mg starch]
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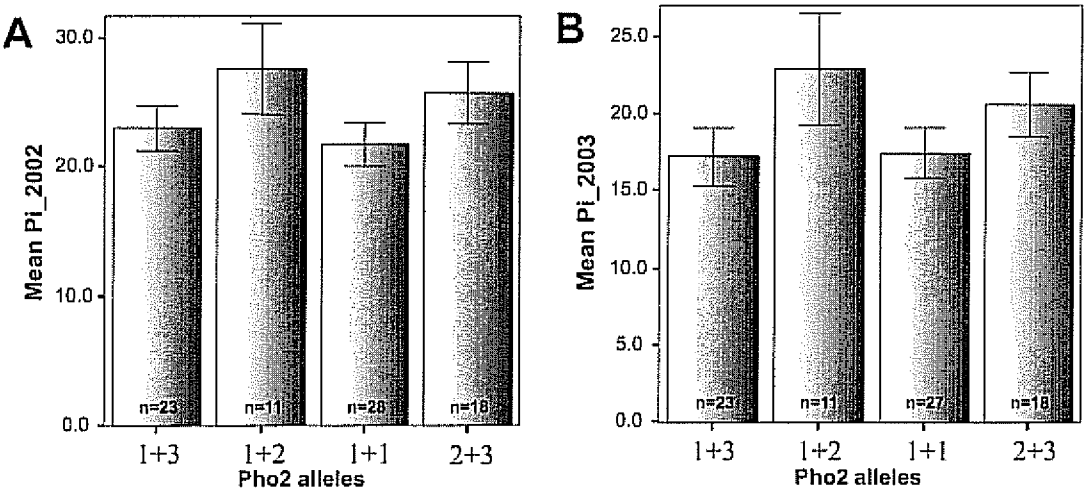
25,0
20,0
15,0
10,0
n=23
n=20
n=24
n=18
Alleles AA
Alleles AF
Alleles AB
Alleles BF
CxE GWD genotypes

Fig. 8


A
Mean Pi_2002
30.0
20.0
10.0
0.0
n=23
n=11
n=28
n=18
1+3
1+2
1+1
2+3
Pho2 alleles
B
Mean Pi_2003
25.0
20.0
15.0
10.0
5.0
0.0
n=23
n=11
n=27
n=18
1+3
1+2
1+1
2+3
Pho2 alleles

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2011/050460

A. CLASSIFICATION OF SUBJECT MATTER
INV. A01H1/04 A01H5/00 C12N15/82
ADD.

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)
A01H C12N

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, BIOSIS, Sequence Search, EMBASE, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	Anonymous: "Books of abstracts", EAPR - EUCARPIA CONGRESS, Wageningen, the Netherlands , 27 June 2010 (2010-06-27), 30 June 2010 (2010-06-30), pages 1-83, XP002659863, Retrieved from the Internet: URL:http://www.eucarpia.org/03publications /EAPR_EUCARPIA2010_abstracts.pdf [retrieved on 2011-09-23] page 43 ----- -/--	1-12

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 September 2011	26/10/2011

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, Fax: (+31-70) 340-3016	Keller, Yves

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2011/050460

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	M. FRISCH: "Selection Theory for Marker-Assisted Backcrossing", GENETICS, vol. 170, no. 2, 1 January 2005 (2005-01-01), pages 909-917, XP055007826, ISSN: 0016-6731, DOI: 10.1534/genetics.104.035451 abstract	1-12
Y	NODA ET AL: "Starch phosphorus content in potato (Solanum tuberosum L.) cultivars and its effect on other starch properties", CARBOHYDRATE POLYMERS, vol. 68, no. 4, 16 March 2007 (2007-03-16) , pages 793-796, XP005938838, APPLIED SCIENCE PUBLISHERS, LTD. BARKING, GB ISSN: 0144-8617, DOI: 10.1016/J.CARBPOL.2006.08.005 abstract figures 1, 2 table 1	1-12
A	SCHWALL G P ET AL: "Production of very-high-amylose potato starch by inhibition of SBE A and B", NATURE BIOTECHNOLOGY, vol. 18, no. 5, 1 May 2000 (2000-05-01), pages 551-554, XP002239695, NATURE PUBLISHING GROUP, NEW YORK, NY, US ISSN: 1087-0156, DOI: 10.1038/75427 abstract	1-12
A	MUHRBECK P ET AL: "DETERMINATION OF THE PHOSPHORYLATION OF STARCH FROM NATIVE POTATO VARIETIES BY PHOSPHORUS-31 NMR", STARCH, vol. 43, no. 1, 1991, pages 25-27, XP002601733, ISSN: 0038-9056 abstract; figures 1, 2 table 1	1-12

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INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2011/050460

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WICKRAMASINGHE H A M ET AL: "Physico-chemical and degradative properties of in-planta re-structured potato starch", CARBOHYDRATE POLYMERS, vol. 77, no. 1, 22 May 2009 (2009-05-22), pages 118-124, XP026019571, APPLIED SCIENCE PUBLISHERS, LTD. BARKING, GB ISSN: 0144-8617, DOI: 10.1016/J.CARBPOL.2008.12.013 [retrieved on 2009-04-21] page 118 - column 2	1-12
Y	WO 2009/000557 A1 (BAYER CROPSCIENCE AG [DE]; FROHBERG CLAUS [DE]; LA COGNATA URSULA [DE]) 31 December 2008 (2008-12-31) example all	1-12

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/NL2011/050460

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009000557 A1	31-12-2008	AR 067143 A1	30-09-2009
		CA 2690489 A1	31-12-2008
		EP 2158320 A1	03-03-2010
		US 2010189870 A1	29-07-2010