The Plant Cell, Vol. 6, 43–52, January 1994

Formation and Deposition of Amylose in the Potato Tuber Starch Granule Are Affected by the Reduction of Granule-Bound Starch Synthase Gene Expression

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The synthesis of amylose in amyloplasts is catalyzed by granule-bound starch synthase (GBSS). GBSS gene expression was inhibited via antisense RNA in *Agrobacterium rhizogenes*-transformed potato plants. Analysis of starch production and starch granule composition in transgenic tubers revealed that reduction of GBSS activity always resulted in a reduction of the production of amylose. Field experiments, performed over a 2-year period, showed that stable inhibition of GBSS gene expression can be obtained. Microscopic evaluation of iodine-stained starch granules was shown to be a sensitive system for qualitative and quantitative examination of amylose formation in starch granules of transgenic potato tubers. In plants showing inhibition of GBSS gene expression, the reduced amylose content in tuber starch was not a consequence of a lower amylose content throughout the entire starch granule. Starch granules of transgenic tubers were found to contain amylose at a percentage similar to wild-type starch in a core of varying size at the hilum of each granule. This indicated that reduced GBSS gene expression results in amylose formation in a restricted zone of the granules. The size of this zone is suggested to be dependent on the GBSS protein level. During development of the granules, the available GBSS protein is thought to become limiting, resulting in the formation of starch that lacks amylose. RNA gel blot analysis of tuber tissue showed that inhibition of GBSS gene expression resulted in a reduced GBSS mRNA level but did not affect the expression level of other starch synthesizing enzymes. Antisense RNA could only be detected in leaf tissue of the transgenic plants.

INTRODUCTION

Starch, the major storage carbohydrate of higher plants, is synthesized for long-term storage in amyloplasts of, for example, potato tubers. In chloroplasts, starch is produced for the transient storage of photosynthetic products. The main components of starch are linear helical amylose (molecular weight between 10^4 and 10^5) and branched amylopectin (molecular weight up to 10^7), which are both glucose polymers. Reserve starch in potato tubers contains 18 to 23% amylose (Shannon and Garwood, 1984). The growth of starch granules occurs via apposition (Badenhuizen, 1963). The structure of starch granules is determined by the crystalline organization of the amylopectin molecules (Oostergetel and van Bruggen, 1989). From cross-linking experiments in potato and maize, it is known that amylose molecules are interspersed among the radially arranged amylopectin molecules (Jane et al., 1992). Transitory and reserve starch are synthesized from glucose by the same set of enzymes of which starch synthases and branching enzymes are key enzymes. Starch synthases catalyze the elongation of amylose and amylopectin by adding ADP-glucose to the nonreducing end of the polymers and can be divided into soluble starch synthases and granule-bound starch synthase (GBSS), the latter being involved in the biosynthesis of amylose (Robyt, 1984).

Recently, GBSS gene expression was shown to be inhibited after the introduction of an antisense GBSS gene construct into the potato genome (Visser et al., 1991a). The antisense RNA–mediated inhibition is found to vary between and within the transgenic clones. In tuber starch of one of these clones, GBSS activity is inhibited up to 100%. Complete suppression of GBSS activity is shown to correlate with the absence of GBSS protein and amylose. Partially suppressed GBSS activity is accompanied by a reduction of GBSS protein level and amylose content.

Antisense gene expression, resulting in varying degrees of reduction of protein level and enzyme activity as observed in the transgenic clones mentioned above, has also been reported for other systems. In potato, inhibition of ADP-glucose-pyrophosphorylase (AGPase) expression results in a varying, but clearly reduced amount of protein and enzyme activity in eight of 35 transformants (Müller-Röber et al., 1992). This led to the inhibition of starch synthesis in tubers and accumulation of soluble sugars. Antisense-mediated inhibition of chalcone synthase (*CHS*) gene expression in petunia results in a reduction of CHS protein level varying for individual transformants

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(van der Krol et al., 1988). Furthermore, variation in flower phenotype is observed both between and within individual transformants (van der Krol et al., 1990).

In this study, the varying antisense RNA–mediated inhibition of GBSS gene expression that has been described before (Kuipers et al., 1991) was further examined in field-grown potato plants. The influence of the introduction of the antisense GBSS gene on GBSS steady state mRNA levels was studied in leaf and tuber tissue. Furthermore, the presence of GBSS antisense RNA was determined in these tissues, and the effect of inhibition of GBSS gene expression on the expression levels of other starch synthesizing enzymes was determined in tuber tissue.

A model is presented to quantify the amylose content in starch using the iodine-staining pattern of starch granules from the transgenic plants, and the effect of reduced GBSS gene expression on the formation of amylose in starch granules is discussed.

RESULTS

Phenotypic Characterization of Transgenic Plants

The level of antisense RNA–mediated inhibition of GBSS gene expression in tubers of the four transgenic potato clones was compared in two successive field trials. In 1990, the field trial was set up with tubers from greenhouse-grown plants that were sprouted in pots in the greenhouse and subsequently transferred to the field. All harvested tubers were stained with iodine to evaluate the presence of amylose, as shown in Figure 1A (for more detailed description, see Kuipers et al., 1991).

The 1991 field trial was set up with field-grown tubers obtained from the 1990 field trial. Tubers obtained from the 1991 field trial were phenotypically analyzed in a random iodine staining test (Figure 1B). This analysis confirmed the results of the 1990 field trial concerning the total and stable inhibition of GBSS gene expression in tubers of WA501. However, in WA511, WA516, and WA517, the relative amount of mixed staining tubers was much higher than in 1990. Tuber staining for these genotypes ranged from almost red (amylose free) via mixed staining, as shown in Figure 2A, to blue (amylose containing). Despite the shift toward a higher percentage of tubers with mixed staining starch, WA511 could be distinguished from WA516 and WA517 because of a higher percentage of red-staining tubers in both years. Most tubers of the genotypes WA516 and WA517 were found to contain mixed staining starch. In both field trials, the phenotype of parental tubers of the genotypes WA516 and WA517 did not determine the ratio of red-, mixed-, and blue-staining tubers.

Distribution of Amylose in Isolated Starch Granules

To gain more insight into the nature of the different staining categories that were observed among tubers with reduced

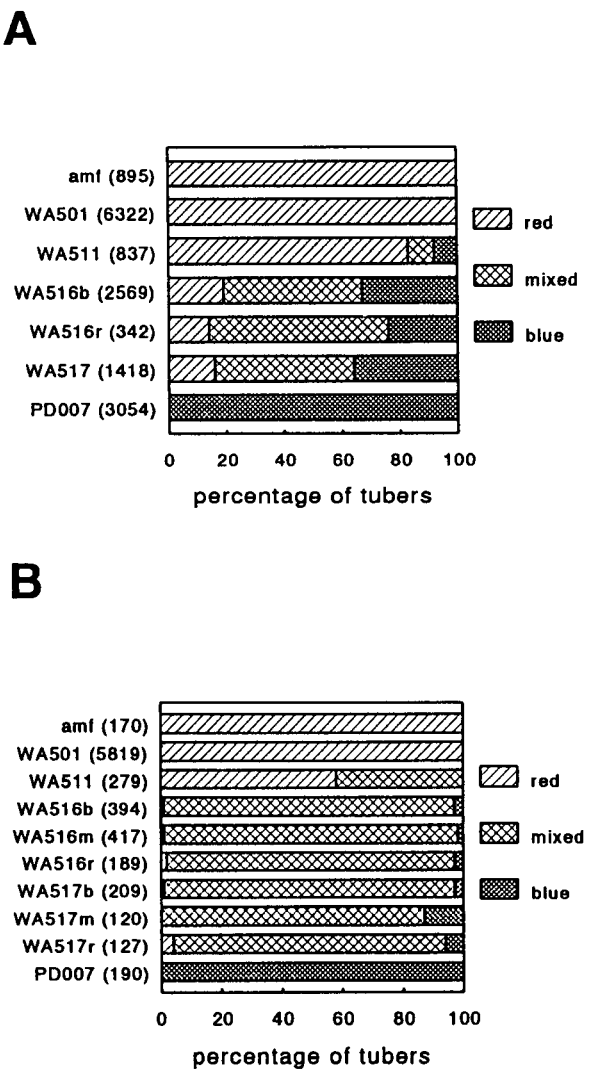

Figure 1. Comparison of the Distribution of Staining Categories of Tuber Sections from Field-Grown Tubers.

(A) Tubers obtained from the field trial in 1990.
(B) Tubers obtained from the field trial in 1991.
amf, *amf* mutant 1031-29; PD007, nontransformed wild type; WA501 and WA511, plants grown from red parental tubers; WA517, plants grown from blue parental tubers; WA516b and WA517b, plants grown from blue parental tubers; WA516m and WA517m, plants grown from mixed-staining parental tubers; WA516r and WA517r, plants grown from red parental tubers. Within parentheses are the numbers of harvested tubers that were stained per genotype.

GBSS expression, iodine-stained starch granules from transgenic and control genotypes were examined microscopically. Completely blue-staining starch was found in wild-type starch granules, whereas starch granules of the amylose-free (*amf*) mutant of potato always were red, occasionally with a faint blue line at a growth ring. Remarkable staining patterns were observed in the starch granules of WA501, WA511, WA516, and

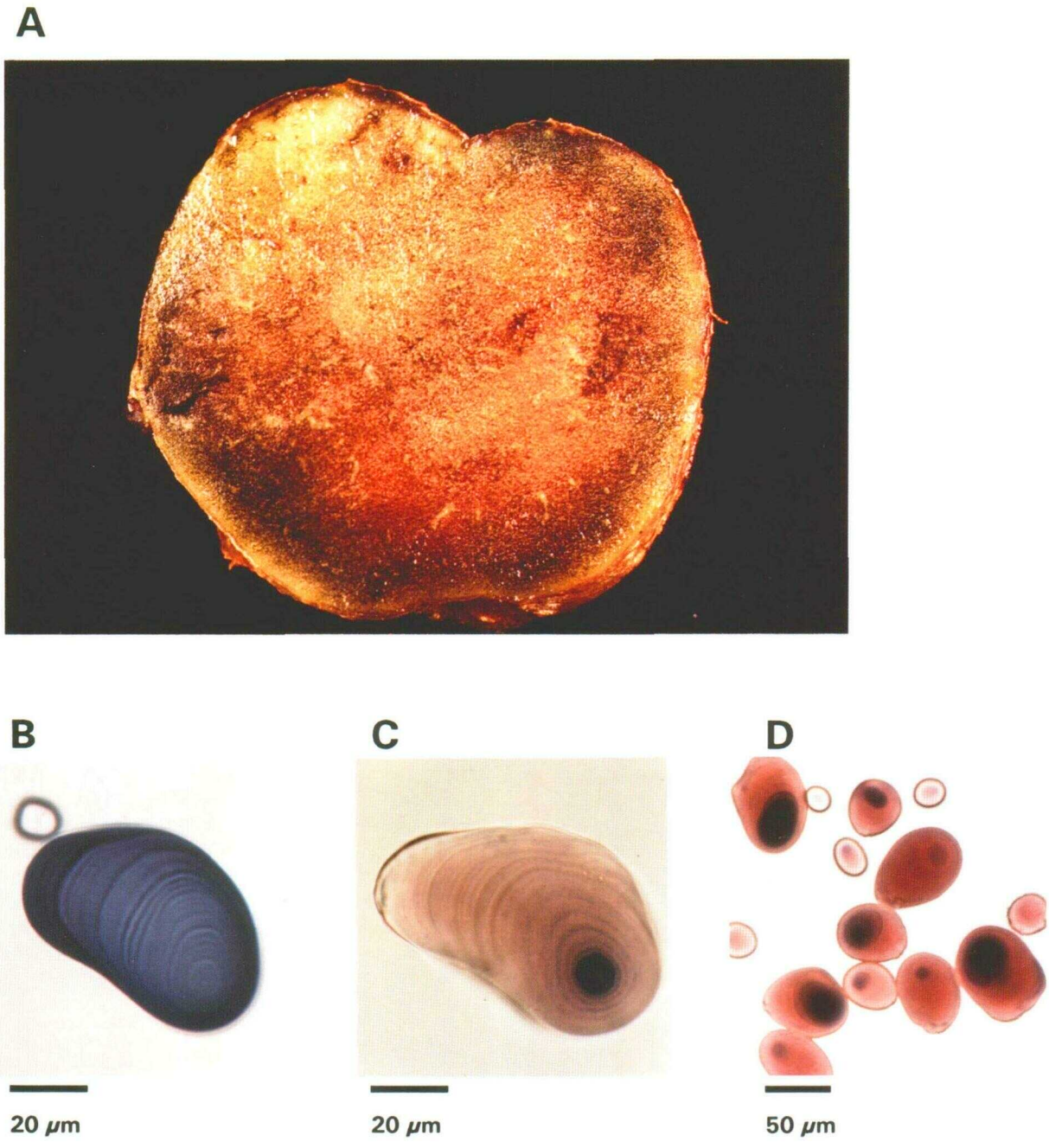

Figure 2. Iodine Staining of Tuber Starch.

(A) Longitudinal section of a WA516 tuber with mixed-staining starch.
(B) Blue-staining starch granules of PD007.
(C) WA501 starch granules with blue-staining core at the hilum and blue lines at the red-staining growth rings.
(D) WA516 starch granules with blue-staining cores of varying size.

WA517, which contained a blue-staining core around the hilum (at the center) of each starch granule, as shown in Figures 2B to 2D. Apparently, the formation of amylose in tubers with reduced GBSS expression is restricted to the hilum of the starch granules.

In the transgenic genotypes, the blue core varied in size but never filled the whole starch granule. At some of the inner red-staining growth rings, concentric blue lines occurred. In WA501 granules, a small blue core was found at the hilum of each granule. The blue-staining core of starch granules from the other genotypes was larger in blue-staining tuber sections than in red-staining tuber sections. In individual cells, the size of the blue core, relative to the size of the complete granule, was found to be comparable for all amyloplasts. Starch granules with a blue core and a red outer part were observed in field-grown and greenhouse-grown tubers as well as in microtubers (data not shown). According to the size of the blue core, three classes of starch granules were observed. They could be related to the macroscopically determined staining types of the tuber sections. The first class consisted of granules with a small blue core, corresponding with red tuber sections. The second class of granules contained a medium-sized blue core, corresponding with mixed-staining tuber sections, and the third class consisted of granules with a large blue core, which corresponded to blue tuber sections.

To quantify the size of blue cores in individual starch granules of each of these classes, we measured the short (R_{width}) and long (R_{length}) radii of the blue-staining core and the total granule using an ocular micrometer. Assuming an ovoid granule shape, the volume of the blue-staining core (V_{blue}) and the total granule (V_{total}) were calculated and used for the calculation of the ratio (V_{blue}/V_{total}) between the volumes of the blue core and the total granule, as shown in Table 1. This ratio represents the relative size of the blue-staining core in starch granules of transgenic tubers.

Table 1. Quantification of the Size of the Blue-Staining Core in Starch Granules from Transgenic Tubers Obtained from the 1990 Field Trial

Observed Size of Blue Core[a]	No. Granules Analyzed[b]	Range V_{blue}/V_{total}[c]	Average V_{blue}/V_{total}[c]
Small (WA501)	42	0.3×10^{-4}–0.5×10^{-2}	0.11×10^{-2}
Small	50	0.2×10^{-3}–0.04	0.80×10^{-2}
Medium	54	0.04–0.70	0.25
Large	58	0.70–0.95	0.85

[a] Small (WA501), blue cores present in starch granules from red-staining tubers of WA501; small, medium, large, blue cores present in starch granules from red-, mixed-, and blue-staining tubers, respectively, of the transgenic clones WA511, WA516, and WA517.
[b] Using an ocular micrometer, the short and long radii of the blue core ($R_{blue,width}$ and $R_{blue,length}$) and the total granule ($R_{total,width}$ and $R_{total,length}$) were measured.
[c] $V_{blue}/V_{total} = (R^2_{blue,width} \times R_{blue,length})/(R^2_{total,width} \times R_{total,length})$.

Table 2. Average Volume Ratio of the Blue-Staining Core in Starch Granules from Tubers of Transgenic Genotypes

Genotype	Classification of Tubers According to Size of Blue Core[a]: % Small (WA501)	% Small	% Medium	% Large	$(V_{blue}/V_{total})_{genotype}$[b]
WA501	100	—[c]	—	—	0.11×10^{-2}
WA511	—	83	9	8	0.97×10^{-2}
WA516b[d]	—	19	48	33	0.40
WA517	—	16	48	36	0.42

[a] Transgenic tubers obtained from the 1990 field trial were stained with iodine and classified according to the size of the blue core, which is related to the staining category of tuber sections, as shown in Figure 1A.
[b] $(V_{blue}/V_{total})_{genotype} = [\%small \times (V_{blue}/V_{total})_{small} + \%medium \times (V_{blue}/V_{total})_{medium} + \%large \times (V_{blue}/V_{total})_{large}]/100\%$.
[c] Dashes indicate the absence of starch granules in the specified class.
[d] WA516b, plants grown from blue-staining parental tubers.

Based on the relative occurrence of red-, mixed-, and blue-staining tubers (Figure 1A), which contained starch granules with small, medium, or large blue cores, respectively, the average volume ratio of the blue-staining core was determined for each of the transgenic genotypes. Table 2 shows that in the transgenic genotypes the average volume of the blue-staining cores was always less than 50% of the total volume of the starch granule. In the genotype WA501, which showed the most pronounced reduction of GBSS expression, 0.1% of the total starch volume contained blue-staining starch. The average volume ratios were used to determine whether the blue cores represented the amylose that is present in the starch granules.

Biochemical Analysis of Tuber Starch

The amylose content and GBSS activity were determined in individual tubers of the transgenic genotypes that were obtained from the 1990 field trial. Both amylose content and GBSS activity could be related to the amount of amylose-containing starch, as surveyed by iodine staining of these tubers, which is shown for WA517 in Figure 3. For WA511, WA516, and WA517, a higher relative radius of the amylose-containing blue core in iodine-stained starch granules was invariably associated with a higher amylose content and GBSS activity.

A larger scale analysis with respect to the amylose content was conducted on tuber samples of all genotypes from the 1990 field trial. Using amperometric titration, amylose could

not be detected in starch from the *amf* mutant, WA501, and WA511 in spite of the presence of blue-staining cores in starch granules of WA501 and WA511, as shown in Table 3.

The determination of the amylose content and the average volume ratio of blue cores in starch granules is based on the formation of an iodine–starch complex. To evaluate the relationship between these two characteristics, a regression analysis was performed for the average volume ratio and the amperometrically determined amylose contents of the transgenic and control genotypes, as shown in Figure 4. This resulted in the least-squares regression curve $Y = 20 \times X^{1.5}$ with an R^2 value of 0.999. When expressed as a function of $(V_{blue}/V_{total})_{genotype}$, the equation is

$$(\text{amylose \%})_{genotype} = (\text{amylose \%})_{PD007} \times [(V_{blue}/V_{total})_{genotype}]^{1.5}.$$

This relationship indicated that in tuber starch granules of the transgenic genotypes, all amylose is located in the blue cores and that the amylose content of the blue cores is similar to that of wild-type starch, irrespective of the relative size of the blue cores. The nonlinearity of the relationship can be explained from the increase of the amylose content that is found during tuber development (Shannon and Garwood, 1984). The equation enabled calculation of the amylose content for transgenic genotypes based on the staining pattern of starch granules, as shown in Table 3. A regression analysis was also performed for $(V_{blue}/V_{total})_{genotype}$ and the GBSS activity, which were shown to be correlated as well. Because of variation in GBSS activity measurements, a reliable least-squares regression curve could not be determined (data not shown).

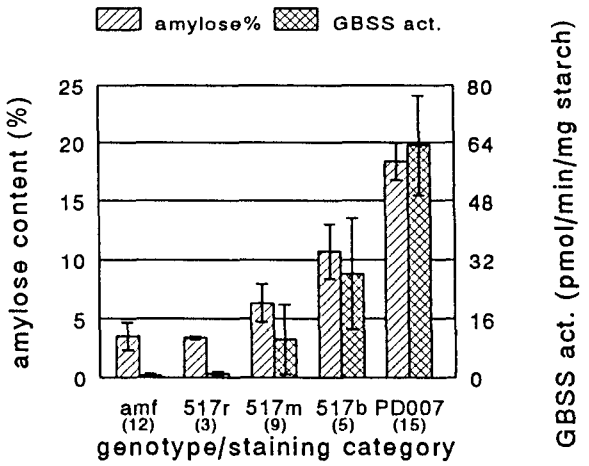

Figure 3. Amylose Content and GBSS Activity in Tubers of WA517.

The amylose content (spectrophotometric method) and GBSS activity (act.) were determined in individual WA517 tubers of different staining categories that were obtained from the 1990 field trial. A comparison was made with the nontransformed wild type and the *amf* mutant. amf, *amf* mutant 1031-29; PD007, nontransformed wild type; r, red-staining tuber starch; m, mixed-staining tuber starch; b, blue-staining tuber starch. Within parentheses are the numbers of tubers analyzed per genotype or staining category. The error bars indicate SDs.

Table 3. Amperometrically Determined Amylose Content and Calculated Amylose Content Based on $(V_{blue}/V_{total})_{genotype}$

Genotype	Amylose Content (%)[a]	Calculated Amylose Content (%)[b]
WA501	0	0.0
WA511	0	0.6
WA516b[c]	5.0	5.0
WA517	5.4	5.5
PD007	20.0	20.0
1031-29	0	0

[a] Determined amperometrically in starch isolated from tubers pooled per genotype.
[b] Using $(V_{blue}/V_{total})_{genotype}$ and the amylose content determined for the nontransformed PD007, the expected amylose content could be calculated for the transgenic genotypes: $(\text{amylose \%})_{genotype} = (\text{amylose \%})_{PD007} \times [(V_{blue}/V_{total})_{genotype}]^{1.5}$.
[c] WA516b, plants grown from blue-staining parental tubers.

RNA Analysis

RNA was isolated from halves of individual tubers that were previously iodine stained and of which the other half was analyzed for amylose content and GBSS activity. RNA gel blot hybridization with a potato rDNA fragment as a probe showed equal amounts of total RNA in each lane. Hybridization of tuber RNA using the GBSS mRNA-specific single-stranded probe revealed a reduced amount of GBSS mRNA in all tubers from transgenic genotypes when compared with the wild type, as shown in Figure 5A. Quantification of the autoradiograms showed a reduction of the GBSS mRNA steady state level varying from 34 to more than 99%. However, there seemed to be no clear relation between the decrease of the GBSS mRNA level and the inhibition of GBSS activity or the reduction of the amylose content in individual tubers.

Using strand-specific probes, it was not possible to detect antisense RNA in tubers. Therefore, RNA was isolated from leaves, in which the GBSS expression level is lower than in tubers, and hybridized with the GBSS cDNA fragment (Figure 5B) and with both strand-specific probes. This hybridization showed that leaf RNA of the transgenic genotypes contained reduced amounts of GBSS mRNA in addition to antisense RNA. The amount of antisense RNA appeared to be highest in WA501: the genotype with the most pronounced suppression of GBSS expression in tubers and lowest in WA516 and WA517. This finding pointed toward a relationship between the amount of antisense RNA present in the leaves and the average

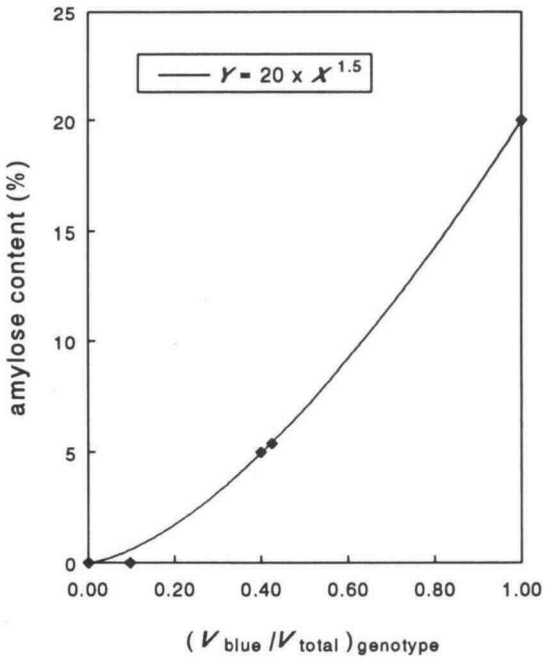

Figure 4. Amylose Content of Transgenic Genotypes Versus Average Volume Ratio $(V_{blue}/V_{total})_{genotype}$ of Blue Cores in Starch Granules.

The amylose content was determined amperometrically (see Table 3). $(V_{blue}/V_{total})_{genotype}$ was determined as described in Table 2. Regression analysis was performed with the curve fit function of SlideWrite Plus 5.00 (Advanced Graphics Software, Inc., Carlsbad, CA).

level of antisense RNA–mediated inhibition of GBSS gene expression in tubers of the four genotypes.

Because the antisense construct pGB50 is based on the full-length GBSS cDNA (~2.4 kb), correct transcription of the antisense gene after insertion into the potato genome should result in an antisense RNA of approximately the same length as the GBSS mRNA. Our hybridization results showed an antisense RNA length of ~1.3 kb, which might be due to a premature stop of transcription.

To verify whether the antisense inhibition of GBSS gene expression affects the expression level of other genes involved in starch biosynthesis, RNA gel blot hybridizations were conducted using probes for several genes. Both sucrose synthase and AGPase are involved in the biosynthesis of ADP-glucose, the precursor for the biosynthesis of amylose and amylopectin. Sucrose synthase catalyzes the cleavage of sucrose into fructose and UDP-glucose, which is converted into glucose-1-phosphate. AGPase subsequently converts glucose-1-phosphate into ADP-glucose. In this analysis, no significant alterations in the expression level of sucrose synthase and AGPase S, encoding one of the AGPase subunits, were observed in tubers with varying levels of inhibition of GBSS gene expression (data not shown). Also for the branching enzyme involved in the biosynthesis of amylopectin, mRNA steady state levels did not differ significantly from wild-type levels.

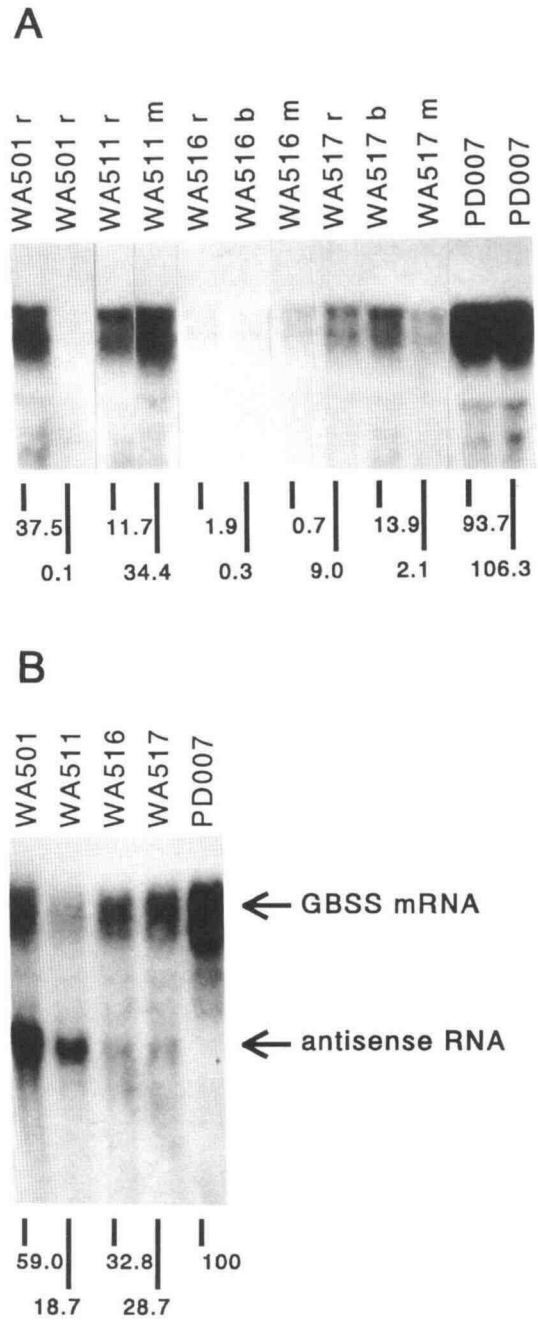

Figure 5. RNA Gel Blot Analysis of Tuber and Leaf RNA of the Transgenic Clones.

(A) Hybridization of RNA from individual tubers using a double-stranded GBSS cDNA probe. r, tuber with red-staining starch; m, tuber with mixed-staining starch; b, tuber with blue-staining starch.

(B) Hybridization of leaf RNA for GBSS mRNA and GBSS antisense RNA using a double-stranded GBSS cDNA probe. (GBSS mRNA and GBSS antisense RNA were detected individually using both strand-specific probes).

Below each lane, the GBSS mRNA steady state level is given as a percentage of the GBSS mRNA steady state level in the nontransformed PD007.

DISCUSSION

Antisense Inhibition in Tubers: Variability and Subcellular Origin

In this study, the effect of inhibition of GBSS gene expression on the production of amylose was analyzed in tuber tissue and subcellularly in starch granules. The macroscopically determined staining patterns of tuber sections and the amylose content and GBSS activity showed that the stable and total suppression of GBSS gene expression, resulting in the production of nearly amylose-free starch in the transgenic clone WA501, was reproducible during two successive generations of field-grown tubers. Variable inhibition of GBSS gene expression among the genotypes WA511, WA516, and WA517 was observed in each of the tuber generations. In 1990 and 1991, a higher percentage of red-staining tubers was found for WA511 compared with WA516 and WA517. For WA516 and WA517, the phenotype of parental tubers did not determine the classification of tubers into staining categories. Therefore, the variability among genotypes is supposed to be caused by position effects, which are known to affect the expression of inserted genes in transgenic plants (Dean et al., 1988).

Surprisingly, the variation in the degree of inhibition as observed in tuber tissue could be related to a distinctive staining pattern of starch granules that consisted of a blue core at the hilum surrounded by red growth rings. The staining pattern is an obvious distinction between the transgenic clone WA501 and the *amf* mutant. This difference is due to the fact that the *amf* mutation directly affects the GBSS gene expression in all starch-synthesizing tissues, whereas the antisense RNA–mediated inhibition is dependent on the relative expression levels of the GBSS gene and the antisense gene in each tissue. A small blue core at the hilum of starch granules has been reported for *waxy* maize (Shannon and Garwood, 1984) and is thought to originate from the complexation of amylose and lipid at the initiation of granule formation (French, 1984).

Tuber starch granules with blue-staining cores of varying size were also observed after the inhibition of GBSS gene expression with an antisense GBSS gene driven by the promoter of the potato GBSS gene (A. G. J. Kuipers, unpublished results). In transgenic clones, the GBSS promoter–driven antisense gene and the endogenous GBSS gene are likely to be expressed in a similar way, although position effects might modulate the expression level of the inserted gene. Therefore, the blue cores are supposed to originate from the reduced expression level of the GBSS gene rather than from a dramatic increase of the antisense effect during tuber development. This assumption is sustained by the observation of starch granules with an identical staining pattern after incomplete complementation of the *amf* mutant with the potato GBSS gene (Flipse et al., 1994).

Because in individual cells, the relative size of the blue core was found to be comparable for all amyloplasts, the available GBSS protein seemed to be equally distributed to all amyloplasts in these cells. The relative volume of the blue-staining core was shown to be related to the amylose content determined for starch synthesized in the transgenic genotypes (Figure 4 and Table 3) and could also be related to the GBSS activity. Furthermore, the amylose content of the blue cores was shown to be similar to the amylose content of wild-type PD007 starch, irrespective of the relative size of the blue cores. This might point toward a GBSS protein threshold value for the production of a fixed percentage of amylose, resulting in the observed "all-or-nothing" pattern. Apparently, the amount of GBSS protein was sufficient for normal amylose production early in tuber development, whereas in later developmental stages the amount of GBSS protein seemed to be below the threshold value or was entirely absent, which led to the formation of amylose-free starch.

The decrease of the GBSS protein content between the core and the surface of the granule might be a consequence of a reduced amount of GBSS protein in transgenic plants in combination with the appositional mode of growth of starch granules (Badenhuizen and Dutton, 1956). Due to the increase in total granule surface during development, the amount of GBSS might be sufficient for the normal level of amylose production until a certain surface size is reached. From this point, which would depend on the level of inhibition of GBSS gene expression, the available GBSS protein may become limiting for adequate synthesis of amylose throughout the outer growth rings of the granule. This hypothesis is sustained by the observation that starch granules of young transgenic microtubers were largely blue and that the relative size of the blue core (V_{blue}/V_{total}) decreased with increasing microtuber age (A.G.J. Kuipers, unpublished results).

To determine the relationship between the staining pattern and the amylose content, the relative volume of the blue cores seemed to be sufficient. This raises the question of whether the blue lines that were frequently observed at the red growth rings surrounding the blue core represent amylose synthesized by GBSS. Because the multiple concentric blue lines were specific for the transgenic antisense GBSS plants and differed from the single blue line that was only occasionally found at the periphery of starch granules of the *amf* mutant, they probably contained a small amount of amylose due to the reduced GBSS gene expression. This granule zone might represent an intermediate between amylose-containing and amylose-free starch and might indicate that the observed transition between amylose-containing and amylose-free starch is not abrupt. The occurrence of a zone with the alternate presence and absence of amylose resembles the specific labeling patterns that were observed in the immunocytochemical localization of AGPase in potato tuber cells (Kim et al., 1989). In this study, AGPase was suggested to be located within specific regions of the starch granule, which were supposed to reflect sites of active starch synthesis. Our findings seem to support this hypothesis.

RNA Characteristics

The analysis of tuber and leaf RNA from the four *Agrobacterium rhizogenes*–transformed genotypes showed an overall reduction

of the GBSS mRNA steady state level when compared with the nontransformed control. The absence in individual tubers of a relationship between the GBSS mRNA steady state level and the extent of inhibition of GBSS gene expression contrasts with the presence in other antisense systems of a relation between the target mRNA level and the protein or enzyme level (van der Krol et al., 1988; Stockhaus et al., 1990; Müller-Röber et al., 1992). Tuber RNA analysis has revealed fluctuations in the GBSS mRNA steady state level in several nontransformed potato genotypes (A.G.J. Kuipers and R.G.F. Visser, unpublished results; Müller-Röber et al., 1992). These fluctuations are likely to occur in the transgenic genotypes as well. They might be the main cause of the lack of a relationship between the GBSS mRNA steady state level and the extent of inhibition, because the amylose content, GBSS activity, and amount of GBSS protein did correlate in several transgenic genotypes (Kuipers et al., 1991; Visser et al., 1991a).

Although no antisense RNA was found in tuber tissue of the transgenic plants, we did find antisense RNA as well as GBSS mRNA in leaf tissue. This is likely to be due to the GBSS gene expression level, which is lower in leaves than in tubers (Visser et al., 1991b) and is comparable to what has been described for antisense RNA–mediated inhibition of patatin gene expression in potato (Höfgen and Willmitzer, 1992). The presence of both antisense RNA and reduced amounts of GBSS mRNA in leaves, as shown in Figure 5B, can be explained from the cauliflower mosaic virus (CaMV)–β-glucuronidase (*GUS*) and GBSS-*GUS* expression patterns in leaves of transgenic potato plants (Visser et al., 1991b). Histochemical analysis of GUS activity showed CaMV-*GUS* expression throughout the leaf and GBSS-*GUS* expression in veins and stomatal guard cells. In the antisense transgenic plants, both GBSS mRNA and GBSS antisense RNA were produced in veins and stomatal guard cells, resulting in a reduced GBSS mRNA steady state level, as was found in tubers of these plants. The GBSS antisense RNA, which could be seen on the RNA gel blots, was likely to have been produced in other parts of the leaf, in which the GBSS gene is either poorly expressed or not expressed at all.

The antisense RNA synthesized in plants carrying the pGB50 T-DNA was expected to be 2.4 kb, based on the GBSS cDNA sequence used for this construct. However, hybridization with a strand-specific probe showed an antisense RNA length of 1.3 kb. Screening of the GBSS cDNA sequence revealed three putative polyadenylation signals in the region between 1.2 and 1.4 kb downstream of the junction of the CaMV promoter and the reversed GBSS cDNA. This points toward a premature termination of transcription, which has also been reported for antisense genes for patatin (Höfgen and Willmitzer, 1992), polygalacturonase (Smith et al., 1988), and CHS (van der Krol et al., 1988).

The specificity at the mRNA level of antisense RNA–mediated inhibition of gene expression has been reported for several antisense systems (Rodermel et al., 1988; Stockhaus et al., 1990; van der Krol et al., 1990), although antisense inhibition of one of the subunits of AGPase in potato was shown to affect the mRNA steady state levels of sucrose phosphate synthase and of storage protein genes (Müller-Röber et al., 1992). Because the inhibition of GBSS gene expression specifically down-regulates the biosynthesis of amylose, we concluded that antisense RNA–mediated inhibition of GBSS gene expression can be effectively applied for the suppression of the biosynthesis of amylose in potato. Furthermore, the correlation between the granule staining pattern and the amylose content (and to a lesser extent the GBSS activity), and the fact that antisense RNA–mediated inhibition ranges from almost complete to no suppression of gene expression, make this system an asset for studying the production of amylose in starch granules.

METHODS

Plant Material and Field Trial

The following potato (*Solanum tuberosum*) genotypes were used: PD007 (HH578; 2n = 2x = 24); 1031-29 (F_2 *amf* mutant; 2n = 2x = 24; Jacobsen et al., 1989); WA501, WA511, WA516, and WA517 (pGB50 transformed PD007; 2n = 4x = 48; Visser et al., 1991a). In the pGB50-transformed genotypes, one to three copies of the antisense granule-bound starch synthase (GBSS) gene were present. This gene consists of a full-length GBSS cDNA fused in reverse orientation between the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase terminator and was inserted into the plant genome using *Agrobacterium rhizogenes*–mediated transformation (Visser et al., 1989a, 1991a).

In vitro–grown plants of the genotypes WA501, WA511, WA516, and WA517 and tubers of the genotypes PD007 and 1031-29 were transferred to the greenhouse for tuber production. Plants were grown under standard greenhouse conditions. Two weeks after harvesting, the tubers were stained at the heel end with Lugol's solution (1% Lugol's solution, I-KI [1:2, v/v]; Merck) and subsequently planted in pots in the greenhouse. Plants were transferred to the field 1 month later.

The field trials were conducted according to governmental guidelines (Anonymous, 1990), as described before for experiments performed in 1990 (Kuipers et al., 1991). In 1991, the field trial was set up with tubers of all genotypes of which the tuber starch color had been determined the year before by means of iodine staining.

Iodine Staining of Starch

The staining of starch in several tissues was performed using Lugol's solution (1% Lugol's solution, I-KI [1:2, v/v]; Merck). Starch in longitudinal tuber sections was stained with Lugol-H_2O (1:1). Starch isolated from individual tubers was suspended in water, stained with a few drops of Lugol's solution, and examined microscopically.

Isolation of Starch

Starch was isolated from tubers that were iodine stained beforehand. Tubers were homogenized in extraction buffer (10 mM EDTA, 50 mM Tris, pH 7.5, 1 mM DTT, 0.1% $Na_2S_2O_5$). The fibrous substance was

washed several times with extraction buffer and subsequently filtered. The filtrate was allowed to set at 4°C. After the starch granules had settled, the supernatant was discarded. The starch granules were subsequently washed with extraction buffer, water, and acetone and dried at 4°C.

Determination of Amylose Content and GBSS Activity

The amylose content of individual tubers was determined spectrophotometrically in 1 to 2 mg isolated starch according to the method described by Hovenkamp-Hermelink et al. (1988). Amperometric titration (Williams et al., 1970) was used for determination of the average amylose content per genotype. For this determination, total starch was isolated from 5 kg of potato tubers per genotype. Using spectrophotometric analysis, low amylose contents are overestimated in comparison with amperometric titration.

The GBSS activity was determined in 1 to 2 mg isolated starch as described by Vos-Scheperkeuter et al. (1986). The individual tuber starch samples were analyzed in triplicate for amylose content and GBSS activity.

Measurements of Blue-Staining Cores in Starch Granules

In iodine-stained starch granules, the radii of the blue-staining core and the total granule were measured microscopically using an ocular micrometer. Due to the ovoid character of the granules, both the long radius (R_{length}) and the short radius (R_{width}) were determined. The radii of the blue-staining core and the total granule were determined in more than 200 individual, randomly chosen starch granules.

From the radii, the ratio (V_{blue}/V_{total}) between the volume of the blue-staining core and the total granule volume was calculated:

$$V_{blue}/V_{total} = (R^2_{blue,width} \times R_{blue,length})/(R^2_{total,width} \times R_{total,length}).$$

RNA Analysis

RNA was extracted from leaves and individual iodine-stained tuber halves of each genotype. Tissue (1 to 2 g) was ground in liquid N_2 and mixed with 3 mL of extraction buffer (50 mM Tris, pH 9.0, 10 mM EDTA, 2% SDS) and 3 mL of phenol. The mixture was centrifuged (5 min at 1400*g*), the supernatant was extracted with 3 mL of phenol/chloroform (1:1), and the mixture was centrifuged again. Nucleic acids were precipitated with 3 mL of isopropanol and centrifuged (10 min at 9000*g*); the pellet was dissolved in 1 mL of H_2O. RNA was precipitated with 1 mL of 4 M LiCl for 3 hr on ice and centrifuged (10 min at 9000*g*). The RNA pellet was dissolved in 0.5 mL of H_2O and precipitated with 50 μL of 3 M NaAc and 1 mL of ethanol. Following centrifugation, the pellet was washed in 70% ethanol, dried, and resuspended in H_2O.

RNA gel blotting and hybridization were performed using 30 μg of tuber RNA or 70 μg of leaf RNA per sample, as described by Sambrook et al. (1989) with some modifications. The membranes were hybridized with the following ^{32}P-ATP–labeled cDNA probes: a 1.3-kb EcoRI fragment of GBSS (Hergersberg, 1988; Visser et al., 1989b); a 1.5-kb EcoRI-HindIII fragment of sucrose synthase (Salanoubat and Belliard, 1987); a 1.7-kb EcoRI fragment of AGPase subunit S (Müller-Röber et al., 1990), a 2.8-kb SstI-HindIII fragment of branching enzyme (Kossmann et al., 1991); and a 2.3-kb EcoRI fragment of a potato 28S rRNA gene (Landsmann and Uhrig, 1985).

Strand-specific ^{32}P-UTP–labeled probes were transcribed from plasmids pT7T3α18GBc and pT7T3α19GBc. These plasmids were obtained by cloning the SpeI-BamHI GBSS cDNA into the XbaI and BamHI sites of pT7T3α18 and pT7T3α19 (Bethesda Research Laboratories). An antisense probe for detecting GBSS mRNA was obtained by restricting pT7T3α19GBc with BamHI and using T7 RNA polymerase for transcription. Sense probes for detecting antisense RNA were obtained either by restriction of pT7T3α19GBc with SalI followed by transcription with T3 RNA polymerase or by restriction of pT7T3α18GBc with SphI followed by T4 DNA polymerase treatment and transcription with T7 RNA polymerase. Probe DNA was removed from the filters by incubation for 15 min in 2 mM Tris, pH 7.5, 0.1% SDS at 65°C; 30 sec in 0.4 M NaOH at 21°C; and 2 min in 0.2 M Tris, pH 7.5, 2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate) at 21°C. The intensity of the RNA signals on the autoradiograms was scanned with a densitometer (Cybertech, Berlin).

ACKNOWLEDGMENTS

The authors would like to thank Herman Masselink and Kees van Beek for their assistance with the field trials and the analysis of the transgenic plants. We gratefully acknowledge Dr. Peter M. Bruinenberg (Aardappelmeel Verkoop Bureau [AVEBE] Research and Development, Foxhol, The Netherlands) for amperometric determination of amylose contents, Dr. Lothar Willmitzer (Inst. für Genbiologische Forschung, Berlin) for supplying some of the probes used for RNA analysis, and Dr. Ir. Will J. Feenstra for helpful discussions and critical reading of the manuscript.

Received September 13, 1993; accepted November 24, 1993.

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