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Expression of a wild-type GBSS gene introduced into an amylose-free potato mutant by *Agrobacterium tumefaciens* and the inheritance of the inserts at the microsporic level

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Abstract Granule-bound starch synthase (GBSS) catalyses the synthesis of amylose in starch granules. Transformation of a diploid amylose-free (*amf*) potato mutant with the gene encoding GBSS leads to the restoration of amylose synthesis. Transformants were obtained which had wild-type levels of both GBSS activity and amylose content. It proved to be difficult to increase the amylose content above that of the wild-type potato by the introduction of additional copies of the wild-type GBSS gene. Staining of starch with iodine was suitable for investigating the degree of expression of the inserted GBSS gene in transgenic *amf* plants. Of the 19 investigated transformants, four had only red-staining starch in tubers indicating that no complementation of the *amf* mutation had occured. Fifteen complemented transformants had only blue-staining starch in tubers or tubers of different staining categories (blue, mixed and red), caused either by full or partial expression of the inserted gene. Complementation was also found in the microspores. The segregation of blue- and red-staining microspores was used to analyse the inheritance of the introduced GBSS genes. A comparison of the results from microspore staining and Southern hybridisation indicated that, in three tetraploid transgenics, the gene was probably inserted before (duplex), and in all others after, chromosome doubling (simplex). The partial complementation was not due to methylation of the *HPA*II/*MSP*I site in the promoter region. Partially complemented plants had low levels of mRNA as was found when the GBSS expression levels were inhibited by anti-sense technology.

Key words Complementation · Granule-bound starch synthase · Amylose content · Gene expression · Inheritance

Introduction

Starch is the major storage carbohydrate in plants. In potato tubers it consists of about 20% amylose, an unbranched glucose polymer and of 80% amylopectin, a branched glucose polymer (Shannon and Garwood 1984). Amylopectin stains red with iodine-potassium iodine, whereas pure amylose stains blue. The monogenic recessive amylose-free potato mutant (*amf*) lacks granule-bound starch synthase (GBSS) activity and GBSS protein, and the starch stains red with iodine (Hovenkamp-Hermelink et al. 1987). The *amf* mutant is similar to the *waxy* mutant of maize but the phenotypic expression of the former has a different tissue specificity. The *waxy* mutation in maize is expressed in microspores, endosperm and embryo sac (Echt and Schwartz 1981), whereas the *amf* mutation in potato is expressed in all tissues containing transitory and reserve starch, including tubers, stomatal guard cells of leaves, columella cells of root tips, and microspores (Jacobsen et al. 1989). For several plant species it was shown that the *waxy* mutation, resulting in loss of GBSS activity, was due to an alteration in the gene encoding GBSS (Wessler and Varagona 1985; Okagaki and Wessler 1988; Okagaki et al. 1991). In potato, van der Leij et al. (1991b) were able to complement the *amf* mutant with the cloned wild-type GBSS gene by using *Agrobacterium rhizogenes*. Transformants with a blue starch colour in the root tips indicated restored amylose synthesis. A sequence analysis of both the mutated and the wild-type allele of GBSS showed a single base-pair deletion in the transit-peptide region of the gene coding for this protein (van der Leij et al. 1991a). Stable expression of an introduced gene is of considerable importance for the application of the gene-transfer technique in plant breeding. Because the expression of the wild-type GBSS

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allele is easily monitored in an amylose-free background by staining with an iodine solution, this system can be used as a model for examining the stability of the expression of an inserted gene. The inheritance of the inserted gene can be determined directly by iodine staining of the starch in microspores. Furthermore, the consequences of chromosome doubling, either before or after the insertion of T-DNA into the genome, can be critically monitored through the detection of either simplex or duplex segregation at the microspore level. In this paper the expression of an *Agrobacterium tumefaciens* – inserted gene in greenhouse and field-grown tubers and the inheritance of the inserted gene according to the segregation pattern of red- and blue-staining microspores is presented.

Material and methods

Plant material

Stem segments were obtained from a diploid (2n = 2x = 24) homozygous *amf* genotype 87.1029-31 of *Solanum tuberosum* L. This mutant, hereafter called genotype B, was the result of a crossing program between the original *amf* mutant 86.040 and two different diploid wild-types. Crosses were made with the aim of improving the fertility and tuber production of the original mutant (Jacobsen et al. 1989). Several wild-types which were genetically related to 87.1029-31 were used as controls. Plants were grown in jars containing MS medium (Murashige and Skoog 1962) with 30 g/l of sucrose (MS 30) and 8% agar, at 22 °C and 16 h light. Transgenic shoots were grown and multiplied on MS30 medium supplemented with 100 mg/l of kanamycin. Microtubers were obtained by transferring stem segments with one or more nodal buds to MS medium with 60 g/l of sucrose and 1.5 mg/l of BAP. A transformant with T-DNA containing the GUS and NPT-II genes was called BAM and transformants obtained after transformation with the construct pWAM100, containing the complete genomic GBSS sequence, were called B followed by the clone number. Ploidy levels were determined by counting the number of chloroplasts in stomatal guard cells (x, 7–9; 2x, 11–14; 4x, 16–22; Frandsen 1968).

In April 1991 the in-vitro plants were planted in pots in the greenhouse, where they were grown under standard conditions. In May 20 plants per genotype were transferred to the field. In the field the plant distance within a row was 40 cm while that between rows was 75 cm. The plot was surrounded with cv Cleopatra, a red-tuberising, non-flowering potato cultivar. The field experiment was set up according to government rules (Anonymous 1990). Flower buds were removed every 1–3 days. Two plants per genotype remained in pots in the greenhouse in order to evaluate starch composition, amylose content, and GBSS activity in the tubers, the segregation pattern of blue- and red-staining starch in the microspores, and the number of inserted GBSS genes.

Vectors and transformation procedure

The vector pWAM100 was used in this experiment. This construct possesses the genomic GBSS sequence driven by its own promotor in opposite orientation in relation to the gene coding for kanamycin resistance in the plant (van der Leij et al. 1991b). pWAM100 was transferred to *A. tumefaciens* LBA 4404 using the direct transformation procedure described by Höfgen and Willmitzer (1988). To test the integration of the mobilized plasmid into *A. tumefaciens*, plasmid DNA, isolated from *A. tumefaciens* was analyzed (Holmes and Quigly 1981). For the control experiment *A. tumefaciens* strain AM 8706 was used harbouring a binary plasmid, which contains the gene encoding β-glucuronidase and the gene encoding kanamycin resistance (Visser et al. 1989b). Transformation was essentially performed as described by Visser (1991a), but 2 or 3 days after inoculation, explants were transferred to MS 20 medium with 1 mg/l of zeatin, 100 mg/l of kanamycin, 200 mg/l of cefotaxime and 200 mg/l of vancomycin and then transferred to a fresh medium every 3 weeks. On this medium, shoot outgrowth took place after 3 to 4 months. Shoots were isolated and placed on MS 30 medium with 75 mg/l of kanamycin. Only transgenic shoots were able to root on the kanamycin-containing medium.

Staining for starch composition in different tissues

Tubers were stained according to Kuipers et al. (1992). The colour of the individual starch granules was determined by spreading a small sample of starch on a microscopic slide, staining it with a water/Lugol's solution (1:1) and screening it under a microscope. Microspores were stained with the same solution to determine the segregation ratio. Two flowers per genotype and three stamens per flower were stained in each case.

Starch isolation, determination of amylose content and GBSS activity

Starch from greenhouse- and field-grown tubers was isolated according to the method described by Kuipers et al. (1992). Amylose content was determined as described by Hovenkamp-Hermelink et al. (1988) and GBSS activity as described by Vos-Scheperkeuter et al. (1986). For measuring the amylose content, three samples, and for measuring GBSS activity, six samples, of about 2 mg of isolated starch were used. A two-sided t-test with a 5% confidence level was used for the statistical analysis.

Molecular analysis of the transformants

DNA was extracted from the leaves of greenhouse-grown plants according to Dellaporta et al. (1983). By digesting the DNA with the restriction enzyme *Eco*RI and using the ^{32}P-random-prime-labelled, distal 1.3 kb of the GBSS cDNA as a probe the minimum number of integrated T-DNA copies could be found by scoring the number of additional bands in a Southern blot (van der Leij et al. 1991b). DNA and RNA of red- and blue-staining tubers were extracted according to Salehuzzaman et al. (1992). To check whether the inserted GBSS gene was methylated the DNA was digested with *HPA*II or *MSP*I and the proximal part of the GBSS gene, which includes the promoter, was ^{32}P labelled and used as a probe. Equal amounts of RNA (checked by hybridisation with potato 28*s* rDNA as a probe) were loaded to allow a comparison between different transformants and between different tuber parts of one transformant. The RNA was hybridised with a 2.4 kb GBSS cDNA. DNA and RNA blot-hybridisation and labelling were performed as described previously (Visser et al. 1989a, b). RNA transcript levels were densitometrically quantified using the Cybertech CS-1 processing system.

Results

The starch phenotype of in-vitro transformants

After inoculation of stem explants with *A. tumefaciens*, 36 regenerated shoots were harvested from the explants and rooted on MS-medium with kanamycin. From each explant only one shoot was isolated to ensure that all

transgenic shoots resulted from independent transformation events. Sixteen transformants (44%) were diploid, like the original genotype, and 20 (56%) were tetraploid. Microtubers appeared on induction medium after 2–3 weeks. Analysis of cut surfaces after staining with iodine showed that 29 transformants (81%) had blue-staining starch in the microtubers similar to the wild-type potatoes (complemented type). Seven transformants (19%) had microtubers with red-staining starch like the transformed *amf* control (BAM) and the untransformed *amf* mutant (B) (Table 1). Nineteen transformants and controls were selected for further analysis.

Characterisation of greenhouse-grown tubers

The two classes of non-complemented and complemented transformants described above were also found in greenhouse-grown tubers when analysed by Lugol staining. However, the class of complemented transformants could be divided into a partially- and a fully-complemented subclass (Table 1).

The transformants B1, B6, B10, B15 and B17 belong to the group of visually partially-complemented transformants. For the transformants B1, B6 and B15 this was shown by the variation in starch colour of greenhouse-grown tubers. The blue and red colours in the mixed tubers were not separated into clear sectors. The red colour was mostly found in the middle of the tuber, but the presence of blue-staining starch was not absent from these regions. Their starch granules had a red-coloured outer layer, showing up as a red-coloured outer ring around a blue core of varying size. The size of the core was related to the macroscopically-observed tuber colour. All greenhouse-grown tubers of B10 stained red, but their starch granules contained a very small blue core indicating partial complementation. All greenhouse-grown tubers of B17 stained blue, but starch granules were found which contained red outer rings.

The amylose contents and GBSS activities of the untransformed control B and the transformed control BAM were similar (Table 1), indicating that the transformation event itself had no direct influence on starch composition and GBSS activity. The amylose contents and GBSS activities of the non-complemented transformants were like those of the *amf* mutant. For each of the partially-complemented transformants the amylose content and GBSS activity was variable throughout the

Table 1 Analysis of microtubers and greenhouse-grown tubers of transgenic clones and their controls for starch colour after Lugol staining, amylose content and GBSS activity (*ND* not determined, *V* variable within one transformant)

Clones	Colour					Amylose content[b]	GBSS activity[b]
	Microtubers	Number of greenhouse tubers			Starch granules[a]	%(SD)	nmol min^{-1} mg^{-1} (SD)
		Red	Mixed	Blue			
Controls							
B	Red	13 (100%)	–	–	Red	3.0 (0.7)	0.8 (0.3)
BAM	Red	12 (100%)	–	–	Red	3.2 (0.9)	0.5 (0.3)
Wild-type	Blue	–	–	15 (100%)	Blue	23.5 (5.5)	73.4 (18.5)
Transformants							
Non-complemented							
B5	Red	24 (100%)	–	–	Red	2.5 (0.4)	2.4 (0.8)
B11	Red	7 (100%)	–	–	Red	3.9 (0.4)	4.6 (4.8)
B18	Red	11 (100%)	–	–	Red	4.5 (0.1)	0.2 (3.1)
B19	Red	9 (100%)	–	–	Red	3.8 (0.4)	1.2 (3.1)
Partially-complemented							
B1	Blue	–	17 (68%)	8 (32%)	Blue/red	V	V
B6	Blue	1 (6%)	9 (56%)	6 (38%)	Blue/red	V	V
B10	Blue	15 (100%)	–	–	Blue/red	V	V
B15	Blue	10 (67%)	1 (6%)	4 (27%)	Blue/red	V	V
B17	Blue	–	–	9 (100%)	Blue/red	V	V
Fully-complemented							
B2	Blue			17 (100%)	Blue	23.9 (4.2)	105.1 (27.3)
B3	Blue			4 (100%)	Blue	23.2 (1.6)	112.2 (4.0)
B4	Blue			11 (100%)	Blue	24.7 (1.6)	74.1 (15.8)
B7	Blue			17 (100%)	Blue	25.5 (1.1)	113.6 (4.3)
B8	Blue			9 (100%)	Blue	22.2 (1.3)	124.8 (20.5)
B9	Blue			19 (100%)	Blue	22.0 (1.0)	125.6 (10.2)
B12	Blue			7 (100%)	Blue	21.1 (0.7)	82.7 (16.4)
B13	Blue			17 (100%)	Blue	17.5 (0.7)	108.7 (21.1)
B14	Blue			7 (100%)	Blue	22.2 (2.4)	33.2 (12.0)
B16	Blue			22 (100%)	Blue	22.7 (1.2)	ND

[a] The starch granules were isolated from greenhouse-grown tubers
[b] The amylose content (n = 3) and GBSS activity (n = 3) was measured in starch isolated from greenhouse-grown tubers. SD is standard deviation

Table 2 The number and percentage of tubers for the different categories observed after staining with Lugol

Clones	Colour of field tubers		
	Red	Mixed	Blue
	Number (%)	Number (%)	Number (%)
Controls			
B	325 (100)	–	–
BAM	116 (100)	–	–
Wild-type	–	–	372 (100)
Transformants			
Non-complemented			
B5	151 (100)	–	–
B11	125 (100)	–	–
B18	232 (100)	–	–
B19	157 (100)	–	–
Partially-complemented			
B1	29 (17)	60 (36)	80 (47)
B6	24 (20)	48 (40)	48 (40)
B10	141 (80)	28 (16)	7 (4)
B15	102 (70)	40 (28)	3 (2)
B17	–	3 (2)	163 (98)
Fully-complemented			
B2	–	–	227 (100)
B3	–	–	77 (100)
B4	–	–	125 (100)
B7	–	–	151 (100)
B8	–	–	140 (100)
B9	–	–	229 (100)
B12	–	–	130 (100)
B13	–	–	167 (100)
B14	–	–	143 (100)
B16	–	–	259 (100)

tuber, depending on the size of the blue core in the starch granules (data not shown). The amylose contents and GBSS activities of the fully-complemented transformants were in the range of that of the wild-type controls. The GBSS activities were highly variable as was also seen for the wild-type controls (Table 1). Using the Spearman rank correlation test, a significant ($P < 0.05$) correlation was found between the amylose content and GBSS activity.

Characterisation of field-grown tubers

The staining results of the field-grown tubers resembled those of the greenhouse grown tubers. Only B17 had more red-staining tuber parts while some tubers of B10 had blue-staining parts. The blue and red colour in the mixed tubers, in contrast to greenhouse-grown ones, was more clearly concentrated in separate parts and different staining patterns could be observed.

Molecular analysis

No difference in restriction pattern could be found when restricting the DNA with *MSP*I or *HPA*II followed by hybridisation analysis, which indicates that methylation in the promoter region of the GBSS gene was not found (data not shown). However, this is not a proof that methylation outside the promoter region may not interfere with the expression of the inserted GBSS gene. RNA was extracted from tubers of different transformants and Northern blots were made. Almost all transformants showed a band of the correct size (Fig. 1 A); no correlation was found between the amount of GBSS mRNA and the starch colour among the different transformants. However, when RNA was extracted from blue- and red-staining tuber parts of a partially-complemented transformant, there seemed to be a relation between the degree of complementation and the amount of mRNA found. A clear GBSS mRNA signal was found in blue-staining parts, a weak signal in red-staining parts and an intermediate signal in the mixed-staining parts (Fig. 1 B). Quantification of the signals showed that there could be three times more transcript present in a blue-staining part of a tuber than in a red-staining part of the same tuber.

Genetical analysis and determinatation of copy number

In Table 3 the transformants that flowered were classified according to their segregation of blue- and red-staining microspores. The transformants B5 and B11 without GBSS expression in their microtubers and in greenhouse- and soil-grown tubers also lacked expression in their microspores which stained red with iodine,

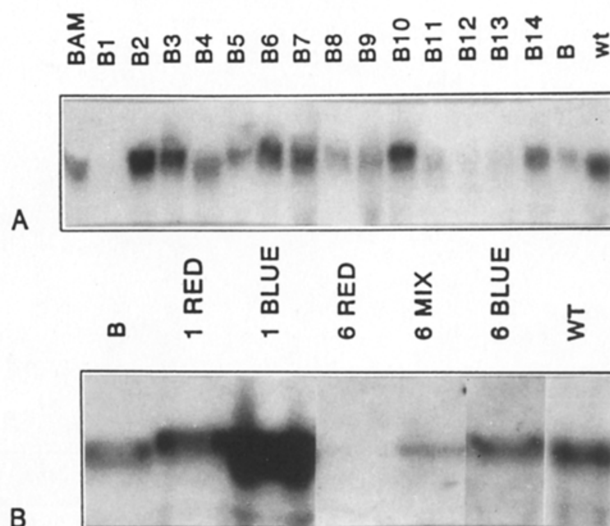

Fig. 1A, B Northern hybridisations of different transformants and controls with a 2.4-kb GBSS cDNA. **A** Expression of GBSS mRNA in greenhouse-grown tubers. **B** GBSS mRNA expression in red-, mixed- and blue-staining tuber parts of the partially-complemented transformants B1 and B6; the values given below were determined by densitometrical scanning of the autoradiograph and are in comparison with the red-staining tuber part: 1 red = 1, 1 blue = 2.5, 6 red = 1, 6 mixed = 1.6 and 6 blue = 3.0

Table 3 The ploidy level, microspore segregation after staining with Lugol, the number of inserts found by Southern hybridisation, and the colour of field-grown tubers of different transgenic potato clones

Clone	Ploidy level	Segregation ratio	T-DNA[a] copies	Starch colour of field tubers
		Blue : Red		
		Class I: no alleles; – – or – – – – (0:1)		
B	2x	0: 825	0	Red
BAM	2x	0:1000	0	Red
B5	4x	0:1324	0	Red
B11	4x	0: 987	1	Red
		Class II: one allele, simplex; *GBSS* – or *GBSS* – – – (1:1)		
B7	2x	445:413 ($\chi^2 = 0.08$)	2	Blue
B9	2x	250:235 ($\chi^2 = 0.35$)	2	Blue
B12	4x	234:210 ($\chi^2 = 1.30$)	1	Blue
B14	4x	337:333 ($\chi^2 = 0.02$)	2	Blue
		Class III: two alleles independently segregating, double simplex; $GBSS_1$–, $GBSS_2$ – or $GBSS_1$ – – –, $GBSS_2$ – – – (3:1)		
B2	2x	583:218 ($\chi^2 = 2.10$)	2	Blue
B8	4x	500:139 ($\chi^2 = 3.59$)	2	Blue
		Class IV: one allele in duplex; $GBSS_1$ $GBSS_1$ – – (5:1)		
B1	4x	188:30 ($\chi^2 = 1.32$)	1	Mixed
B4	4x	347:80 ($\chi^2 = 1.30$)	3	Blue
B6	4x	887:181 ($\chi^2 = 0.06$)	1	Mixed

[a] The number of additional inserts according to the Southern hybridization

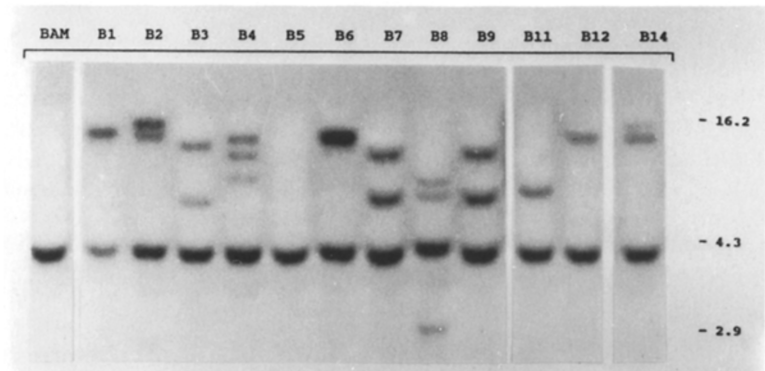

Fig. 2 Southern hybridisation of total DNA from transformants with the distal part of the GBSS cDNA as a probe. The plant DNA was cut with *Eco*RI, which gives an endogenous band of 4.3 kb with this probe. The band of 2.9 kb in lane B8 indicates the insertion of a truncated GBSS gene

like the controls. Southern hybridisation (Fig. 2) of the non-complemented clone B11 indicated that one inactive GBSS gene was present in the genome. For B5 no inserts could be detected. A segregation according to one active insertion was found in the diploid transformants B7 and B9 (*GBSS* –) and in the tetraploid B12 and B14 (*GBSS* – – –; simplex). According to the Southern hybridisation one or two inserted genes appeared to be present. Because of this monogenic inheritance the second gene in B7, B9 and B14 is expected to be inactive or else closely linked to the first one. A segregation of 3:1, indicating that two genes are segregating independently, was found for the diploid B2 ($GBSS_1$ –, $GBSS_2$ –) and the tetraploid B8 ($GBSS_1$ – – –, $GBSS_2$ – – –) and was confirmed by Southern analysis (Fig. 2). B8 contained a truncated GBSS gene as well. The tetraploid transformants B1, B4 and B6 had a segregation of 5:1 indicating a duplex situation (*GBSS GBSS* – – –). One band was found by Southern hybridisation (Fig. 2) for B1 and B6, supporting this duplex situation. For B4 three bands were found. The duplex segregation suggests that two of them are inactive, or else placed on the same chromosome as the gene in duplex.

Discussion

Using the *A. tumefaciens* system, the introduction of the GBSS allele into the *amf* mutant lacking GBSS activity leads to a restoration of GBSS activity and amylose synthesis (Table 1). This is in agreement with the observations of van der Leij et al. (1991b) after using *A. rhizogenes* as a vector. With respect to starch colour in subterranean tubers three different groups of transform-

ants were found: non-complemented, fully complemented, and partial complemented. Transformants of the latter class contained starch granules which had a blue core of varying size and a red outer ring. This has also been observed after transforming a wild-type potato with an anti-sense GBSS gene (G. J. Kuipers, unpublished results). The amylose contents and GBSS activities of these transformants varied depending on the size of the blue core in the starch granules.

The GBSS gene was also expressed in microspores. According to the segregation of red- and blue-staining microspores, up to two independently-segregating active inserts were present. Comparison of the results of the microspore segregation and the Southern hybridisation indicated that sometimes more than one insert could be present on one chromosome or else not all inserts were active (Table 3). The tetraploid transformants B1 and B6, with unstable expression of the inserted gene, were probably duplex (Table 3). This means that insertion had taken place here before chromosome doubling, leading to a duplex situation. The active inserts in the fully-complemented tetraploid plants were either situated on two non-homologous chromosomes or at different positions on homologous chromosomes. The amount of GBSS mRNA varied among the different transformants and was not correlated to the expression of the inserted GBSS gene (Fig. 1A). This could be expected because Visser et al. (1989b) reported the presence of normally-sized GBSS mRNA in the *amf* mutant. However, within a single transformant, a relation between the degree of expression and amount of mRNA could be found based on a densitometric quantification of the bands. A small amount of mRNA was present in the amylose-free red-staining parts of the tubers and this amount increased with an increase in amylose content (Fig. 1B).

Unstable expression of an inserted gene has been described earlier and, amongst others, methylation or sense inhibition are mentioned as possible causes. Unstable gene expression was found in a petunia mutant transformed with the A1 cDNA of maize (Meyer et al. 1987) and was due to methylation of the promoter; this was positively correlated to the number of integrated genes and also influenced by the chromosomal position of the inserted genes (Linn et al. 1990). Similar results were reported by Matzke et al. (1989) who found methylation and inactivation of a first inserted T-DNA after the integration of a second T-DNA gene and presumed, therefore, that the methylation was the result of an interaction of homologous sequences. In the present study, however, no indication was found for methylation of the GBSS gene in the red sectors of the mixed-staining tubers.

Another possible explanation for the unstable gene expression is sense- or co-suppression. Co-suppression is the phenomenon whereby the mutant phenotype can be obtained after introduction of one or more copies of the wild-type gene into wild-type plants.

Grierson et al. (1991) suggested that the anti-sense RNA was formed because of a simple read-through of the kanamycin resistance gene which was placed in opposite orientation. GBSS gene expression in wild-type potato has been inhibited in previous work by using anti-sense RNA (Visser et al. 1991b) or by introducing an extra GBSS gene (van der Leij et al. 1990). In the present research the construct pWAM100 which was used possesses the gene for kanamycin resistance in opposite orientation and in relation to the GBSS gene, so that the kanamycin resistance gene could have read-through. Another explanation could be that a strong promoter in the plant DNA near the site of T-DNA insertion caused the formation of anti-sense RNA. However, the main difference with the above mentioned previous research on anti-sense genes is that we introduced wild-type genes in a mutant background. Although the observations in the present investigation were similar to those obtained after inhibiting GBSS activity in wild-type potato with anti-sense GBSS RNA, we were not able to detect anti-sense RNA in the transformants (data not shown). The fact that the partially-complemented plants showed a decrease in GBSS mRNA upon an increase of red-staining starch in tubers might indicate that co-suppression plays a role in these plants. This research shows that a wild-type gene inserted into the mutant lacking the activity of this particular gene is not always fully expressed. For the application of gene manipulation in plant breeding, stable expression of an inserted gene is important. Selection of plants with stable gene expression should be performed before using them in plant breeding.

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