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Complementation of the amylose-free starch mutant of potato (*Solanum tuberosum.*) by the gene encoding granule-bound starch synthase

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Summary. *Agrobacterium rhizogenes*-mediated introduction of the wild-type allele of the gene encoding granule-bound starch synthase (GBSS) into the amylose-free starch mutant *amf* of potato leads to restoration of GBSS activity and amylose synthesis, which demonstrates that *Amf* is the structural gene for GBSS. Amylose was found in columella cells of root tips, in stomatal guard cells, tubers, and pollen, while in the control experiments using only vector DNA, these tissues remained amylose free. This confirms the fact that, in potato, GBSS is the only enzyme responsible for the presence of amylose, accumulating in all starch-containing tissues. Amylose-containing transformants showed no positive correlation between GBSS activity and amylose content, which confirms that the former is not the sole regulating factor in amylose metabolism.

Key words: *Solanum tuberosum.* – *Agrobacterium rhizogenes* – Starch – Amylose-free – *Waxy*

Introduction

Starch in tubers of wild-type potato consists of about 20% amylose, the helical linear glucose polymer, and about 80% amylopectin, the larger, branched glucose polymer (Shannon and Garwood 1984). An amylose-free potato mutant, isolated after X-irradiation, was described as having neither granule-bound starch synthase (GBSS) activity nor the protein in the starch granules of its tubers (Hovenkamp-Hermelink et al. 1987).

The recessive, monogenic *amf* mutation shows similarities with the well-known *waxy* (or *glutinous*) mutations in monocots and in *Amaranthus*, except for a difference in tissue specificity. The *amf* mutation is not only expressed in generative tissue (pollen), but also in vegetative transitory and reserve starch-containing tissues like columella cells of root tips, leaf cells, stomatal guard cells, and tubers (Jacobsen et al. 1989). The *waxy* mutations in maize and rice are known to be expressed only in the pollen, endosperm, and embryosac (Shannon and Garwood 1984). Several of these *waxy* mutations have been investigated at the molecular level (Wessler and Varagona 1985; Okagaki and Wessler 1988) and alterations of the gene encoding GBSS were often detected. It seems probable that in potato the *Amf* gene is the structural gene for GBSS. Since no large lesion could be detected in the GBSS gene of the amylose-free potato mutant, while a GBSS transcript is still formed (Visser et al. 1989a), we carried out a complementation experiment to prove that the *Amf* locus of potato is the gene encoding GBSS. The most suitable method of stably introducing the GBSS gene in the mutant for our purposes was an *Agrobacterium rhizogenes*-mediated gene transfer system (Visser et al. 1989b), which allowed very rapid screening of the starch composition in columella cells of transformed roots.

Materials and methods

Plant materials

Three diploid (2n = 2x = 24) homozygous *amf* genotypes from the *Solanum tuberosum.* $F_1 \times F_1$ families 87.1029 and 87.1031, obtained after crossing the *amf* mutant with wild-type plants (Jacobsen et al. 1989), were propagated in vitro on MS medium (Murashige and Skoog 1962) with 3.0% (w/v) sucrose (MS30) and 0.8% (w/v) agar. Plants 87.1029-32, 87.1031-29, and 87.1031-47 will henceforth be denoted 32, 29, and 47, respectively, followed by the number of the construct introduced and the clone number. As control plants, the diploid PD 007 ($H^2$578) and the tetraploid cultivar Bintje were used.

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Standard methods and reagents

Standard techniques of DNA manipulation were performed as described in Maniatis et al. (1982), and plasmid isolations from *Escherichia coli* were according to Birnboim and Doly (1979). Restriction enzymes, T_4 DNA Ligase, calf intestinal phosphatase (CIP), and the digoxigenin DNA detection kit were from Boehringer, Mannheim, Germany. CIP treatment was performed for 30 min at 37 °C and stopped by adding nitrillo triacetate and heat inactivation (30 min, 70 °C), followed by agarose gel electroelution and ethanol precipitation.

Construction of the binary plasmids pWAM 100, pWAM 101, and pWAM 150 containing the GBSS gene

The lambda EMBL4 derivative LGBSS^{wt}-6 (Visser et al. 1989a; see also Fig. 2) was used as source of the genomic GBSS gene for cloning in the binary vector pBIN19 (Bevan 1984). The 6.5-kb GBSS gene fragment, obtained after digestion with BglII, was ligated into the BamHI site, resulting in the recombinant plasmids pWAM 100 and pWAM 101. In pWAM 100, the GBSS gene is oriented in the opposite direction towards the plant kanamycin resistance gene and, in pWAM 101, both genes have the same orientation. Plasmid pWAM 150 was obtained following a two-step procedure. A partial HindIII digest was cloned in the HindIII site, resulting in pWAM 120, which contains the two distal HindIII fragments. The first of these two fragments bears an EcoRI restriction site, allowing the additional cloning of the 4.2-kb proximal EcoRI fragment with the promoter and upstream sequences (Visser et al. 1989a). All transformations of ligation products were performed with the *E. coli* strain JM83 (Vieira and Messing 1982) to permit easy detection of insertion in the *LacZ* gene of pBIN19.

Triparental mating and stem segment transformation

Mobilization of the pWAM vectors from *E. coli* to *A. rhizogenes* LBA 1334 (Offringa et al. 1986) was done with the helper plasmid pRK 2013 (Ditta et al. 1980) in *E. coli* HB101 (Boyer and Roulland-Dussoix 1969). Control plasmid pBIN19 was mobilized from strain MC 1022 (Cassadaban and Cohen 1980). Kanamycin-resistant *A. rhizogenes* conjugants were tested by Southern hybridization of isolated plasmids (Holmes and Quigley 1981) to digoxigenin-labeled potato GBSS cDNA. Potato stem segments were inoculated with *A. rhizogenes* cultures, as described by Visser et al. (1989b). The formation of hairy roots was allowed to take place on solid MS 30 with 200 mg/l cefotaxim, without kanamycin.

Initial screening and regeneration

Independently formed hairy roots were selected for kanamycin resistance (Km^R) twice on MS 30 with cefotaxime and kanamycin (100 mg/l). Screening of kanamycin-resistant roots for the presence of amylose was performed by staining the root tips with a freshly made mixture of Lugol's solution:chloral hydrate (2.5 g/ml H_2O), generally in a ratio of 4:5 (v/v). The proximal parts of these roots were used for callus induction and the subsequent regeneration of plants (Visser et al. 1989b). One shoot per callus was propagated and a part of each resulting clone was transferred to a greenhouse (16 h light, 18 °C/16 °C day/night temperature).

Determination of GBSS and amylose

Subterranean tubers from different plants of each clone were harvested separately and stored at 4 °C until use (max. 1 month). Before starch was isolated, the tubers were cut in pieces and slices were exposed to iodine vapor. Starch isolations were according to Nelson et al. (1978); samples were frozen and stored at −20 °C.

Western analysis was done with 5 mg starch per slot, applied without boiling in a denaturing buffer on a SDS-PAA gel containing 12% polyacrylamide. Blotting and immunodetection were performed as described by Vos-Scheperkeuter et al. (1986). GBSS activity was measured in samples less than 2.2 mg, according to Nelson et al. (1978), adapted as described by Vos-Scheperkeuter et al. (1986), with ^{14}C-ADP-glucose as substrate. The assay was carried out at pH 9.1 instead of pH 8.5 (Ponstein et al. 1990).

Amylose percentages of starches were determined spectrophotometrically (Hovenkamp-Hermelink et al. 1988). For comparison, the amylose percentages of a few samples were measured using the colorimetric method as described by Williams et al. (1970).

For screening of stomata for the presence of amylose with Lugol's reaction, leaves were harvested at the end of the day, kept in 96% ethanol to extract the chlorophyll, and washed with tap water. Amylose in pollen was detected with 25% lugol in H_2O (v/v).

Southern analysis

DNA of greenhouse-grown plants was extracted from young leaves according to Dellaporta et al. (1983) and digested with the restriction enzymes EcoRI and BglII. Southern hybridization with ^{32}P-labeled potato GBSS cDNA was performed as described previously (Visser et al. 1989a, c).

Results

Iodine staining of columella cells of root tips

After transformation of *amf* stem segments with *A. rhizogenes* transmitting either pWAM 100, 101, or 150, blue-staining starch was observed in about one-half of the number of kanamycin-resistant hairy roots (Table 1). The blue color was in most cases very clear (Fig. 1 A). Blue-staining starch could also be observed in young roots that were not even set on kanamycin yet (Fig. 1 B), indicating the usefulness of the staining method for rapid screening without the use of kanamycin. From the 287 independently derived, kanamycin-resistant roots 59 plants were regenerated. In 88% of the pWAM transformed plants, blue-staining starch was observed at least once in the root tips. However, the staining of root tips of these plants was not always reproducible. Ten clones contained next to plants with blue-staining root tips one or more plants that stained red, whereas the original hairy root had a blue-staining root tip. The opposite effect (initially red and, after regeneration, blue) was found in 19 clones. Several regenerated plants had red- as well as blue-staining root tips (Fig. 1 C). In the root tips of two primary transgenic roots, both blue- and red-staining starch was observed (Fig. 1 D). All of the starch-containing primary roots and root tips of regenerated plants of the control experiment with pBIN19 stained red with iodine.

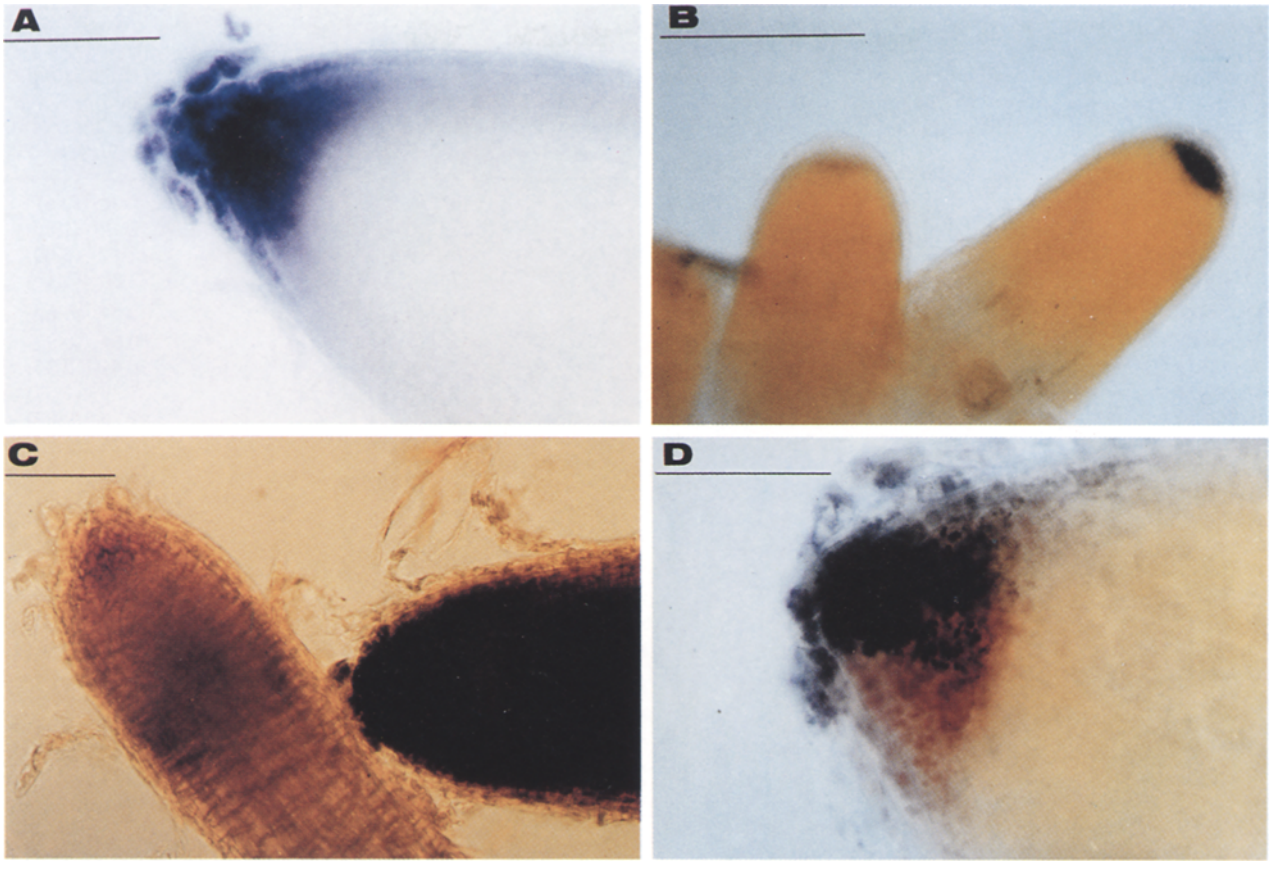

Fig. 1 A–D. Staining of columella cells of root tips by coloring the starch with a mixture of chloral hydrate and Lugol's solution. Bars represent 1 mm. **A** Amylose containing tip of a kanamycin-resistant root from a pWAM-transformed mutant. The clear blue color in this particular case is due to the use of an excess of chloral hydrate. **B** An amylose-free (*left*) and a complemented root tip (*right*) taken before selection on kanamycin. Detection of amylose can be achieved within 10 days of inoculation. About 10% of the root tips stain blue in this stage. **C** Two roots of one regenerant: one without amylose (*left*) and one with amylose (*right*). **D** A primary hairy root showing an amylose-containing as well as an amylose-free sector

Table 1. Transformation percentages and screening of hairy roots by staining with iodine

Binary plasmid	pBIN19	pWAM100	pWAM101	pWAM150	Total pWAM
Number of hairy roots	132	345	112	259	716
Number of Km^R hairy roots	25	141	56	90	287
Percentage Km^R hairy roots	19	41	50	35	40
Number of Km^R starch-containing roots	23	122	45	77	244
Number of blue-staining Km^R roots	0	62	32	38	132
Percentage blue-staining Km^R roots	0	51	71	49	54
Number of plants regenerated from all Km^R roots	9	35	9	15	59
Percentage of plants with blue-staining root tips	0	89	89	88	88

Analysis of tubers

Fifteen tuber-bearing clones were further investigated. The amylose contents of the different tubers with blue-staining starch (Table 2) were comparable to the wild-type contents, although in a few cases up to 4.6% more amylose was measured. Absorbance ratios of red-staining tubers were in the range of those found for the *amf* control and indicated low amylose contents. However, from previous colorimetric measurements (unpublished results) we know that the amylose content of tubers of *amf* F_2 plants, like the original mutant, is 0%. This was corroborated by the same colorimetric measurement result reported here (Table 2).

Table 2. Characteristics of regenerated transformants

Genotype	Minimum no. of integrated GBSS genes	Iodine staining of tubers	GBSS immuno-detection	Amylose % according to		GBSS activity pmol/min/mg starch $n=2$ (SD)[a]
				Method 1 $n=3$ (SD)	Method 2 $n=1$	
1029-32 (*amf*)	0	red	−	4.3 (0.2)	0	0.76 (0.64)
29-BIN19 (*amf*)	0	red	−	3.0 (0.6)		2.12 (1.85)
cv Bintje (wt)	0	blue	+	18.2 (0.6)	19.5	27.17 (3.00)
007-BIN19 (wt)	0	blue	+	17.3 (0.7)	15.6	22.30 (5.17)
32-100-1	2	blue	+	21.1 (0.1)		37.28 (3.08) w
47-100-8	3	red	−	5.9 (0.4)		0.64 (0.37) m
47-100-11 a	1	blue	+	22.2 (0.5)		36.91 (2.35) w
47-100-11 b	1	blue	+	22.8 (0.7)	23.8	37.29 (1.57) w
47-100-27	1	blue[b]	+	14.2 (0.0)		41.26 (7.05) w
47-100-34	4	blue	+	20.0 (0.2)		34.26 (2.55) w
47-100-42 a	2	blue	+	21.2 (0.4)	21.7	28.15 (8.80) w
47-100-42 b	2	blue	+	20.4 (0.6)		32.44 (4.68) w
47-100-42 c	2	blue	+	21.7 (0.2)		34.47 (15.37) w
47-100-45	1	blue	+	17.2 (0.8)		20.71 (4.05) w
47-100-48	1	red	−	7.9 (0.5)		2.01 (0.76) m
29-101-7	1	blue[b]	+	18.4 (0.3)		33.63 (6.64) w
29-101-12	5	blue	+	22.2 (0.5)		33.84 (0.10) w
29-101-14	1	blue	+	22.3 (0.5)		34.69 (2.81) w
32-150-1	2	blue	+	15.5 (0.2)		44.08 $n=1$
47-150-4 a	1	blue	+	17.4 (1.6)		26.40 $n=1$
47-150-4 b	1	blue	+	18.0 (0.4)		9.40 (0.54) w
47-150-5	1	blue[b]	+	22.2 (0.7)		
47-150-9	4	blue	+	20.0 (0.3)		32.24 (2.34) w

Method 1 = Absorbance ratio method (Hovenkamp-Hermelink et al. 1988)
Method 2 = Colorimetric method (Williams et al. 1970)

[a] w = not significantly different from wt controls, significantly different from *amf* controls
m = significantly different from wt controls, not significantly different from *amf* controls
Each transformant was tested for a significant difference with respect to the two wt controls and the two *amf* controls (two-sided *t*-test, significance level $\alpha = 5\%$, 2 *df*)

[b] Tubers stained faintly blue and contained little starch

Western blotting of the starches revealed the presence of GBSS in all amylose-containing tubers, whereas the amylose-free transformants did not show detectable levels of GBSS (Table 2). The GBSS activities of the amylose-containing transformants were not significantly different from the activities of the wild-type control plants and differed significantly from amylose-free plants. Transformants with red-staining starch in the tubers did not differ from the *amf* control plants, but were significantly different from wild-type plants (Table 2).

Spearman rank order correlation coefficients (r_s) for GBSS activity and amylose percentage were determined for all possible combinations of plants of the amylose-containing clones. Since three plants of one clone and two plants of two other clones had been investigated, 12 combinations were analyzed. At the 5% level of significance, the correlation coefficient must be larger than 0.503 in order to indicate a positive association between amylose percentage and GBSS activity (Siegel and Castellan 1988). The correlation coefficients found were all below this critical r_s value (0.042–0.399).

Plant number 32-100-1 gave a tuber that remained colorless in the middle and stained only peripherically blue. Some other tubers stained only faintly blue with iodine vapor. From these tubers only small amounts of starch could be isolated, compared to the amounts of starch we isolated from the other blue-staining tubers. Amylose-free tubers with little starch were also observed.

Analysis of stomatal guard cells and pollen

The number of chloroplasts per pair of stomatal guard cells was higher than 20 in all regenerated plants. This means that all regenerants had become at least tetraploid (Frandsen 1968), probably due to a relatively long callus phase. Most of the plants with amylose-containing tubers showed blue-staining chloroplasts in the guard cells of the stomata. In two cases (47-100-27 and 47-150-5) only red-staining chloroplasts were seen. These two plants both formed tubers with amylose-containing starch (Table 2). In a number of cases, differently staining chloroplasts of different stomata within one leaf were also observed.

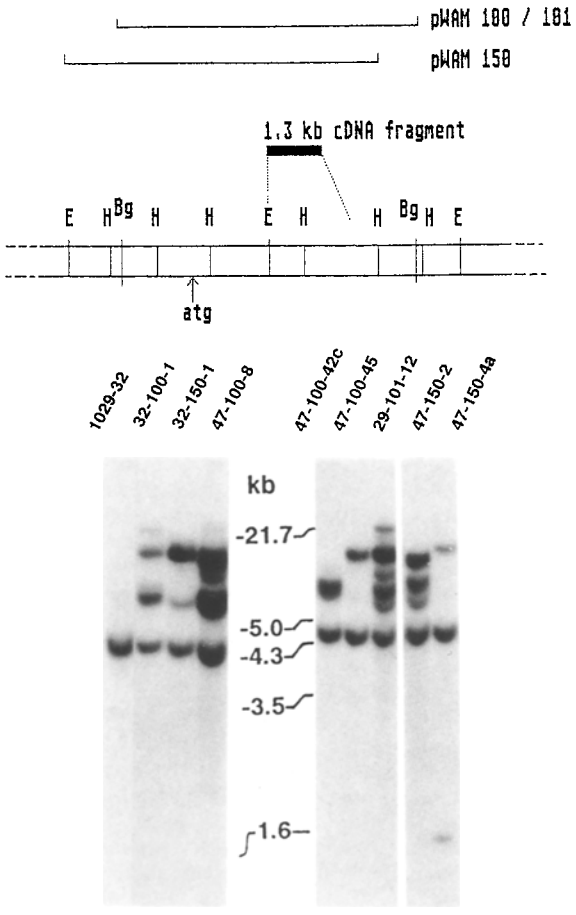

Fig. 2. Southern hybridization of total DNA from transformants with the distal part of the GBSS cDNA as probe. The plant DNA was cut with EcoRI, which gives an endogenous band of 4.3 kb with this probe. *Lane 2* shows some faint bands probably dure to a partial restriction

Less than 10% of the pollen, taken in stages of flowering that normally give about 50% pollen with starch, did stain with iodine. Blue-staining pollen was seen, but in several cases in fewer numbers than red-staining pollen (data not shown), indicating that no clear reflections of segregation ratios were observed, since at least a 1:1 ratio would be expected.

Southern analysis

By using the distal 1.3-kb GBSS cDNA fragment as probe (see Fig. 2), an easy detection of the minimum number of integrated copies could be accomplished by scoring the number of additional bands obtained after EcoRI digestion. This method revealed minimum numbers of integrated copies between one and five (Table 2). However, the actual numbers of integrated copies may be higher in cases of tandem integrations. The latter results in stronger hybridization signals of certain bands, which was sometimes observed.

Discussion

The Amf locus is the gene encoding GBSS

Introduction of the wild-type GBSS allele into the amylose-free potato mutant *amf* lacking GBSS activity leads to restoration of amylose formation and of GBSS activity. The latter was shown by measuring ^{14}C-ADP-glucose incorporation in the starches from subterranean tubers of the transformants. Since all nine control pBIN19-transformed regenerants remained amylose free, whereas eight out of nine pWAM-transformed regenerants showed amylose (Table 1), the presence of amylose must be a consequence of the introduction of the GBSS gene, and the possibility of reversion of the endogenous *amf* allele must therefore be ruled out.

By the complementation of the monogenic recessive *amf* mutation with the GBSS gene, we provide clear evidence that the *Amf* locus in potato is the structural gene for this enzyme. Since the *amf* mutant lacks amylose in all starch-containing tissues (Jacobsen et al. 1989), GBSS is the only enzyme in potato involved in the synthesis of detectable amounts of amylose. The expression of the GBSS gene is not restricted to tissues with reserve starch or to generative tissues, as is the case for the cereals maize, rice, and barley (Shannon and Garwood 1984), and the dicot *Amaranthus* (Okuno and Sakaguchi 1982; our own unpublished results). This is in agreement with the detection of GBSS transcripts by means of dot-spot hybridization of RNA from several tissues of the potato plant (Visser et al. 1989a), and with the phenomena found after introduction of antisense GBSS gene constructs (Visser et al. 1991a, b).

Analysis of transformants

Nearly 90% of the kanamycin-resistant transformants turned out to be complemented, although amylose was not always detectable in all tissues investigated (see, e.g., Fig. 1C). The percentage of complementation was irrespective of the pWAM construct used. The high percentage of coupling between kanamycin resistance and complementation is in agreement with the results found earlier for the introduction of CaMV-*GUS* (β-glucuronidase gene behind the cauliflower mosaic virus 35S promoter) constructs via the same transformation procedure as described here (Visser et al. 1989b).

In general we observed in tubers of complemented plants the occurrence of amylose in percentages of wild-type levels. The highest percentage of amylose measured (23.8%) is comparable to percentages found for "starch-cultivars," e.g., cv Astarte (Hovenkamp-Hermelink et al. 1988). As was found earlier (Visser et al. 1991a, b), there is no correlation between GBSS activity and amylose content but, since GBSS activity was absent or very low only in amylose-free tubers, the appearance of amy-

lose is in all cases correlated with a detectable GBSS activity and with the immunodetection of GBSS. Southern analysis made clear that the minimum number of integrated copies of the GBSS gene was between one and five. However, insertion of one or more copies of the wild-type (wt) GBSS gene does not necessarily lead to complementation (Table 2, 47-100-8). We attribute this phenomenon to a position effect (see, e.g., Vaucheret et al. 1990), since *A. rhizogenes*-mediated integration is often confined to one region of the recipient genome (Visser et al. 1989c). A negative effect on gene expression could be caused by "sense-inhibition" (Linn et al. 1990), to which the GBSS gene has shown to be susceptible (van der Leij et al. 1990). Epigenetic effects may also play a role in the unstable appearance of amylose, which was seen in root tips of single plants, and even within single root tips, leaves, and tubers.

To date, there have only been a few reports of functional complementation of mutants of higher plants by introducing the homologous wild-type alleles. Van Tunen (1991) reported the complementation of a recessive *Petunia Po* mutant with a structural gene for chalcone flavanone isomerase, and Vaucheret et al. (1990) described the complementation of nitrate reductase-deficient *Nicotiana* mutants by introducing the *Nia* genes. In the latter case the enzyme activities of the transformants were much lower than the wild-type levels.

All the transformants described in this paper had become polyploidized, which made them less suitable for crossings and analysis of progenies. Analogous *Agrobacterium tumefaciens*-mediated introduction of the pWAM constructs is currently in progress and will presumably provide more suitable plant material for further genetic and agronomic studies.

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