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(54) **PLANT DE POMMES DE TERRE PRODUISANT DE L'AMIDON
ESSENTIELLEMENT EXEMPT D'AMYLOSE**

(54) **POTATO PLANT PRODUCING ESSENTIALLY AMYLOSE-
FREE STARCH**

(57) A potato plant which has a genome containing, as a result of genetic engineering, at least one gene construct containing a potato granule-bound starch synthase (PGBSS) cDNA or genomic DNA sequence in reverse or functional orientation in an expression cassette which is functional in potato plants, said gene construct giving rise to tubers containing essentially amylose-free starch. In one embodiment, said gene construct contains a PGBSS cDNA sequence in reverse orientation which results in the production of PGBSS antisense RNA. In another embodiment, said gene construct contains a PGBSS genomic DNA sequence in functional orientation which results in co-suppression of PGBSS enzyme activity.

ABSTRACT

Title: Potato plant producing essentially amylose-free starch

A potato plant which has a genome containing, as a result of genetic engineering, at least one gene construct containing a potato granule-bound starch synthase (PGBSS) cDNA or genomic DNA sequence in reverse or functional orientation in an expression cassette which is functional in potato plants, said gene construct giving rise to tubers containing essentially amylose-free starch. In one embodiment, said gene construct contains a PGBSS cDNA sequence in reverse orientation which results in the production of PGBSS antisense RNA. In another embodiment, said gene construct contains a PGBSS genomic DNA sequence in functional orientation which results in co-suppression of PGBSS enzyme activity.

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Title: Potato plant producing essentially amylose-free starch

Field of the invention

The invention is in the field of genetic engineering by recombinant DNA technology, more particularly the genetic engineering of potato plants in order to change the starch
5 composition in the tubers towards essentially amylose-free starch.

Background of the invention

Starch is the major storage carbohydrate in potato and
10 consists of two components, a linear $(1\rightarrow4)\alpha$ -D-glucan polymer and a branched $(1\rightarrow4)(1\rightarrow6)\alpha$ -D-glucan called amylose and amylopectin, respectively. Amylose has a helical conformation with a molecular weight of 10^4 - 10^5 . Amylopectin consists of short chains of α -D-glucopyranose units primarily linked by $(1\rightarrow4)\alpha$ bonds with
15 $(1\rightarrow6)\alpha$ branches and with a molecular weight up to 10^7 . In plants starch is found in two types of plastids: chloroplasts and amyloplasts. In both types of organelles the starch occurs as granules. In chloroplasts so-called transitory starch is accumulated for only a short period of time, whereas starch in
20 amyloplasts is accumulated for long term storage and hence is named reserve starch. Generally, amylose makes up 11%-37% of the total reserve starch and variation in the amylose content is not only found among different plant species, but also among different cultivars of the same species. In potato the amylose
25 content in the tuber varies from 18% to 23%. Furthermore, in a

number of plant species mutants are known with a starch composition which deviate significantly from the above mentioned percentages.

Transitory and reserve starch are generally considered to
5 be synthesized by the same enzymes. Starch metabolism in leaves follows a diurnal rhythm: synthesis and accumulation occur during the light period while hydrolysis occurs during the night. In storage tissue, starch synthesis occurs during a specific phase of tissue development; the synthesis being the
10 predominant function of amyloplasts. The amount of amylose found in storage tissue of potato is about twice as high as that in leaves.

Sucrose is considered to be the major substrate for starch biosynthesis which involves the following steps: initiation,
15 elongation, branching and granule formation. In the pathway of conversion of sucrose into amylose and amylopectin at least 13 enzymes play a role. Three groups of enzymes are directly involved in the formation of starch. These enzymes are phosphorylase, starch synthases and branching enzymes.
20 Phosphorylase is active in starch breakdown, branching enzyme converts amylose into amylopectin by the breakage of (1→4)α-bonds and the synthesis of (1→6)α-bonds. Starch synthases are responsible for the synthesis of starch by the addition of ADP (UDP) glucose subunits to the non-reducing end of an (1→4)α-D-
25 glucan polymer. Starch synthase has been identified in two forms: one form is soluble while the other is tightly associated with starch granules. The soluble enzyme uses only ADP-glucose as the D-glucosyl donor, whereas the granule bound starch

synthase (GBSS) utilizes ADP-glucose and UDP-glucose.

Solubilization of the GBSS protein from starch granules of various plants has been reported. Although in maize there are thought to be at least two forms of GBSS, potato seems to have
5 only one form. The presence and activities of the different starch synthases are important to starch biosynthesis not only because they have an effect on the amylose/amylopectin ratio in starch, but also because they can have a large impact on total starch content. In general, it appears that complete suppression
10 of the enzymes producing amylose can be achieved with almost no change in the total amount of starch laid down, whereas suppression of the enzyme system producing amylopectin leads to a marked decrease of the amount of total starch.

Starch as such or in modified form is widely used in the
15 food, paper and textile industries. With the depletion of natural oil resources starch could also become a substitute for oil as a raw material for the chemical industry. Therefore, it could become of major interest to produce starch which meets special requirements for certain applications. Although special
20 forms of starch are already available from mutants of maize and rice and starches from other sources might have certain advantages, genetical engineering could be an option in order to obtain tailor-made starches in plants in which (recessive) mutants are not easily obtained. Selection of mutants is
25 especially difficult in vegetatively propagated crops which are mainly crosspollinators and/or polyploids, such as the potato.

Although recently in a laborious isolation procedure a mutant of potato (amf) was isolated which, in analogy to the wx

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mutants in maize, lacks GBSS protein, GBSS activity and amylose (Hovenkamp-Hermelink et al. 1987), the breeding of such a mutant into a cultivar will take another number of years. One cause for the long duration of the procedure is the fact that a haploid clone had to be used for the isolation of the recessive mutant. To circumvent problems of isolating recessively inherited mutants in a polyploid crop like potato and to speed up the introduction of such a mutant character in potato varieties, the antisense approach would be a very important alternative, because an antisense gene would act as a dominant suppressor gene. The great advantage is that eventually it will become possible to mimick such a mutant phenotype directly in a tetraploid variety. With the availability of GBSS sequences, both from maize (Shure et al 1983) and potato (Hergersberg 1988; Visser et al 1989d) and an efficient transformation system for potato (Visser et al 1989a, 1989b) this approach could be tested.

It has been shown that antisense RNA transcripts can be used to mimic mutations in pro- and eukaryotes (for review see van der Krol et al. 1989). Antisense RNA was originally found as a naturally occurring mechanism used to control gene expression in bacteria (Tomizawa et al. 1981; Mizuno et al. 1984). Izant and Weintraub (1984, 1985) proposed that antisense RNA could be used to inhibit the expression of eukaryotic genes. By inhibiting the expression of specific target RNAs, this approach has led to the generation of mutant phenotypes in a number of different eukaryotic systems. In plants the use of antisense RNA proved to be successful in effectively inhibiting the activity of nopaline

synthase (Rothstein et al. 1987; Sandler et al. 1988),
chloramphenicol acetyltransferase (Ecker and Davis 1986; Delauney
et al. 1988), chalcone synthase (van der Krol et al. 1988),
polygalacturonase (Smith et al. 1988; Sheehy et al. 1988),
5 phosphotricin acetyl transferase (Cornelissen and Van de Wiele
1989) and β -glucuronidase (Robert et al. 1989).

Visser (1989) tested whether the antisense approach could be
used to inhibit the expression of the gene for granule-bound
starch synthase in potato using heterologous antisense
10 constructs, i.e. an antisense gene constructed from a maize
genomic GBSS gene.

The antisense gene was fused between the 35S cauliflower
mosaic virus promoter and the nopaline synthase terminator in the
binary vector pROK-1, which also carries a plant selectable
15 kanamycin resistance gene. Since it was known from the amf-mutant
that the mutation is expressed in all tissues in which starch is
formed, including columella cells of the root cap, it was
expected that also antisense effects would be visible in roots.
The presence or absence of amylose could be easily detected
20 because amylose forms a blue staining complex with the iodine
present in Lugol's solution (I-KI). Starch without amylose, i.e.
only containing amylopectine, forms a reddish-brown staining
complex with iodine. In order to efficiently test the introduced
antisense gene in potato for a biological effect a transformation
25 system was developed in which the binary antisense vector was
incorporated into Agrobacterium rhizogenes. The binary vector was
present next to the wildtype Ri-plasmid of A. rhizogenes which is
responsible for the formation of so-called hairy roots on plant

explants. Agrobacterium rhizogenes was used instead of Agrobacterium tumefaciens because it is possible to screen for an effect of the introduced constructs already after 10 days by staining hairy roots with Lugol's solution and because plants can be easily regenerated from hairy roots. In this way heterologous (maize) binary antisense GBSS plasmids were transferred by A. rhizogenes to stem segments from potato.

Both in untransformed or otherwise transformed wildtype roottips never anything else than blue staining roottips were present. Hairy roots obtained after transformation with A. rhizogenes carrying heterologous binary antisense GBSS plasmids were analyzed for the presence or absence of amylose in their starch by staining the roottips with Lugol's solution. The majority of the roots stained blue as wildtype untransformed roots did. However, some roots (1-15% of the stained roots) had a colour pattern different from that of wildtype roots in that the central cells of the root cap were blue and the cells towards the outside of the rootcap were red. These intermediate colouring roots were indications that the inserted antisense genes had some effect on the amylose content. Root clones were established and subcultured and roottips were investigated every fortnight during six weeks of culture. The results of these experiments showed that instability of colour patterns occurred at a rather high frequency. The instability of the effect in columella cells was the reason to regenerate plants from kanamycin resistant hairy roots irrespective of their colour.

On plants, regenerated from kanamycin resistant hairy roots, microtubers as well as soil grown tubers were induced. Analysis

of these tubers showed that none of them had red or intermediate staining starch. All tubers showed blue staining (= amylose containing) starch. Starch isolated from these tubers was analyzed for the presence of GBSS protein and GBSS activity and for the presence of amylose. In all tubers tested GBSS protein was, seemingly unaltered, present. However, GBSS activity in particular and to a much lesser degree amylose content were affected in starch preparations from a number of transformed plants. As shown in fig. 2A, the untransformed wildtype (PD007) and a pBI121 transformed wildtype (Ri-007) had similar GBSS activities, while the amf-mutant had no detectable GBSS activity. GBSS activity was inhibited significantly in the antisense GBSS transformants down to only 10% of that found in wildtype plants. Total inhibition of GBSS activity was not obtained in any of the transformants analyzed. The amylose content measurements gave a different picture. Although in almost all cases there was a somewhat lower amylose content, the difference was significant in only two cases (R-196 and R-227, Figure 2B). The maximum reduction of the amylose content was found in transformant R-196, which also had the lowest GBSS-activity; a reduction down to 78% of the wildtype amylose content. Molecular analyses of the antisense transformants revealed that the number of integrated antisense copies was 1 to 4, but only those plants which contained three or more copies of the antisense GBSS construct showed a pronounced effect on GBSS activity. It is evident from these observations that the effect of a lower GBSS activity on the amylose/amylopectin ratio is not straightforward.

The results described resemble very closely the situation obtained in tomato when using antisense poly-galacturonase genes. A reduction of 90% of the polygalacturonase activity does not have a great effect on the lycopene content (Sheehy et al. 1988, Smith et al. 1988).

The above results were not too encouraging, but it was nevertheless decided to expand the investigations to homologous constructs derived from a full-length potato GBSS cDNA.

Surprisingly, it was found that it is possible to inhibit the expression of granule-bound starch synthase (GBSS) in potato, and thus affect the amylose content of potato tuber starch, by stably introducing homologous antisense constructs. The results described show that it is possible using the antisense approach to interfere with enzymes in biosynthetic pathways such as starch biosynthesis. In using this technique loss of function mutations, such as the amf mutation, which are principally inherited recessively can be mimicked, because antisense genes act as dominant (hemizygous) genes suppressing translation of mRNA.

Surprisingly, it was subsequently found that the effect of essentially amylose-free tuber starch could also be obtained by stably introducing homologous sense constructs, e.g. based on potato GBSS genomic DNA. A phenomenon known as co-suppression appears to occur; it is not yet possible to give an explanation of it.

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Summary of the invention

The invention provides a potato plant which has a genome containing, as a result of genetic engineering, at least one gene construct containing a full length potato granule-bound starch synthase (PGBSS) cDNA sequence in reverse orientation in an expression cassette which is functional in potato plants, said gene construct giving rise to tubers containing essentially amylose-free starch.

In one preferred embodiment, said gene construct contains a PGBSS cDNA sequence in reverse orientation which results in the production of PGBSS antisense RNA.

In another preferred embodiment, said gene construct contains a PGBSS genomic DNA sequence in functional orientation which results in co-suppression of PGBSS enzyme activity.

The invention further provides cells, parts and tubers of said potato plant, and essentially amylose-free starch from it.

Detailed description of the invention

The invention will be illustrated by means of examples which are given for illustrative purposes only and may not be construed as limiting the scope of the invention. For example, the transformation system used in example 1 (Agrobacterium rhizogenes) may be replaced by any suitable alternative, such as the Agrobacterium tumefaciens transformation system (see ex. 2) or the direct gene transfer technique (DGT). Such alternatives are well known to the person skilled in the art. A survey of transformation systems suitable for potato is given in chapter I of Visser (1989).

Similar remarks apply to the choice of the transformation vector (if any), the elements of the expression cassette, the selection markers, etc. For example, the PGBSS promoter may be used to regulate the transcription of the sense or anti-sense
5 PGBSS DNA, instead of the CaMV promoter used in example 1.

The sense or anti-sense PGBSS cDNA or genomic DNA sequence does not have to cover the complete coding sequence but should cover a sufficient part of it to be effective for obtaining tubers containing essentially amylose-free starch. At present, the use
10 of anti-sense PGBSS cDNA is preferred above using anti-sense PGBSS genomic DNA. The gene construct used may contain the PGBSS DNA (preferably genomic DNA) in its functional orientation and yet result in essentially amylose-free tuber starch.

15 Description of the Drawings

Fig. 1 shows the construction of the sense and antisense granule-bound starch synthase (GBSS) vectors. The original GBSS cDNA which contained an internal EcoRI site was subcloned as two fragments in pUC9, denoted pWx 1.1 and pWx 1.3. The 1.3 kb GBSS
20 cDNA fragment from pWx 1.3 was ligated into the partial EcoRI-restricted plasmid pWx 1.1 yielding pGB2. Plasmid pGB2 was restricted with SpeI, made blunt ended with Klenow enzyme, BamHI linked and restricted with BamHI. The GBSS cDNA fragment was ligated into BamHI-restricted pUC18 yielding pGB6 and into
25 BamHI-digested calf intestinal phosphatase (CIP) treated pROK-1 yielding pGB50 (antisense) and pGB60 (sense). Abbreviations: B, BamHI; E, EcoRI; H, HindIII; S, SpeI; LB, RB, left and right T-DNA border repeats; Km^r, kanamycin resistance marker expressed

at bacterial level; NPT-II (neomycin phosphotransferase II gene) kanamycin resistance marker expressed at plant level; P_{CaMV}, 35S cauliflower mosaic virus promoter; T_{Nos}, nopaline synthase terminator.

5 Fig. 2 compares the antisense effects on GBSS activity and amylose content of tuber starches in the case of heterologous constructs (2A, 2B) and homologous constructs (2C, 2D).

2A. GBSS activities of control (PD007, Ri-007 and amf) potato and antisense transformed potato tuber starches.

10 GBSS activity of wildtype PD007: 86.2 pMol ADP glucose min⁻¹ mg starch⁻¹

GBSS activity of mutant amf: 1.3 pMol ADP glucose min⁻¹ mg starch⁻¹.

2B. Apparent amylose content of control potato and anti-
15 sense transformed potato tuber starches

wildtype PD007: 18.4%

mutant amf : 0%

2C. GBSS activity of control (PD007, Ri-007 and amf) potato and class I (WA 501, WA 511), class II (WA 504) and
20 class III (WA 507, WA 514) antisense transformed tuber starches.

wildtype PD007: 66.9 pMol ADP glucose min⁻¹ mg starch⁻¹

mutant amf : 0 pMol ADP glucose min⁻¹ mg starch⁻¹

2D. Apparent amylose content of control and antisense
25 transformed potato tuber starches

wildtype PD007: 20.3%

mutant amf : 0%

EXAMPLE 1

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Materials and methodsPlant materials

In vitro shoot cultures of the Solanum tuberosum clones
5 PD007 (HH 578, $2n=2x=24$), Ri007 (pBI121 transformed PD007,
 $2n=2x=24$; Visser et al. 1989a, b) and the doubled amf mutant
($2n=2x=24$; Hovenkamp-Hermelink et al. 1987) were used. The
shoots were grown at 21°C with a regime of 14 h light (3200 lux)
per day on basal MS medium (Murashige and Skoog 1962)
10 supplemented with 30 g/l sucrose.

Standard methods and reagents

Standard techniques of DNA manipulation were performed as
described by Maniatis et al. (1982). All DNA-mediated
15 transformations were carried out with Escherichia coli strains
JM83 (Vieira and Messing 1982) and MH1 (Casadaban and Cohen
1980). Plasmid isolation was according to Birnboim and Doly
(1979) and inserts were isolated from the restriction enzyme-
digested plasmids using the "freeze-squeeze" method (Tautz and
20 Renz 1983). Plant DNA was isolated according to Dellaporta et
al. (1983). DNA blot hybridizations and radioactive labelling of
the isolated 1.3 kb GBSS cDNA fragment from pGB6 (Fig. 1) were
as described previously (Visser et al. 1989 b,c,d). Enzymes
required for the DNA constructions were from Gibco-BRL and/or
25 Boehringer Mannheim and were used according to the
manufacturers' recommendations.

Construction of vectors

Two subclones encompassing a full-length cDNA clone from potato GBSS isolated from a lambda NM1149 library (Hergersberg 1988; Visser et al. 1989d) were used as indicated in Fig. 1 for the construction of the antisense and sense binary vectors. The antisense (pGB50) and sense (pGB60) vectors obtained after the ligation of the 2.3 kb cDNA in the alkaline phosphatase-treated BamHI site of the binary plant transformation vector pROK-1 (Baulcombe et al. 1986) were introduced into Agrobacterium rhizogenes LBA 1334 (Offringa et al. 1986) as described by Visser et al. (1989a). Verification of the integrity of the plasmids in Agrobacterium was as described previously (Visser et al. 1989a).

15 Iodine staining of hairy roots

Starch granules in root cap cells were stained with a 1:1 (v/v) mixture of Lugol and chloral hydrate as previously described by Jacobsen et al. (1989).

20 Transformation of potato

Inoculum preparation of Agrobacterium cells carrying the antisense or sense constructs, inoculations of stem segments of S. tuberosum PD007 and isolation of binary vector-transformed hairy roots were as described (Visser et al. 1989a). Callus induction on hairy roots, followed by the subsequent regeneration of shoots was as described previously (Visser et al. 1989a). Plants grown in vitro were transferred to the greenhouse to obtain soil-grown tubers.

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Assays for amylose content and GBSS activity

The amylose/amylopectin ratio in starch from potato tubers was determined by the method described previously (Hovenkamp-Hermelink et al. 1988). By using the equation postulated there:
5 $P = (3.5 - 5.1 R) / (10.4 R - 19.9)$ where R is the ratio of the absorbance at 618 nm and 550 nm, the amylose fraction (P) can be easily determined. Since, when P was calculated for artificial mixtures of amylose and amylopectin of known composition, slight deviations were found between the calculated ratio and the
10 actual ratio, a correction factor was used based on the ratio of calculated and actual values of P in these artificial mixtures. Stock solutions of amylose and amylopectin were made as described by Hovenkamp-Hermelink et al. (1988), mixed to obtain starch solutions containing 0% to 40% amylose and diluted with
15 water to obtain final starch concentrations of 6.25 mg/100 ml.

GBSS activity was measured in 50 µl assay buffer containing 1.0-2.0 mg of enzymatically active starch granules using ^{14}C -labelled ADP-glucose as substrate, as described by Vos-Scheperkeuter et al. (1986). Suspensions were incubated for
20 30 min and 90 min at 37°C to get a linear incorporation of ^{14}C -ADP-glucose.

Gel electrophoresis and immunoblotting

Proteins were extracted from starches as described by Vos-Scheperkeuter et al. (1986). Analysis on 10% SDS polyacrylamide
25 gels and subsequent immunoblotting or silver staining of proteins were performed as described by Vos-Scheperkeuter et al. (1986) and Hovenkamp-Hermelink et al. (1987).

Results

Transformation, hairy root isolation and shoot regeneration

Wild-type strains of *A. rhizogenes* and those harbouring the antisense (pGB50) and sense (pGB60) DNA constructs of GBSS (Fig. 1) were used to inoculate stem segments of wild-type *S. tuberosum* PD007. *A. rhizogenes* was used because columella cells of root tips contain starch and because the mutation in GBSS leads to the absence of amylose in columella cells in the *amf* mutant. Since the presence of amylose can be easily monitored by staining hairy roots with iodine (starch staining blue when amylose is present and reddish-brown when amylose is absent), this provides an easy screening method.

Hairy roots, when induced and grown on kanamycin-free medium were obtained in about 10 days after inoculation. Numerous root tips of untransformed PD007 shoots and hairy roots obtained on PD007 stem segments after inoculation with wild-type and pGB60 *A. rhizogenes* strains were tested for their starch composition in columella cells. These preparations always contained blue staining starch when stained with Lugol's solution. Roots inoculated with pGB50 could be classified into three staining groups, i.e. blue, intermediate and reddish-brown. In the intermediate class blue and red staining columella cells were found within the same root tip. Over 50% of the pGB50 inoculated roots showed an altered starch composition, staining either intermediate or red with iodine (Table 1).

To study intra-clone variation 10 root clones, which were established on kanamycin-containing medium, were investigated for starch composition every fortnight during 6-weeks (Table 2).

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It was found that the antisense effect occurred at a high frequency, but mostly in an unstable fashion. Young root clones with red staining amylose-free starch in the columella cells tended to change into intermediate ones and at a later time
5 after inoculation changed again into amylose-containing, homogenously blue staining root clones. Although one red root clone appeared to be stably amylose-free, the majority of the root clones was unstable for this antisense effect. All these observations clearly demonstrated that antisense effects for
10 this character could be obtained in columella cells of root tips of transformed potato, but that suppression of amylose synthesis is unstable in such root tips.

Because of the instability problems it was decided to regenerate plants, irrespective of the antisense effect in their
15 columella cells, from kanamycin-resistant hairy roots containing vector T-DNA in order to investigate these effects in other starch-containing parts of transgenic plants. Hairy roots transformed with pGB50 were isolated from stem segments and cultured on medium containing 50 mg/l kanamycin and 200 mg/l
20 claforan. After two more rounds of subculturing, hairy root clones which still grew on kanamycin-containing medium were considered transformed. Each independently derived hairy root was subcultured separately on MS 30 medium with claforan and kanamycin to increase root mass. Root pieces obtained from 46
25 independently transformed PD007 hairy root clones (designated WA 500 to WA 546) were transferred to callus induction medium. All root clones formed callus, and shoot regeneration was observed in 25 (=54%) of the WA clones. Of these 16 were analysed

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further; after in vitro multiplication 5 plants from each of these 16 clones, as well as control plants (pBI121 transformed PD007 and untransformed PD007 plants), were transferred to the greenhouse and soil-grown tubers were harvested.

5

Tuber starch analysis

From all 16 clones subterranean tubers were harvested. Two to three randomly picked tubers from every plant were cut in slices and the cut surfaces were stained with Lugol's solution. Based on the staining reaction three classes were discerned. Eleven plants formed tubers with red staining (amylose-free) starch and three plants formed tubers with only blue staining (amylose-containing) starch, whereas two formed tubers with a mixed staining type of starch. The mixed staining tubers were different from the intermediate staining root tips in that individual cells, which were grouped in a certain zone of the tuber, contained either red or blue staining starch. All tubers from such plants were of mixed staining phenotype. The sizes of the blue and red zones varied. Always the heel side of the tuber (the side attached to the stolon) contained blue staining starch.

Analysis of isolated starch from tubers of the three different staining classes revealed that activity and/or amount of GBSS protein were affected in all the different plants transformed with antisense constructs. Table 3 shows that all plants staining red for tuber starch, which were investigated for those characters, had strongly decreased GBSS activities comparable to that of the amf mutant, whereas all plants with

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only blue staining tuber starch had significant GBSS activities, which, however, were lower than that of the wild type PD007. The GBSS activity in pBI121 transformed Ri007 plants was equal to that of the wild type. The amylose/amylopectin ratio was
5 determined in tuber starch and the apparent percentage of amylose was calculated. The relative amylose content of tubers with blue staining starch was in some transgenic plants comparable to that of the wild type, PD007 or Ri007, while in other plants it was much lower. In tubers with red staining
10 starch the amylose level of the amf mutant was achieved. The mixed staining tubers had amylose percentages which varied between those seen for the tubers with red and blue staining starch respectively, mainly because these tubers consisted of a mixture of amylose-free and amylose-containing starch.

15 The results are also shown in fig. 2C and 2D to facilitate a comparison with the heterologous transformants. As can be seen in Figure 2C, two out of the three different classes of tubers from homologous transformants contained GBSS activities which correspond with their colour when stained with Lugol's solution.
20 Levels similar to that of the amf-mutant for red staining tubers and detectable but clearly lower than wildtype GBSS activities for blue staining tubers. The amylose content for these two classes is also in agreement with the colours of the tubers (fig. 2D). No amylose for the red staining tubers and rather
25 high amylose contents comparable to those found in wildtype tubers for the blue staining tubers. The latter group also closely resembles the heterologous transformants. Starches isolated from the 'intermediate' colouring class gave a

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completely different result. An example of such a transformant is WA504, which has a higher GBSS activity than both transformants from the 'blue colouring' class, WA507 and WA514, but the apparent amylose content of this transformant is much lower than that of the other two transformants.

GBSS protein analyses were performed by running protein extracts from a number of tuber starch samples on SDS polyacrylamide gels followed by subsequent silver staining of the gels or by transferring them onto nitrocellulose filters and immunoblotting with antibodies raised against GBSS from potato. From both silver stained gels or from the immunoblots it is evident that only blue and intermediate staining tubers contain GBSS protein in their starch granules. Although the extraction of protein from starch is difficult to quantify it seems that in the blue staining tubers little reduction of the level of GBSS protein has occurred. As demonstrated by the weaker signal on the immunoblot for clones WA 504 and WA 517, a reduced level of GBSS protein is clearly found in the intermediate staining tubers.

20 Molecular analysis of the transformants

Southern blot analyses were performed to determine the number of correctly introduced antisense GBSS constructs in the transgenic plants. Genomic DNA from a number of independently obtained transformants was isolated and digested with EcoRI and BglII. These restriction endonucleases excise the construct in such a way that the number of different bands corresponds to the number of different integrations. In this way it was found that the number of integrations in the independent transformants

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varied from 1 to 5. In untransformed PD007 or pBI121 transformed plants only the hybridization pattern from the resident GBSS genes could be discerned. No relationship was found between the antisense effects and the number of integrated copies. Both
5 plants with tubers with red or blue staining starch could contain either one or more than one antisense copy.

Stability of expression of the antisense genotype

The antisense GBSS gene is expressed and the antisense-
10 derived phenotype is visible, as is the amf phenotype, in all tissues where starch is formed; apart from columella cells of root tips and tubers this also includes stomatal guard cells. However, although the composition of the starch in the tuber of a given plant seemed to be quite stable, there was always a
15 variable expression of the antisense genotype in stomatal guard cells and in columella cells. Root tips and guard cells with red and with blue staining starch could be found in one and the same plant, irrespective of its tuber starch reaction. The only
20 variability in tuber starch colour was found in the transformants with mixed staining starch. These always had a varying zone of blue and red staining starch in their tubers; no variegated patterns or differently oriented zones were ever observed in the mixed staining tubers. Vegetative propagation of
25 tubers with red staining starch from three transformants showed that the antisense trait is transmitted after multiplication and thus is apparently stably integrated into the genome.

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Discussion

In this example we describe effects of the introduction of GBSS antisense constructs into potato on the amount and activity of GBSS and on the amylose/amylopectin ratio in potato starch.

5 Analysis of the hairy roots revealed that the introduction of pGB50 antisense constructs resulted in phenotypic changes, which did not occur when pGB60 sense constructs were used, as judged by the staining of starch in columella cells with Lugol's solution. However, because of the instability of the observed
10 effect in columella cells this system is only suitable for testing constructs for their potential biological effects. Therefore, plants were regenerated from kanamycin-resistant roots irrespective of the root tip phenotype with respect to starch composition. In contrast to the hairy root clones
15 investigated which were mostly blue staining, all but three plants formed tubers with stably red (11 plants) staining starch. None of the investigated transgenic plants, not even the blue ones, had a GBSS activity which was comparable to that of the wild type controls. In this respect the antisense approach
20 was successful in all kanamycin-resistant plants.

The fact that variable expression of the antisense genotype occurs in stomatal guard cells, in which transitory or metabolic starch is deposited, as well as in columella cells of root tips is difficult to explain. Since such metabolic starch, at least
25 in leaves, contains less amylose than reserve starch (Hovenkamp-Hermelink et al. 1988), it might be expected that an effect could be accomplished more easily in leaves. Since less amylose and less GBSS mRNA (Visser et al. 1989) are present a

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suppressing effect in leaves would be more likely and would also be more stable. If the starch in roottips were also of metabolic origin identical results would be expected. However, our results contradict this expectation. In the guard cells of leaves the

5 results obtained could point to the involvement of a physiological component such as photosynthesis, in roots such an involvement is more difficult to imagine. Another reason for the differences between effects obtained in tubers and in other organs of the plant can perhaps be found in the more constant

10 expression of GBSS mRNA in tubers. The promoter used in this study could also be the reason for the observed phenomenon. The 35S CaMV promoter is considered a constitutive promoter in all tissues, but reports from Benfey and Chua (1989) and Benfey et al. (1989) have shown that this need not always be true. There

15 are at least two domains in the promoter region (Benfey et al. 1989) which, when introduced separately into transgenic plants, can confer different developmental and tissue-specific expression patterns. In our case the complete 35S CaMV promoter perhaps allows specific expression levels in various tissues at

20 different times during the day like those reported for the two different domains of the 35S CaMV promoter (Benfey et al. 1989). More research in the field of variable expression is necessary before one can hope to explain the observed results.

Here, however, we can only draw conclusions from the

25 results derived from tuber starch analysis, since only in tuber starch does the expression appear to be stable and is measurable quantitatively. The data show a reduction of GBSS activity in all the investigated transgenic plants. In those cases where

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there is total absence of GBSS activity, GBSS protein and amylose are also absent. In all other cases there is an inhibitory effect on activity and possibly also on the amount of GBSS protein. The effect of a lower GBSS activity on the amylose/amylopectin ratio is not straightforward, as was also seen in heterologous GBSS antisense experiments (Visser 1989). The strong reduction in GBSS activity required to evoke only a rather small reduction in the amylose/amylopectin ratio in transformants WA 507 and WA 514 are in contrast to that found in transformant WA 516, which has a higher GBSS activity but a lower amylose content. The interpretation of the effect on amylose/amylopectin ratio is complicated further by the fact that no data on absolute amounts of starch are available.

The introduction of antisense GBSS constructs clearly has an effect on a number of related parameters in starch metabolism. The fact that there is variation among different plants transformed with the same antisense construct is not surprising. Similarly variable responses of plants to particular antisense constructs have also been obtained for antisense chalcone synthase (CHS) in Petunia hybrida and Nicotiana tabacum (van der Krol et al. 1988), antisense chloramphenicol acetyl transferase in tobacco (Delauney et al. 1988) and antisense polygalacturonase (PG) in tomato (Smith et al. 1988). Moreover, in the animal systems variable inhibition of the target genes by antisense vectors has also been observed (Holt et al. 1986).

It is assumed that the variations in response arise from differential influence on antisense expression of the chromosomal region in which it is integrated, i.e. so-called

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position effects (Van der Krol et al. 1988; Smith et al. 1988). All GBSS antisense transformed plants contained variable numbers of integrated antisense constructs, but no relationship between GBSS copy number and the observed antisense effect could be found. These results are similar to those observed by Van der Krol et al. (1988), where there was no correlation between antisense CHS copy number, antisense CHS mRNA level and phenotypic effects on flower pigmentation. These results differ from those obtained using heterologous GBSS constructs in potato (Visser 1989) where a correlation was found between GBSS copy number and phenotypic effect. A relationship between copy number and antisense effect was also found for antisense polygalacturonase genes in tomato (Schuch et al. 1989) and for antisense chloramphenicol acetyl transferase (CAT) in constitutively expressing chloramphenicol acetyltransferase (CAT⁺) tobacco plants (Delauney et al. 1988).

In conclusion, this example shows that the introduction of GBSS antisense cDNA constructs results in a strongly reduced GBSS activity, which in most cases is accompanied by an equally large reduction in the amount of GBSS protein and amylose content. However, in those cases where the antisense effect is not absolute the degrees of reduction in the amount of GBSS activity, GBSS protein and the amylose content are unpredictable. The antisense approach can be a very effective alternative technique to mutagenesis programmes for enzymes involved in the metabolic pathway of starch formation, especially in vegetatively propagated (polyploid) crops. Moreover, the availability of antisense plants should make

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normally recessively inherited mutations more easily obtainable, because antisense genes themselves act as dominant (hemizygous) suppressor genes, thus enabling plant breeders to shorten their breeding programmes.

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TABLE 1

Iodine staining of starch in columella cells of hairy roots formed on stem segments of Solanum tuberosum PD007 after inoculation with Agrobacterium rhizogenes pGB50.

5

	Experiment	No. of roots stained	Percentages of roots staining		
			Blue	Intermediate	Red
10	I	43	40	21	39
	II	49	41	21	38
	III	103	34	33	34
	IV	34	41	18	41
	V	78	33	31	36
15	Total	319	38	24	38

20

Table 2

Iodine staining behaviour of starch in columella cells of kanamycin-resistant hairy roots containing pGB50 and analysed over a period of 42 days.

Root clone	Days after oculation			
	1	15	30	42
1	R————>	R————>	R————>	R
2	R————>	R* I————>	B————>	I B
3	I————>	R* I B————>	I* B————>	I B
4	I————>	I* B————>	B————>	B
5	B————>	B————>	B————>	B
6	R————>	I————>	R* I————>	I
7	R————>	R————>	R* I B————>	I B
8	I————>	R* I————>	I* B————>	I B
9	I————>	B————>	I* B————>	I B
10	B————>	B————>	B————>	B

R. red staining; I, intermediate staining; B, blue staining.

For every measurement obtained 3-8 root tips were stained. Of

root clones with different staining root tips the clones

25 marked with an asterisk were transferred to fresh medium.

Table 3

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Tuber starch colour, GBSS activity and amylose content of antisense GBSS transformed potato plants and their controls.

5	Plant clone	Tuber starch colour after iodine staining	GBSS activity (as % of the content of PD007)	Apparent amylose content
10	<hr/>			
	Class I			
	WA 501	red	0	0
	WA 505	red	3	2
15	WA 506	red	0	2
	WA 508	red	0	2
	WA 511	red	0	0
	WA 512	red	2	3
	Class II			
20	WA 504	blue/red	18	26
	WA 517	blue/red	6	22
	Class III			
	WA 507	blue	9	83
	WA 514	blue	10	84
25	WA 516	blue	32	49
	Controls			
	PD007	blue	100	100
	Ri007	blue	96	106
	amf	red	0	0
30	<hr/>			

The tuber starch colour was determined by staining a cross-section of a tuber with Lugol's solution as described in the Materials and methods. GBSS activities measured as the incorporation rate of ^{14}C -labelled ADP-Glucose, ranged from 0 to 8.1 pmol/min per mg starch in individual tubers with red staining starch, from 8.7 to 70 pmol/min per mg starch in individual tubers with blue staining starch and from 6.2 to 32.7 pmol/min per mg starch in individual tubers with mixed staining starch. The values of untransformed or pBI121 transformed PD007 ranged from 66.9 to 98.9 pmol/min per mg starch and for the amf mutant were approx. 0 pmol/min per mg starch. Amylose percentages varied from 0% to 1.9% for tubers with red staining starch, from 14% to 27% for tubers with blue staining

starch and from 5% to 7% for tubers with mixed staining starch. Transgenic plant clones WA 502, WA 503, WA 509, WA 513 and WA 515, all containing red staining tuber starch, were regarded as class I transformants but were not included in the above analysis.

5

EXAMPLE 2

This example investigates if it is possible to increase the amylose content in potato tubers. This might be achieved by overexpressing granule-bound starch synthase if this enzyme is
10 rate limiting to amylose biosynthesis. In order to investigate this question a full length genomic, actively transcribed (Visser et al. 1989, van der Leij et al. 1991), GBSS gene was introduced in a number of different wildtype and heterozygous (Amfamf) potato genotypes. The results of these experiments show
15 that no significant increase of the amylose content could be obtained in any of the transformants. Surprisingly it was found that the introduction of this gene can instead block the expression of both the introduced and endogenous GBSS genes.

Materials and methods

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Plant materials

In vitro shoots of the Solanum tuberosum genotype cv. Astarte ($2n=4x=48$), PD 007 (HH 578, $2n=2x=24$), the Amfamf genotype 871024-2 ($2n=2x=24$) and the amfamf genotype 871029-31 ($2n=2x=24$, Jacobsen et al. 1989) were used. The shoots were grown at 21°C with a regime of 14h light per day on basal MS medium (Murashige and Skoog 1962) supplemented with 30 g/l sucrose (MS 30).

Standard methods and reagents

Standard techniques of DNA manipulation were performed as described by Maniatis et al. (1982). Plasmid DNA isolations from Escherichia coli were according to Birnboim and Doly (1979) and inserts were isolated from the restriction enzyme digested plasmid using the method described by Tautz and Renz (1983). Enzymes were from Gibco/BRL or Boehringer Mannheim and were used according to the manufacturers recommendations.

Construction of vectors and transformation of potato

The construction of the binary plasmid pWAM 100 containing a full length GBSS genomic gene, capable of complementing an amylose-free mutant, was described before (van der Leij et al. 1991). The binary plasmid was introduced into Agrobacterium rhizogenes LBA 1334 using triparental mating (Visser et al. 1991b). The binary vector pWAM 100 was also introduced into Agrobacterium tumefaciens LBA 4404 using the direct transformation method of competent Agrobacterium cells described

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by Höfgen and Willmitzer (1988). Integrity of the plasmids in Agrobacteria was verified according to Holmes and Quigly (1981).

Potato stem segments were inoculated with A. rhizogenes as described by Visser et al. (1989a). The formation of hairy roots was allowed to take place on solid MS 30 medium with 200 mg/l cefotaxim, without kanamycin. Regeneration of shoots from hairy roots was as described before (Visser et al. 1989a). Transformation of potato stem segments with A. tumefaciens followed by subsequent regeneration of shoots from these explants was as described by Visser (1991).

Microtubers were obtained by transferring nodal buds to MS medium containing high amounts of sucrose (80 g/l) as described by Hovenkamp-Hermelink et al. (1987).

Plants grown in vitro were transferred to the greenhouse to obtain soil-grown tubers.

Screening of tubers

Subterranean tubers obtained from A. rhizogenes or A. tumefaciens transformants (respectively R-n or T-n) were cut and the surface was stained with Lugols solution.

Assays for GBSS activity and amylose content

GBSS activity was measured in 50 µl assay buffer containing samples of 1-2 mg of enzymatically active starch as described by Vos-Scheperkeuter et al. (1986) using ¹⁴C labelled ADP-glucose as substrate.

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The amylose/amylopectin ratio in starch from potato tubers was determined by the method described previously (Hovenkamp-Hermelink et al. 1988).

5 Gel electrophoresis and immunoblotting

Proteins were extracted from starches as described by Vos-Scheperkeuter et al. (1986). Analysis on 10 % SDS polyacrylamide gels and subsequent immunoblotting or protein staining were performed as described by Vos-Scheperkeuter et al. (1986) and
10 Hovenkamp-Hermelink et al. (1987).

Southern and northern analysis

DNA of greenhouse grown plants was isolated from young leaves according to Dellaporta et al. (1983) and digested with
15 the restriction enzymes EcoRI and BglII. Southern blot hybridizations with radioactive labelled GBSS cDNA was performed as described previously (Visser et al. 1989b,d).

RNA isolation from tubers and leaves, followed by northern blotting and hybridization were as described (Visser et al.
20 1989d).

Results

The diploid genotype PD007 and the tetraploid cv. Astarte were used in transformation experiments using the Agrobacterium
25 rhizogenes strain harbouring the binary vector pWAM100.

The diploid genotype 871024-2 was used in transformation experiments harbouring the vector pWAM100. Table 4 shows the

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number of individual transformants obtained using either transformation approach.

The transformants were allowed to tuberize in the greenhouse. All A. tumefaciens transformants produced tubers
5 whereas only 65% of the A. rhizogenes transformants produced tubers. From all tubers starch was isolated to determine amylose/amylopectin ratio and from most of the tuber starch samples also the GBSS activity was measured (Table 5).

From the results of Table 5 it is obvious that none of the
10 transformants had a significantly higher amylose content as compared to the wildtype controls. However, both in the A. rhizogenes and the A. tumefaciens transformants clones were found containing red staining starch in their tubers. Also in the case of the tetraploid cv Astarte where 12 transformants
15 produced tubers (Table 4), one clone was found with red staining sectors in the tubers (results not shown). This was also the case in one of the 871024-2 transformants (T-21). In all transformants with red staining tuber starch the GBSS activity proved to be very low or undetectable. In these particular
20 transformants no detectable amounts of amylose could be measured (Table 5, clones R-11, R-30a and T-40), thus resembling the amf-mutant for these characters.

The phenotype from these transformants in other starch containing tissues was different from that of the amf-mutant;
25 stomata and roottips had blue and only occasionally red staining starch. In this respect these transformants resembled more some anti-sense GBSS transformed clones (Visser et al. 1991a).

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These results were due to the fact that transformants contained extra copies of the GBSS gene, as was confirmed both by polymerase chain reaction experiments as well as by Southern blot analysis. The number of integrated copies varied from one
5 to four per haploid genome and no relation between copy number and inhibitory effect was found (results not shown).

Discussion

In this example, the effect of the introduction of
10 additional homologous GBSS gene copies in diploid (both heterozygous and homozygous for the amylose-free character, respectively Amfamf and AmfAmf) and tetraploid potatoes on GBSS activity and amylose percentage is investigated.

Although it was the intention to increase expression of the
15 GBSS gene leading to an increased activity of the enzyme, something which was reported for sense alfalfa glutamine synthetase in transgenic tobacco (Eckes et al. 1989) no such phenomenon was observed in our experiments. Unexpectedly the introduction of extra copies of the GBSS gene led to a (almost)
20 complete inhibition of the GBSS enzyme activity in 8% to 22% of the transformants (Table 5), irrespective of their ploidy level or genetic constitution for the amf-character which in the heterozygous diploid means that only one copy of the gene has to be suppressed to achieve an effect. The lower or absent GBSS
25 activity was accompanied with lower amounts or total absence of GBSS protein when analysed by Western blot (cf. Table 5). The absence of GBSS protein and activity in turn led to an almost complete absence of amylose (<1% of the wildtype level) and

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could be made visible by staining cut tuber surfaces with Iodine: presence of amylose gave blue staining, absence of amylose red staining starch (Table 5).

The phenomenon that a mutant phenotype can be obtained after introduction of one or more copies of a wildtype gene in a wildtype host has been described before (Napoli et al 1990, Smith et al. 1991) and is known as co-suppression. One common feature of co-suppression in different plant systems seems to be the occurrence of instable or reversible phenotypes. An indication for this phenomenon in the five sense GBSS transformants showing an effect in the tuber, might be the starch composition in other starch containing organs of these plants. Another example of such an unstable or reversible phenotype might be transformant T-21 which contained partly blue and red staining starch in its tuber. It is thought that these phenotypes are related to natural cases of gene expression like flower colour patterning, and epigenetic effects as in paramutations and other modulating mechanisms of transposition (Jorgensen 1991, Matzke and Matzke 1991).

At present two explanations for the feature of co-suppression are in favour: methylation or anti-sense RNA effects. Methylation is thought to be a result of interactions of homologous sequences at different sites in the genome. Several examples which share similarities with co-suppression are known to be related to methylation. In these cases homology between promoters seems to be essential and suppression acts at the level of transcription (Matzke et al. 1989, Matzke and Matzke 1991). In our case homology with the complete sequence

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including the promoter region with resident sequences exists. However, none of the 12 potato clones transformed with a chimaeric gene consisting of the GBSS promoter and the β -glucuronidase (GUS) gene contained amylose-free starch (Visser et al. 1991b, unpublished results).

A second explanation for co-suppression involves the so called anti-sense RNA interaction. It was postulated by Grierson et al. (1991) that anti-sense RNA could be generated because of simple read through of the kanamycin resistance gene which is cotransferred for selection and used so far in all the systems known to show the phenomenon of co-suppression (van der Krol et al. 1990, Napoli et al. 1990, Grierson et al. 1991, Matzke and Matzke 1991). However, the fact that the GBSS promoter, which was used in our case, is a much more powerful promoter than for instance the 35S (CaMV) promoter (Visser et al. 1991b) makes this unlikely. Rather the place of integration, also known as position effect, seems to play a more important role. It is known from experiments with promoterless constructs that most of the integrations take place in regions of the genome which are transcriptionally active (Koncz et al. 1989, Goldsbrough and Bevan 1991). In potato about 10% of the plants transformed with a promoterless GUS-gene showed GUS activity in tubers and/or leaves.

According to this example, additional copies of the structural gene granule-bound starch synthase (GBSS) were transferred into different potato genotypes using either Agrobacterium tumefaciens or Agrobacterium rhizogenes as a vector to investigate the possibility of increasing the amylose

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content in potato tubers. Out of eighteen transformants, only two had a higher GBSS expression, but this did not lead to increased amounts of amylose in tuber starch. Surprisingly, however, in five transformants a strongly reduced GBSS activity was found. In the three transformants which showed the highest reduction in GBSS activity, this severe reduction was accompanied by the absence of GBSS protein and amylose in the starch granules. The specific inhibition of GBSS expression involved both the introduced and the endogenous gene. The effect obtained was not related to the ploidy level of the plant nor to the number of extra gene copies introduced into the plant.

In conclusion, this example shows that the introduction of an actively transcribed granule-bound starch synthase gene into potato may lead to suppression of the expression of both the introduced and the endogenous gene.

Table 4

The number of (tuberizing) transformants obtained either by using A. rhizogenes or A. tumefaciens harbouring the binary vector pWAM 100.

Plant genotype		Bacterium	No. of transformants	with tubers
25	PD007	R	19	9
	Astarte	R	16	12
	871024-2	T	30	30

R= A. rhizogenes, T=A. tumefaciens

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Table 5

Comparison of GBSS activity, presence of GBSS protein, amylose content and starch colour of pWAM 100 transformants with the untransformed controls (PD007, 871024-2 and 871029-31).

5	Plant	GBSS activity as % of wt*	Amylose content as % of wt**	GBSS protein	Tuber starch colour
10	PD007 (AmfAmf)	100	100	+	blue
	R-5	nd	84	+	blue
	R-6	nd	86	+	blue
	R-11	0	<1	-	red
15	R-19	nd	79	+	blue
	R-20	nd	86	+	blue
	R-24	129	102	+	blue
	R-27	nd	73	+	blue
	R-29	nd	95	+	blue
20	R-30a	4	0	-	red
	871024-2 (Amfamf)	100	100	+	blue
	T-4	18	75	+	blue
25	T-7	131	95	+	blue
	T-11	49	75	+	blue
	T-13	46	95	+	blue
	T-21	10	30	-	red/blue
	T-25	81	100	+	blue
30	T-26	56	95	+	blue
	T-34	44	95	+	blue
	T-40	3	0	-	red
35	881029-31 (amfamf)	0	0	-	red

nd = not determined

GBSS protein presence determined by Western blot analysis,

+ = present, - = absent

40 * PD007 and 87 1024-2 activity ranged from 65 to 100 pMol/
min/mg starch, 871029-31 activity from 0 to 4 pmol/min/
mg starch

** PD007 amylose % 18 to 27 %

871024-2 amylose % 18 to 24 %

45 871029-31 amylose % 0 to 4 %

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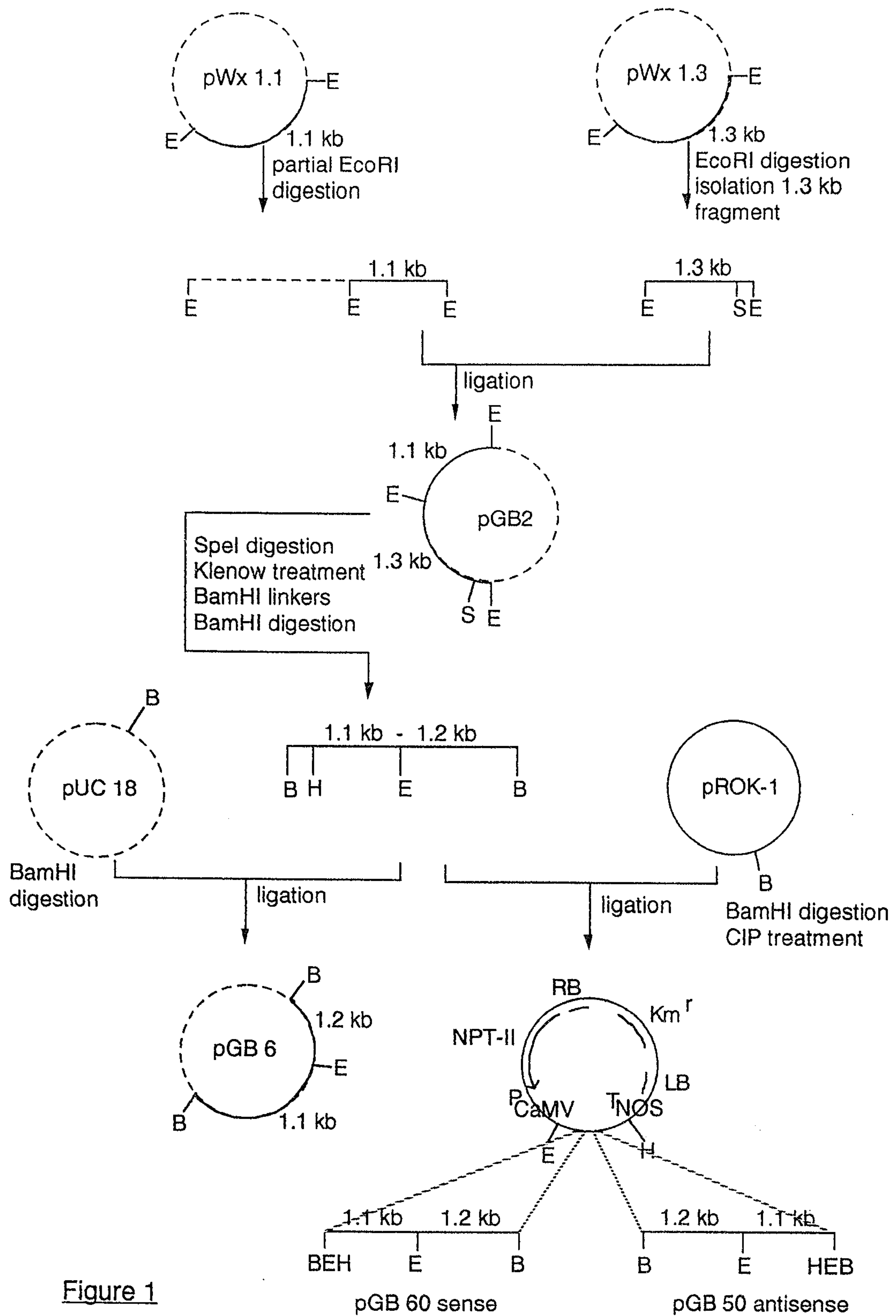
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- 25

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A potato plant cell which, as a result of genetic engineering, has a genome containing at least one gene construct containing a full length potato granule-bound starch synthase (PGBSS) cDNA in reverse orientation in an expression cassette which is functional in potato plants, said gene construct giving rise to tubers containing essentially amylose-free starch.
2. The potato plant cell of claim 1, wherein said expression cassette contains the cauliflower mosaic virus 35S promoter (P_{CaMV}).
3. The potato plant cell of claim 1, wherein said expression cassette contains the PGBSS promoter.
4. The potato plant cell of claim 1, wherein said expression cassette contains the nopaline synthase terminator (T_{nos}).
5. The potato plant cell of claim 1, wherein said expression cassette contains the PGBSS terminator.
6. The potato plant cell of claim 1, wherein said gene construct contains the neomycin phosphotransferase II gene (NPT-II) kanamycin resistance marker.



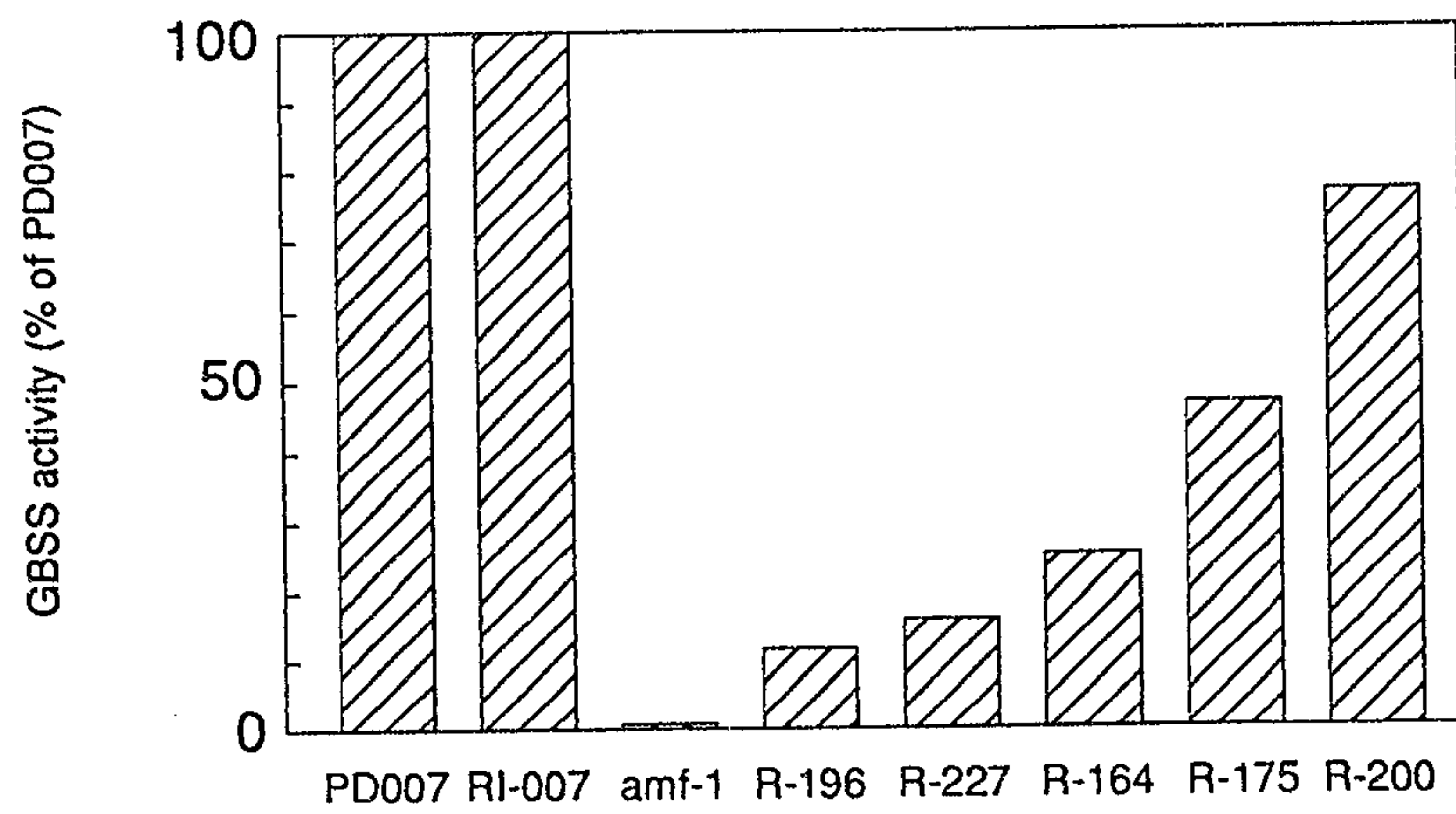


FIG. 2A

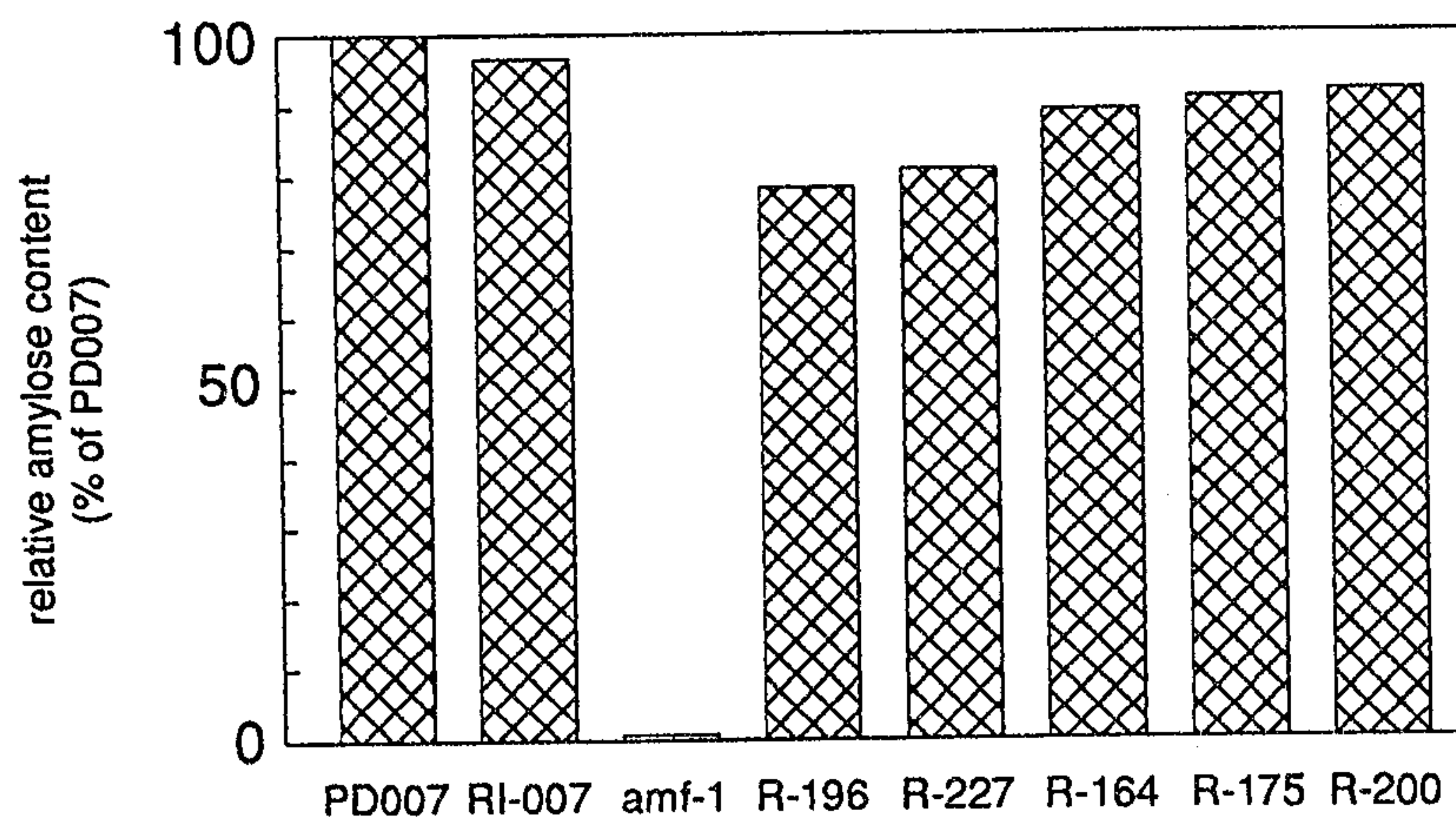


FIG. 2B

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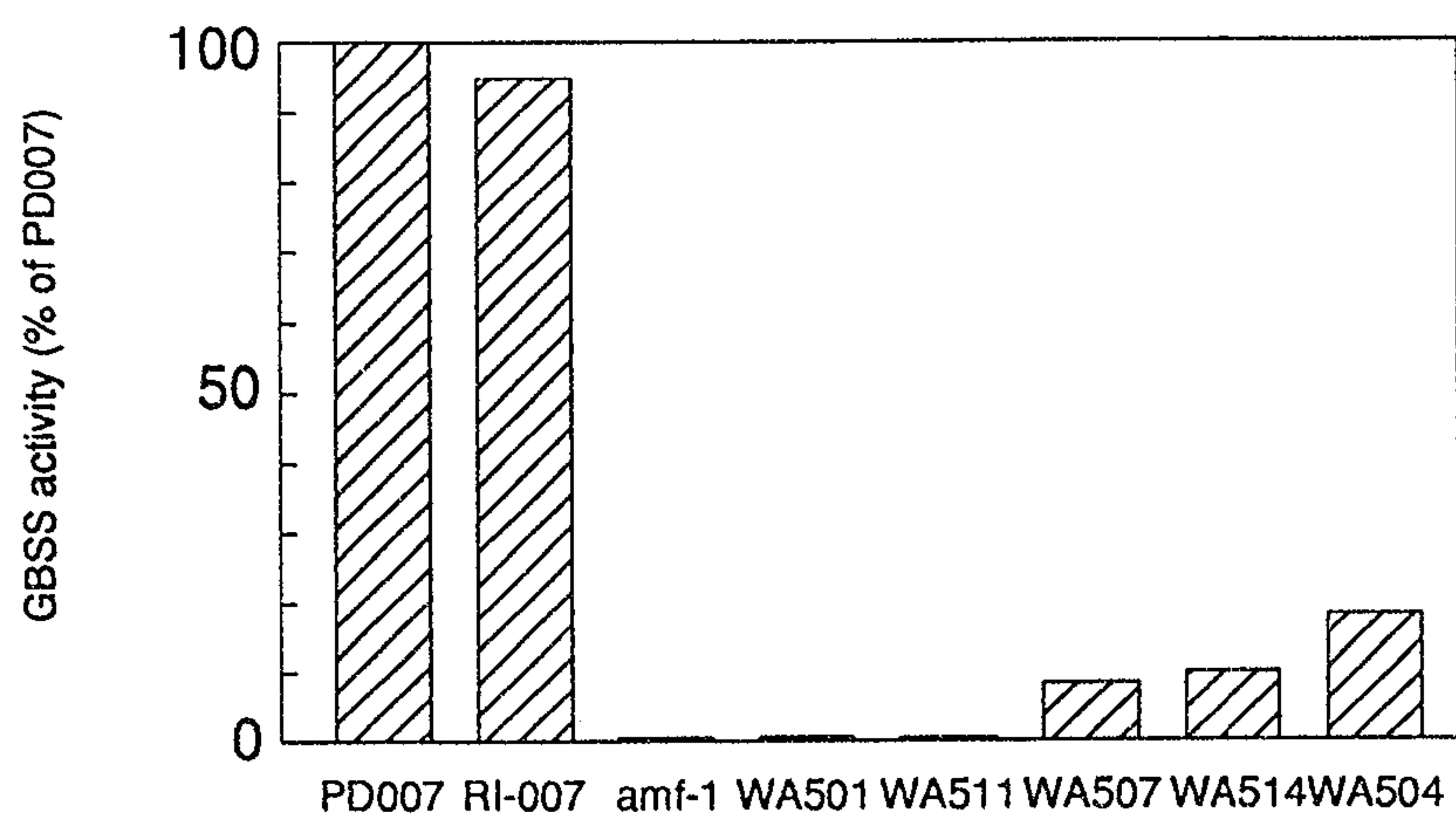


FIG. 2C

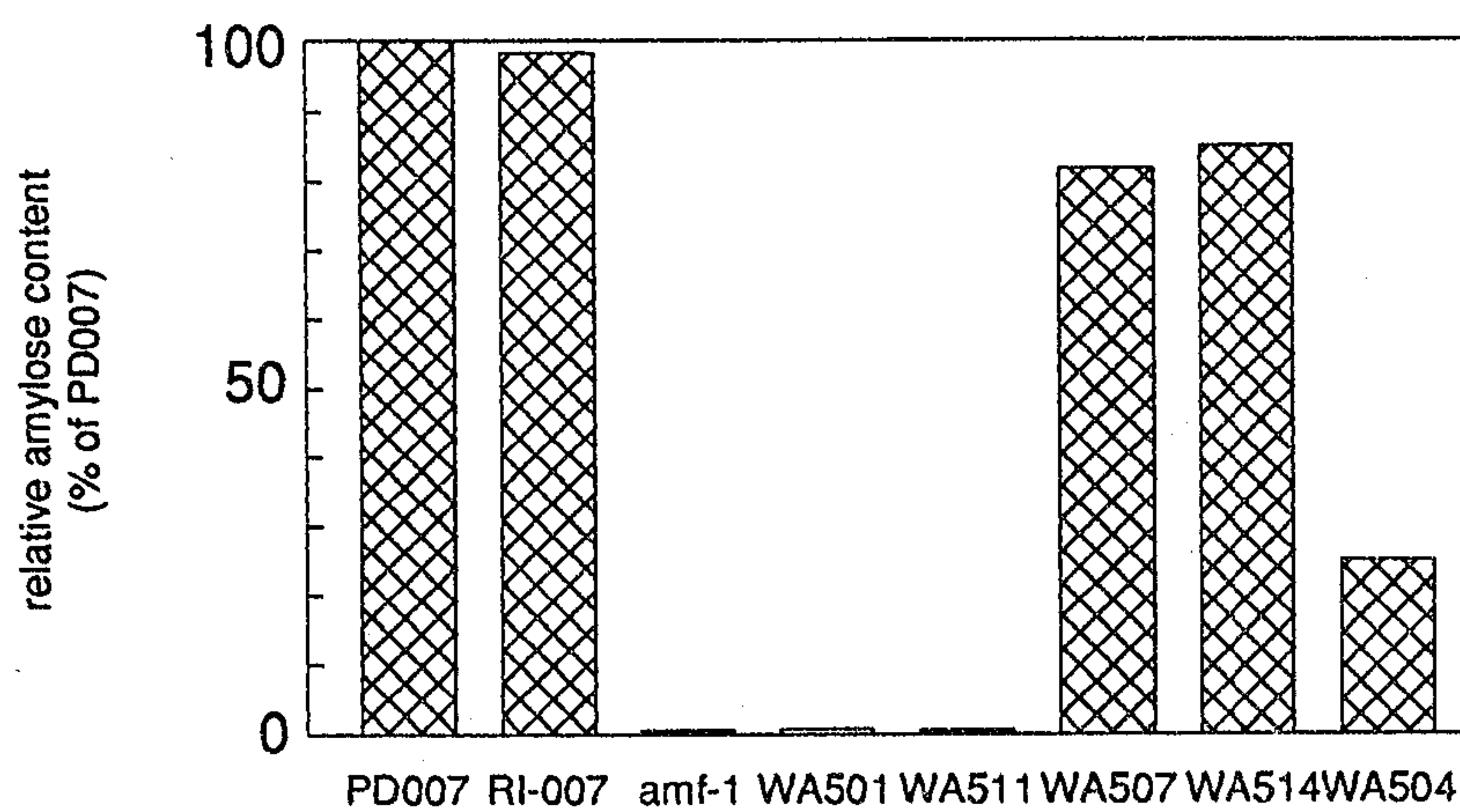


FIG. 2D