

Inhibition of the ADP–glucose pyrophosphorylase in transgenic potatoes leads to sugar-storing tubers and influences tuber formation and expression of tuber storage protein genes

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Transgenic potato plants were created in which the expression of ADP–glucose pyrophosphorylase (AGPase) was inhibited by introducing a chimeric gene containing the coding region of one of the subunits of the AGPase linked in an antisense orientation to the CaMV 35S promoter. Partial inhibition of the AGPase enzyme was achieved in leaves and almost complete inhibition in tubers. This resulted in the abolition of starch formation in tubers, thus proving that AGPase has a unique role in starch biosynthesis in plants. Instead up to 30% of the dry weight of the transgenic potato tubers was represented by sucrose and up to 8% by glucose. The process of tuber formation also changed, resulting in significantly more tubers both per plant and per stolon. The accumulation of soluble sugars in tubers of antisense plants resulted in a significant increase of the total tuber fresh weight, but a decrease in dry weight of tubers. There was no significant change in the RNA levels of several other starch biosynthetic enzymes, but there was a great increase in the RNA level of the major sucrose synthesizing enzyme sucrose phosphate synthase. In addition, the inhibition of starch biosynthesis was accompanied by a massive reduction in the expression of the major storage protein species of potato tubers, supporting the idea that the expression of storage protein genes is in some way connected to carbohydrate formation in sink storage tissues.

Key words: ADP–glucose pyrophosphorylase/antisense inhibition/starch biosynthesis/transgenic *Solanum tuberosum*/tuber development

Introduction

Starch is the major reserve carbohydrate in most higher plants. It is stored in the form of water-insoluble, osmotically inactive granules in amyloplasts and chloroplasts, which are the major if not the only subcellular organelles where starch biosynthesis takes place. There is ample evidence suggesting that starch synthase and branching enzyme are responsible for the formation of amylose and amylopectin (the two compounds of which starch is composed), by the addition of one molecule of ADP–glucose to an α -1,4-glucosyl chain and by cleavage of an α -1,4-glucan chain and religation via an α -1,6 linkage, respectively (see Preiss, 1991, for a recent review). ADP–glucose is formed from glucose-1-phosphate and ATP by the action of the

enzyme ADP–glucose pyrophosphorylase (ATP: α -glucose-1-phosphate adenylyl transferase, EC 2.7.7.27), hereafter referred to as AGPase. In higher plants, AGPase is supposedly a heterotetramer with subunits belonging to two different classes (see Preiss, 1991). The evidence that ADP–glucose is the sole precursor for starch biosynthesis in higher plants originates mainly from mutants containing a lower AGPase activity. Thus, maize mutants (*brittle-2* and *shrunk-2*), which contain only 25–30% of the wild-type amount of starch in their endosperm, show only 5–10% of residual activity of the AGPase (Tsai and Nelson, 1966; Dickinson and Preiss, 1969). The same holds true for the *rb* embryo mutant of pea and the *adg1* and *adg2* mutants of *Arabidopsis thaliana* where a reduced level of AGPase activity (3–5% in pea, and <2% and 5% respectively in *Arabidopsis*) results in reduced starch formation (38–72% in the pea embryo mutant, and 2 and 40% respectively in the *Arabidopsis* leaf mutants) (Smith *et al.*, 1989; Lin *et al.*, 1988a,b). Nevertheless since another enzyme, starch phosphorylase, can both degrade and synthesize starch starting from glucose-1-phosphate, and since UDP–glucose is also accepted by starch synthase (see Preiss, 1991), the role of the ADP–glucose pathway as the exclusive pathway has often been questioned, even more so as only two of the mutants mentioned above (the maize mutants *brittle-2* and *shrunk-2*) can unequivocally be assigned to mutations of structural genes of the AGPase (Bae *et al.*, 1990; Bhavé *et al.*, 1990). In these mutants, however, the residual amount of starch is still significant. In the pea and *Arabidopsis* mutants, the possibility of a more pleiotropically acting mutation being responsible for the observed reduction in starch content cannot be excluded.

We are interested in the roles of sucrose and starch in photoassimilate partitioning and allocation as well as in the growth and development of plants. We chose potato as a model plant since tubers of potato represent typical starch-storing storage sinks that accumulate high levels of starch in their amyloplasts.

Potato tubers originate from stolons, which are lateral underground shoots normally arising from the basal nodes of the plant. Tuber initiation involves a change in the growth behaviour of the stolon, i.e. a cessation of the initial extension growth and the onset of radial growth, finally leading to the formation of tubers. Tuberization is accompanied by a sharp increase in starch formation in the apical part of the stolon (Reeve *et al.*, 1969; Sowokinos, 1976; Hawker *et al.*, 1979) and a simultaneous rise in AGPase activity (Sowokinos, 1976). In addition to the increase in starch content, the concentration of a major storage protein of potato tubers, namely patatin, increase strongly in the very early stages of tuber development (Park *et al.*, 1985). It is not known whether the process of tuberization is a prerequisite for starch accumulation or whether the plant's capability to form starch determines the actual initiation of tubers and their further development. All the data available do not allow a clear

conclusion to be drawn concerning the role of starch biosynthesis for the development of potato tubers.

In order to investigate the influence of starch biosynthesis on the tuberization process, we wanted to eliminate starch synthesis of transgenic potato plants *in vivo*. Therefore, we decided to try to inhibit the AGPase using an antisense RNA approach. A chimeric gene containing the coding region of one of the subunits of the potato AGPase linked in reverse orientation to the CaMV 35S promoter, and given a polyadenylation signal from the octopine synthase gene, was introduced into potato plants.

In addition to analysing the importance of starch formation for growth and development of potato plants and tubers we wanted to answer the following questions. (i) Does inhibition of AGPase influence the expression of other genes involved in starch biosynthesis and in sucrose biosynthesis/degradation? (ii) Is AGPase the only enzyme able to support starch biosynthesis in a vegetative storage organ of higher plants *in vivo*? (iii) What is the influence of inhibiting AGPase on the expression of genes encoding the major storage proteins of potato tubers?

Results

Construction of a chimeric 'antisense' AGPase gene

In order to down-regulate AGPase activity in transgenic potato plants via an antisense RNA approach, a 1.6 kb cDNA (~85% of the full size) encoding subunit B (Müller-Röber *et al.*, 1990) of potato AGPase was inserted in reverse orientation between the CaMV 35S promoter and the terminator of the octopine synthase gene of pTiACH5 (Figure 1a). The resulting antisense gene, AntiAGP-1, was ligated into the plant transformation vector Bin19 (Bevan, 1984) to create pBin-AntiAGP-1. This construct was used to transform potato plants (*Solanum tuberosum* L. cv. Désirée) via *Agrobacterium tumefaciens* (Rocha-Sosa *et al.*, 1989). Transgenic plants were screened for integration of the chimeric gene by DNA blot analysis and only plants containing the intact gene were used for further analysis (data not shown).

Screening for transgenic potato plants having reduced amounts of AGPase B protein in their leaf tissue

Transgenic potato plants maintained in tissue culture were visually indistinguishable from non-transformed control plants. 35 transgenic plants were randomly selected, transferred to soil and grown under standardized greenhouse conditions (see Materials and methods). In order to screen for plants having reduced levels of AGPase B protein, leaf samples were taken from transgenic and non-transformed control plants and subjected to Western blot analysis using an antibody raised against the homologous AGPase (i.e. brittle-2) polypeptide of maize. Almost no variation in the amount of cross-reacting protein was detected in several control plants. The size of the cross-reacting protein was ~50 kDa, which is in agreement with the subunit size reported for the potato tuber and leaf enzyme (Sowokinos and Preiss, 1982; Anderson *et al.*, 1990; Okita *et al.*, 1990). From the 35 transgenic plants selected for analysis, eight plants [subsequently called R (reduced) plants] had clearly reduced, but still detectable, amounts of AGPase B protein in their leaf extracts (data not shown). Analysis of AGPase enzyme activity revealed that these plants were highly deficient for this activity, displaying only 5–30% of the

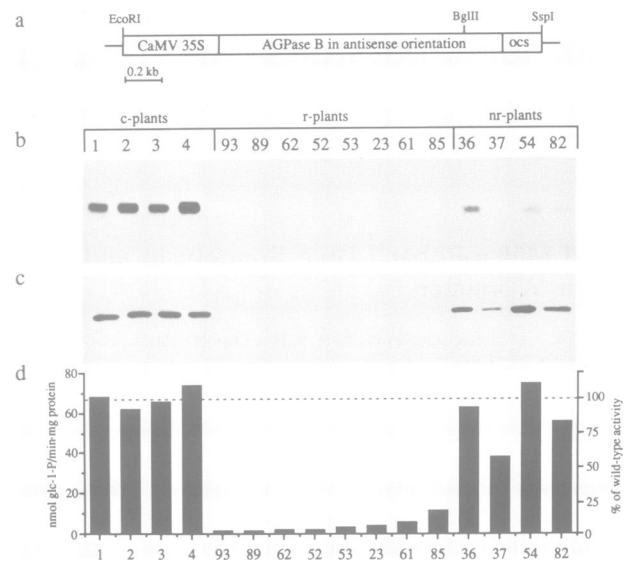


Fig. 1. Structure of the chimeric gene and analysis of tubers of transgenic potato plants inhibited for expression of ADP-glucose pyrophosphorylase. (a) Structure of the chimeric AGPase B antisense gene used for transformation of potato plants (for details see Materials and methods). (b) Analysis of AGPase B mRNA expression in tubers of transgenic and control plants. Total RNA (50 µg/lane), extracted from tubers, was hybridized to a multiprimer labelled AGPase B specific probe. The transcript size was ~1.8 kb. (c) Western blot analysis of tuber extracts. Total protein was extracted from tubers, separated by SDS-PAGE (20 µg/lane) and analysed by immunoblotting using an antiserum raised against the homologous maize brittle-2 protein. The size of the cross-reacting protein was ~50 kDa. (d) AGPase activity in tuber extracts in nmol glucose-1-phosphate/min/mg protein (left) and as a percentage of wild-type activity (right). Values represent the average of 6–12 determinations obtained for 4–6 different tubers each. The SEM was <20% in each case. The average activity measured in tubers of untransformed control plants was taken as 100%. c-plants, untransformed control plants; r- and nr-plants, transgenic plants with reduced (r) and wild-type (nr) AGPase activity in leaf extracts. Each number refers to an independent plant.

activity of untransformed potato plants [C (control) plants], or of transformed plants which did not show any reduction of the AGPase B protein in Western blot experiments [NR (not reduced) plants] (data not shown). A reduction in starch content of up to 10-fold (as compared with wild-type plants) in starch content was observed in leaves from R plants harvested at 16.00 h. Despite this strong reduction in both AGPase activity and starch in the leaves of the transgenic plants, no dramatic change in the phenotype of the transgenic potato plants was observed when grown in the greenhouse under a light–dark regime of 16 h light–8 h dark. The only differences identified by visual scoring were a higher tendency to form axillary shoots, an earlier onset of flowering of ~2–4 weeks and a slightly higher accumulation of anthocyanins in the upper part of the stem in highly inhibited plants (see Figure 2).

Analysis of the expression of AGPase B in tubers of potato plants containing reduced AGPase in leaves
Plants from the C, R and NR groups were allowed to tuberize and the tubers were harvested at the end of the tuberization period (i.e. when the aerial parts of the plants were senescent) and analysed for AGPase B gene expression and AGPase activity.

Figure 1b shows the result of an RNA blot experiment



Fig. 2. Inhibition of AGPase activity in leaves leads to only small changes in the aerial part of the plant. Aerial parts of a transgenic potato plant showing a strong reduction in AGPase activity in leaf extracts (R-transformant no. 93; left) and a control plant (right). The transgenic plant shows a higher tendency to produce axillary shoots and a slightly higher accumulation of anthocyanins in the upper part of the stem.

using an AGPase B specific cDNA fragment as a probe. A strong signal is obtained with RNA isolated from tubers of wild-type plants which is consistent with observations described earlier (Anderson *et al.*, 1990; Müller-Röber *et al.*, 1990). In contrast, a significant reduction in transcript level is observed in NR plants and essentially no AGPase B specific signal could be detected in RNA from tubers of R plants, demonstrating that the concentration of both sense and antisense RNAs in these tissues is below the detection level.

The strong reduction in the level of AGPase B specific RNA in tubers of R plants was reflected at the protein level (see Figure 1c). Almost no cross-reactive AGPase B polypeptide was detected in tuber protein extracts of R plants in protein blot experiments using the AGPase B specific antibody. In contrast, strong signals were observed in C plants. Interestingly, with the exception of plant no. 37, no significant reduction in the amount of AGPase B protein was observed in extracts from tubers of NR plants (Figure 1c). This indicates that a rather strong reduction in AGPase B mRNA amount is necessary to achieve a detectable reduction in AGPase B protein.

Finally protein extracts of the tubers of R, NR and C plants were assayed for AGPase activity by determining the rate of production of glucose-1-phosphate (glc-1-P; see Materials and methods). In extracts from tubers of untransformed plants the average activity was 67.2 nmol glc-1-P/min/mg protein which is in agreement with previous measurements (see e.g. Sowokinos, 1976). In R plants, activities between 1.0 and 11.6 nmol glc-1-P/min/mg protein were detected, corresponding to 1.5–17% of wild-type activity (see Figure

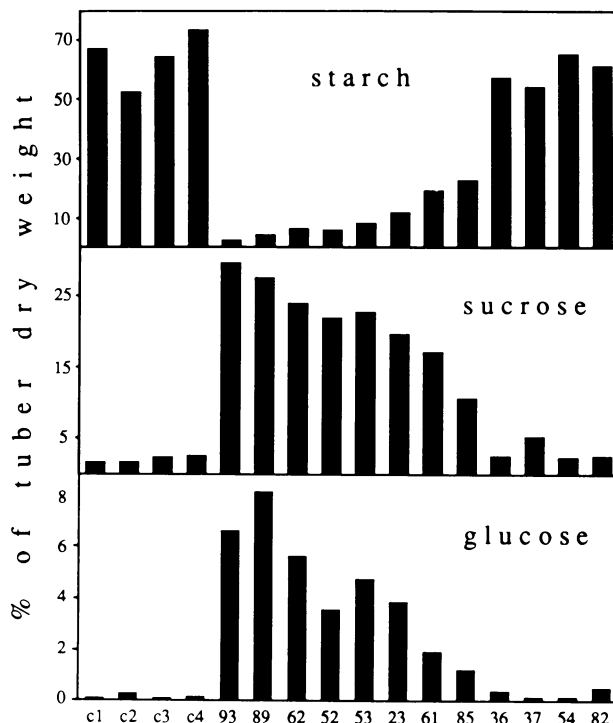


Fig. 3. Inhibition of AGPase in tubers leads to a decrease in starch and an increase in soluble sugars. Tubers were harvested at the end of the tuberization period (i.e. when the aerial parts of the plants were senescent) from plants grown under standardized greenhouse conditions (see Material and methods). Values for starch, sucrose and glucose are given as percentages of tuber dry weight. At least 10 determinations from three to five tubers of comparable fresh weights (9–15 g) were performed for each value. Considerable variation in the sucrose and glucose content was observed for the different tubers of C and NR plants (maximal factor: 4). The highest values observed for sucrose and glucose were 3 and 0.6%, respectively, of the tuber's dry weight. In tubers of R plants, the variation between different tubers was lower, the SEM being maximally $\pm 25\%$ for sucrose, and maximally $\pm 40\%$ for glucose.

1d). No clear reduction in activity was observed in tubers of NR plants with the exception of transformant no. 37 (37.9 nmol glc-1-P/min/mg protein; corresponding to 56% of the wild-type level).

Thus, introduction of a chimeric gene encoding an antisense RNA specific for the AGPase B gene of potato results in nearly complete elimination of AGPase activity in tubers of transgenic plants.

Tubers devoid of AGPase are reduced in starch but store massive amounts of sucrose and glucose

In order to analyse the influence of inhibiting AGPase in tubers of transgenic potato plants on carbohydrate metabolism, tuber extracts from inhibited and wild-type plants were analysed for their carbohydrate content with respect to starch, sucrose, glucose and fructose. The starch content of tubers deficient in AGPase is severely reduced and amounts to between 4% (transformant no. 93) and 35% (transformant no. 85) of levels seen in control plants (see Figures 3 and 4d). Surprisingly, the tubers that are largely deficient of starch contain a significantly increased level of soluble sugars, specifically sucrose and glucose (Figure 3). The level of fructose was near the detection limit in both inhibited and wild-type tubers (data not shown). The highest level of sucrose was found in transformant no. 93. Tubers

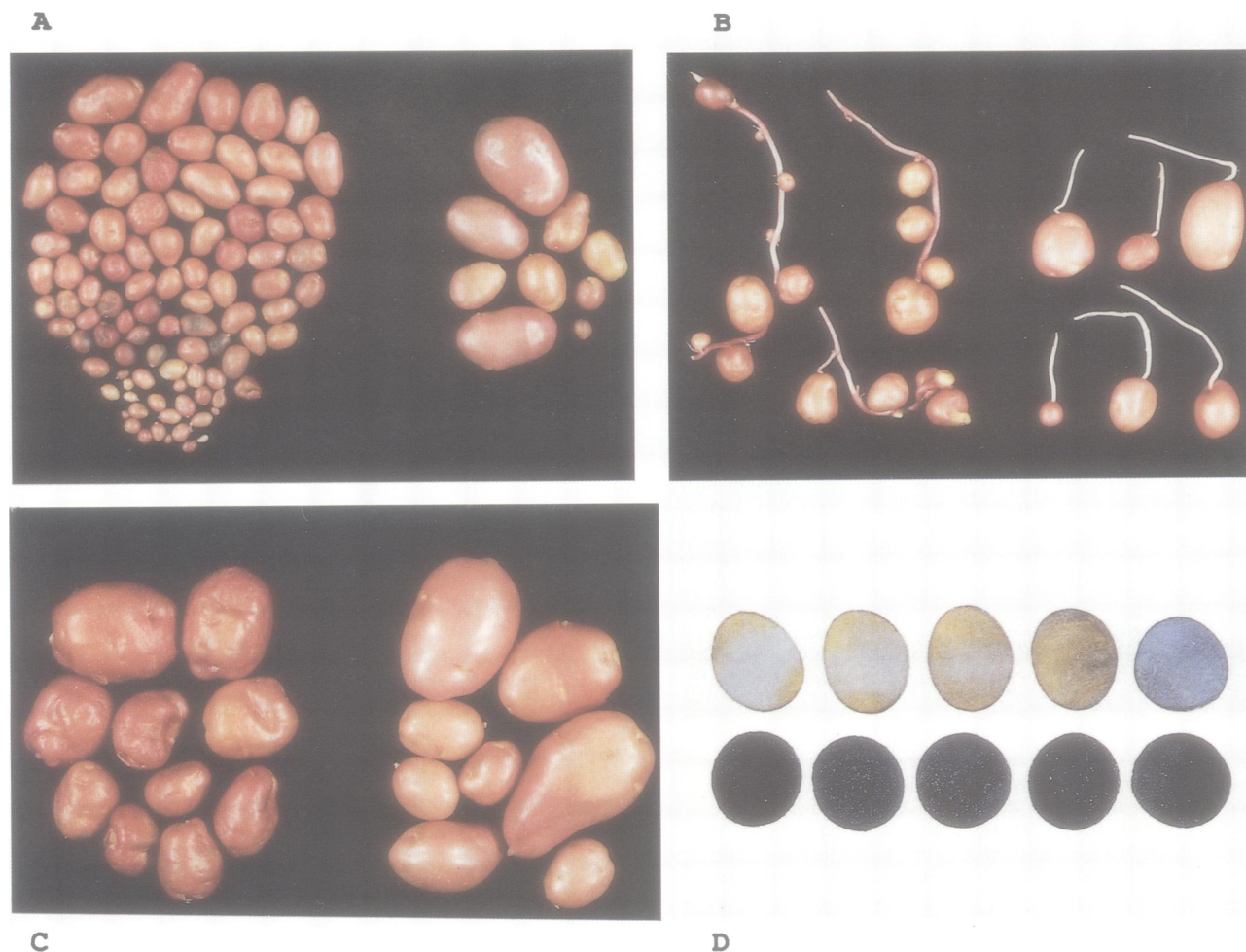


Fig. 4. Inhibition of AGPase leads to significant changes in the tuberization process. (A) Change in tuber number and size. Tubers collected from R transformant no. 89 (left) and control plant c1 (right) demonstrate the effect of inhibition of starch biosynthesis on the number of tubers formed by a single plant. (B) Changes in the number of tubers formed per stolon. In potato plants with a strong reduction in AGPase activity, a large proportion of the tubers is arranged in groups originating from the tip and lateral parts of the stolon (left; tubers/stolons selected from R transformant no. 93). In untransformed plants and in transformed plants showing no or a weak reduction in AGPase activity, almost all of the stolons formed no more than a single tuber on the stolon tip (right; tubers/stolons collected from control plant c3). (C) Formation of tubers displaying a shrunken phenotype. Inhibition of the AGPase activity frequently leads to shrunken tubers [compare tubers selected from R transformant no. 62 (left) with tubers harvested from control plant c2 (right)]. (D) Iodine staining of tuber discs from wild-type C plants (bottom row) and different R transformants (top row; from left to right: transformant nos 93, 89, 53, 61 and 85).

of this plant contained 30% of the tuber dry weight in the form of sucrose, which is >10-fold above the level of sucrose in wild-type controls and NR plants. The total level of soluble sugars in tubers of R plants is further increased by the presence of glucose which contributes another 1.2–8.3% to the tubers' dry weight (compared with 0.12% in wild-type control plants). These results demonstrate that a reduction in AGPase activity is paralleled by a decrease in starch content and is positively correlated with the amount of soluble sugars accumulating in the tubers.

Inhibition of starch biosynthesis leads to an increase in both total tuber number per plant and number of tubers formed per stolon

The onset of the tuberization process is accompanied by a massive increase in starch biosynthesis (see e.g. Hawker *et al.*, 1979; Sowokinos, 1976). It was therefore of interest to analyse if the inhibition of starch biosynthesis would lead to any changes with respect to the tuberization process. As shown in Figure 4a–c, this is indeed the case.

First the number of tubers formed on a single plant

increases in proportion to the decrease of the AGPase activity. Whereas under greenhouse conditions C and NR plants produced 8–16 tubers, this number increased to between 41 and 91 tubers for different R plants (see Figure 4a for a comparison between tubers harvested from transformant no. 89 and control plant c1).

A second significant change relates to the number of tubers formed per stolon. As a rule, in C and NR plants only one tuber forms per stolon, usually at its end. As evident from Figure 4b this is not the case for the AGPase deficient plants of the R series. In these plants, a large proportion of stolons produced a significantly higher number of tubers (2–10 tubers per stolon). Thus inhibition of AGPase not only leads to inhibition of starch biosynthesis but also to significant changes in the tuberization process.

Conversion of the tuber from starch- to sugar-storing does not significantly influence sprouting and further plant development

Tubers are the major storage sink organs central to the vegetative reproduction of potato plants. In wild-type plants,

carbohydrates are allocated into tubers in the form of starch, which subsequently get mobilized during sprouting due to the action of starch-degrading enzymes (amylases and phosphorylases). Thus starch serves as the major reserve carbohydrate supporting the growth of the new plant. It was therefore of interest to analyse whether or not the replacement of the reserve carbohydrate starch by sucrose and glucose would have any significant influence on formation of new sprouts and further development of the plant. Starchless tubers from plants containing the antisense gene were therefore harvested in parallel with tubers from control plants and, after a prolonged storage at 8°C, planted into soil and grown in the greenhouse. No significant differences were observed in the timing or intensity of the sprouting process or in the further development of the plants. Tubers harvested from this second generation of antisense plants were devoid of starch but again contained large amounts of sucrose and glucose similar to those found in tubers of direct regenerants, thus demonstrating the stability of the trait over at least one generation (data not shown).

Inhibition of starch biosynthesis leads to an increase in fresh weight but a decrease in dry weight of total tubers per plant

The increase in the number of tubers per plant observed for the AGPase deficient potato plants results in a 40% increase in total tuber fresh weight (241 ± 19 g on average in R plants compared with 169 ± 12 g in C and NR plants). However, the average size and fresh weight per single tuber was clearly reduced in these plants (see Figure 4a for size of tubers). A reduction was also evident for the average tuber dry weight, which ranged from 9.8% (transformant no. 89) to 15.8% (transformant no. 85) of the tuber fresh weight in strongly inhibited antisense plants, and was 23.9 and 23.0% in C and NR plants, respectively. Consequently the dry weight of the combined tubers of starchless transgenic plants reached only 60–70% of the dry weight of the combined tubers of control plants.

The fact that the starchless tubers have a lower dry weight, and therefore contain more water than wild-type plants is also reflected in the appearance of the tubers: significantly more tubers showed a shrunken phenotype (see Figure 4c).

Inhibition of AGPase expression does not influence the expression of other genes involved in starch biosynthesis but leads to an increased expression of the sucrose phosphate synthase gene in tubers

The expression of genes encoding enzymes for a complex biosynthetic pathway can be controlled by the first enzyme of this pathway with the signal controlling the induction of the other enzymes being one of the metabolites produced from this first enzymatic step. In order to see whether or not a similar type of control is exerted on genes encoding enzymes directly involved in starch synthesis or degradation, total RNA was extracted from tubers of control and different AGPase antisense transformants, and Northern blots were probed for the expression of several genes.

As shown in Figure 5A, no significant changes were found in the level of RNAs coding for the second subunit of AGPase (AGPase S), the granule bound starch synthase (GBSS), the branching enzyme (BE) or the starch phosphorylase (STP; plastid-localized isoform). Thus the expression of the corresponding genes at the RNA level is

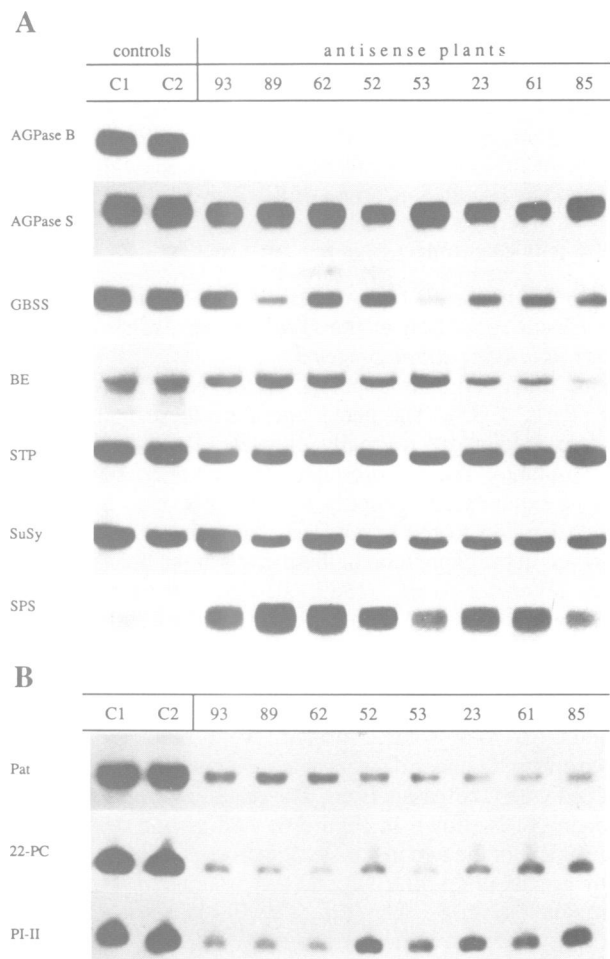


Fig. 5. Northern analysis of RNA isolated from tubers of untransformed and AGPase-inhibited potato plants. 50 µg total RNA from each sample was loaded in each lane. The blot was probed with ³²P-labelled cDNA fragments. (A) RNA expression of genes encoding enzymes involved in starch and sucrose metabolism. AGPase B, ADP-glucose pyrophosphorylase, subunit B; AGPase S, ADP-glucose pyrophosphorylase, subunit S; GBSS, granule bound starch synthase; BE, branching enzyme; STP, plastidic starch phosphorylase; SuSy, sucrose synthase; SPS, sucrose phosphate synthase. (B) RNA expression of genes encoding the most abundant tuber proteins. Pat: patatin; 22-PC: one protein of the 22 kDa protein complex; PI-II: proteinase inhibitor II. For details see Materials and methods. For assignment of the different plants, see Figure 1.

not influenced by any of the metabolites produced by the AGPase. The variation observed in the case of the BE and the GBSS does not deviate significantly from the variation seen in RNA extracted from wild-type tubers (data not shown). Protein blots probed for the presence of the BE or the starch phosphorylase proteins using specific antibodies show that inhibition of the starch biosynthesis did not result in a significant change in the amount of these two proteins (data not shown).

Sucrose synthase is the major sucrose-metabolizing enzyme present in potato tubers. It converts sucrose into UDP-glucose and fructose, UDP-glucose subsequently being converted into glucose-1-phosphate, the precursor for ADP-glucose. Due to the reduced flow of glucose-1-phosphate to ADP-glucose in the transgenic plants, it was therefore of interest to analyse the expression of the sucrose synthase (SuSy) gene in these plants. As shown in Figure 5A, no significant change in the levels of RNA was observed.

Finally, as the starchless tubers contain strongly elevated levels of sucrose, we tested the expression of the key enzyme in sucrose synthesis, sucrose phosphate synthase (SPS) on Northern blots. In contrast to all other genes tested, there was a dramatic and significant increase in SPS mRNA in tubers of starchless potato plants compared with control plants. The possible meaning of the increased level of SPS mRNA and the nearly unchanged level of sucrose synthase RNA will be discussed later.

Inhibition of starch biosynthesis is accompanied by a significant reduction in the level of expression of the most abundant tuber proteins

Potato tubers contain a specific set of major proteins (see e.g. Park, 1983). The genes encoding these major tuber protein species are normally only expressed in tubers of potato plants. Their expression can, however, be induced in leaf explants upon supply with high levels of sucrose, which has led to the hypothesis that sucrose is somehow involved in the expression of these genes at the transcriptional level (Wenzler *et al.*, 1989; Rocha-Sosa *et al.*, 1989; Johnson and Ryan, 1990; Jefferson *et al.*, 1990).

In order to determine whether or not the dramatic changes in the carbohydrate composition of tubers of antisense plants would influence the expression of these proteins in tubers, extracts of total soluble protein were separated by gel electrophoresis under denaturing conditions and subsequently stained with Coomassie Blue. The result of a representative protein gel is shown in Figure 6. A dramatic decrease in the amount of the two major storage proteins of potato tubers, namely patatin (~40 kDa) and the proteins of the 22 kDa complex (see e.g. Suh *et al.*, 1990), is readily seen in extracts of starch deficient tubers.

The expression of these major protein species was also analysed at the RNA level. Radioactively labelled cDNA probes specific for patatin, one protein of the 22 kDa complex and the proteinase inhibitor II were hybridized in blot experiments against total RNA extracted from tuber tissues. As evident from Figure 5B, the inhibition of starch biosynthesis results in a significant reduction of the amount of the different RNAs encoding these three major tuber proteins.

Discussion

Starch biosynthesis via ADP-glucose is the predominant, if not the only, pathway operating in plants

The introduction of a chimeric gene containing the coding region of the B-subunit of potato AGPase in a reversed (antisense) orientation with respect to the CaMV 35S promoter into potato plants, results in a dramatic reduction of the expression of the corresponding sense RNA in both leaf and tuber tissue. In leaf tissue, even in the plant showing the highest level of inhibition, some AGPase protein and corresponding enzymatic activity was still present. In tubers of several transgenic plants, however, the levels of both RNA and protein were reduced below the detection limit and the corresponding AGPase activity was <2% of the wild-type activity. The reason for this difference in the level of antisense inhibition in these two tissues is presently unclear, but it has also been observed in several other cases (Kossmann, J. and Sonnenwald, U., unpublished observations). In parallel with the reduction of the RNA, protein and

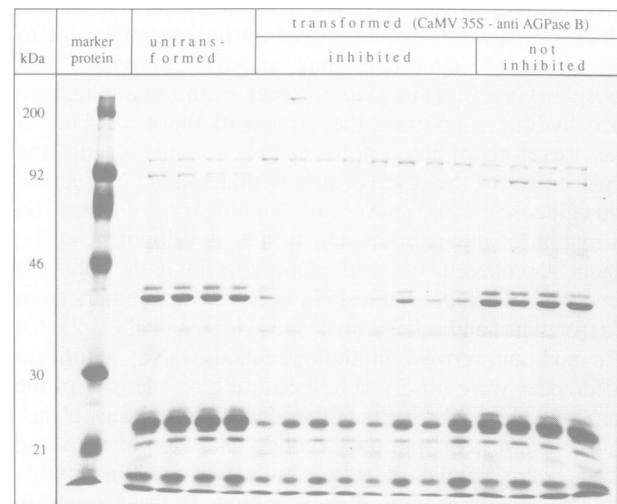


Fig. 6. SDS-PAGE analysis of total soluble protein extracted from tubers of untransformed and AGPase-inhibited potato plants. Proteins were separated on 10% SDS-polyacrylamide gels (20 µg/lane) and subsequently stained with Coomassie Blue. 'Untransformed' denotes wild-type control (C) plants; 'inhibited' denotes plants of the R collection, and 'not inhibited' denotes plants of the NR collection (same order as in Figure 1). A strong reduction in the amount of the patatin protein (major band at ~40 kDa) and of the 22 kDa protein complex is clearly visible in tuber extracts from AGPase-inhibited potato plants.

enzymatic activity, a great reduction in starch was observed both in tuber and leaf tissue. More importantly, the relative reduction in starch followed the relative reduction in the AGPase B RNA and protein levels.

The hypothesis that AGPase controls the major pathway for starch biosynthesis is based mainly on the analysis of *Arabidopsis thaliana*, pea and maize mutants with decreased AGPase activities (Lin *et al.*, 1988a,b; Smith *et al.*, 1989; Tsai and Nelson, 1966; Dickinson and Preiss, 1969). Only in the case of the two maize mutants *brittle-2* and *shrunk-2* was unequivocal molecular evidence presented that the structural gene encoding one of the subunits of the AGPase had been mutated (Bae *et al.*, 1990; Bhave *et al.*, 1990). A more pleiotropic effect caused by a mutation of a regulatory locus cannot be excluded with absolute certainty in the case of the pea and *Arabidopsis* mutants. A 90–95% reduction of the maize endosperm AGPase activity in the two maize mutants results in a loss of 70–75% of wild-type starch content in the endosperm (Tsai and Nelson, 1966; Dickinson and Preiss, 1969). The dramatic loss of starch observed as a direct result of the inhibition of AGPase via antisense RNA described here therefore supports and extends the previous hypothesis that ADP-glucose is the major, and probably sole, precursor for starch biosynthesis in higher plants. Other pathways for starch biosynthesis, e.g. via the action of starch phosphorylase which has both a degradative and a biosynthetic capacity with respect to starch, are therefore only of minor if any importance *in vivo*. These data also exclude the possibility that a different, recently proposed pathway based exclusively on *in vitro* experiments is of major physiological significance. According to this model, ADP-glucose would be formed in the cytosol by the action of sucrose synthase and subsequently transported into chloroplasts/amyloplasts via an adenylate translocator (Pozeueta-Romero *et al.*, 1991).

Whereas AGPase from prokaryotic sources consists of only one type of subunit, two subunit types have been observed in all higher plants so far analysed (see Preiss, 1991 for a review). Most of the available evidence suggests that the active enzyme is a heterotetramer containing two molecules of each subunit. However, it is still unclear whether a homotetramer could also be active. In the case of the two structural mutants of maize, where either one of the two subunits was mutated, a residual activity of 5–10% could still be measured (Dickinson and Preiss, 1969). Along the same lines the *adg2* mutant in *A. thaliana*, which lacks only one of the two subunits, displays a residual activity of 5% resulting in a reduction of the starch content down to only 40% of wild type levels (Lin *et al.*, 1988b). In the antisense potato plants described here, again only one of the two subunits of the AGPase enzyme has been eliminated. The plant with the highest level of inhibition, i.e. plant no. 89, exhibited <2% of the wild-type level of activity. These data therefore further support the notion that a heterotetramer is needed for full activity. They do not, however, exclude the possibility that a homotetramer could also be partially active, as the residual activity observed could either be due to the low specific activity of the presumed homotetramer or due to a residual activity of the low amounts of a heterotetramer still being formed.

Inhibition of starch biosynthesis transforms a starch-storing storage sink into a sugar-storing storage organ and leads to changes in the developmental process of tuber formation

Starch biosynthesis and tuber formation are two closely connected processes. Upon induction of tuberization a great increase in starch synthesis is observed at the tip of the stolon (see e.g. Reeve *et al.*, 1969). Carbohydrates have even been discussed as potential inducers of the tuberization process (Wellensiek, 1929; Forsline and Langille, 1976). The fact that inhibition of starch biosynthesis does not lead to abortion of tuber development excludes the speculative possibility that the tuberization signal is somehow directly linked to starch or one of the metabolites of starch biosynthesis.

Despite the unchanged ability of the plants to form tubers, major changes are observed with respect to the tuber forming process. As described in the results section, potato plants inhibited for the AGPase activity contain both more tubers per plant and more tubers formed per stolon, with the size of an individual tuber being smaller than those of wild-type plants. Thus phases of tuber growth alternate with phases of stolon growth and furthermore tubers stop growing ('mature') at an earlier stage of development.

In wild-type plants, only a few (usually one) of the many tuber initiation sites present on a stolon finally yield mature tubers. One possible explanation for the observed changes in tuber development is based on the hypothesis that the different tubers of a plant (and a stolon) compete for a limited supply of photoassimilates. Due to non-synchronous tuber formation the first tubers formed would represent the strongest sink, outcompeting the tubers initiated later. If this is true one would predict that inhibition of AGPase activity, and thus starch formation, would eliminate a major determinant of sink strength. Therefore the differences in sink strength between tubers initiated at different times during development would be smaller, thus allowing the tubers initiated later to develop further.

A second possible explanation of the changes in the tuberization process assumes that tuberization is controlled by the relative concentrations of two or more factors. Indeed, tuber formation and cessation can be controlled very precisely by, respectively, decreasing and increasing the nitrogen supply in a hydroponic culture of potato plants, resulting in alternating tuber–stolon–tuber–stolon structures (Krauss and Marschner, 1976) very similar to the ones seen in Figure 4b. Alternating tuber–stolon structures, as well as the early cessation of tuber growth, could therefore be due to a change in the nitrogen:carbon ratio during growth of an individual tuber which could be caused by an inhibition of starch formation. Experiments are presently under way to answer these questions.

At the metabolite level, inhibition of starch biosynthesis has converted a starch-forming storage organ into a sugar-storing storage organ. This has resulted in a loss of ~30–40% of the dry weight allocated in the combined tubers of a transgenic plant when compared with wild-type plants. As a result of the accumulation of the osmotically active sugars sucrose and glucose, the tuber cells have to cope with a high osmotic stress which is reflected in the increased water content of the tubers (as evidenced by the decrease in dry weight content).

It seems plausible (though by no means proven in this case) to assume that sucrose is stored in the vacuole. Assuming that the volume of the vacuole of a tuber parenchyma cell is equivalent to the total cellular volume and that this is equivalent to the total water content, the concentration of the sucrose in the vacuole would amount to ~50–100 mM. This concentration is well below that assumed to exist in the phloem (~0.8–1 M) (see e.g. Riens *et al.*, 1991) which demonstrates that there is still a steep downhill gradient from the phloem sap to the vacuoles of potato tubers. Nevertheless, the mere fact that sucrose concentrations >10-fold higher than those found in wild-type potato tubers can accumulate in (probably) the vacuoles of the starchless mutants indicates that there must exist carrier systems of high enough activity for transporting sucrose into vacuoles in a plant which would normally store starch in the amyloplasts.

Similar changes to those described here have been observed in maize mutants containing a reduced AGPase activity. A decrease in starch content from 75 down to 20% was accompanied by an increase of sucrose content from 3 to 25% (both as a percentage of dry weight) and a concomitant decrease in dry matter content from 44 to 25% (Creech, 1965). A similar picture also emerges in the case of the pea *rb* mutant which displays only a 3–5% residual level of AGPase activity in seeds. As a result, the amount of starch decreases from 50% of the final dry weight in wild-type plants to 30% in the mutant plants, and sucrose content increases from 5% to 9% (Kooistra, 1962). Thus, in all three cases where the AGPase activity was decreased in a sink storage organ (like seeds or tubers), the decrease in starch was partly compensated for by an increase in sucrose. This is in contrast to the situation found in photosynthetically active tissues. In *A. thaliana* mutants a decrease in the AGPase activity to 7% of wild-type levels led to an increase in the ratio of sucrose synthesis to starch synthesis by a factor of six when kept at low irradiances. However, this is due mainly to the decrease in starch synthesis as the absolute sucrose synthesis increased only by a factor of 1.5. Under high irradiances this slight increase in sucrose synthesis was

completely lost and, in contrast, a decrease in sucrose synthesis of 50% accompanied by a reduced rate of photosynthesis was observed (Neuhaus and Stitt, 1990). Thus decreasing the AGPase activity in photosynthetically active tissue under high light does not lead to a simple change in partitioning of the photoassimilates between sucrose and starch, but rather to a decreased photosynthetic activity which is therefore different from the situation in sink tissues.

Inhibition of AGPase does not influence the expression of other genes involved in starch biosynthesis, but has a profound effect on the expression of sucrose phosphate synthase

The introduction of the chimeric gene encoding an antisense RNA directed towards the AGPase B-subunit has resulted in efficient repression of the AGPase. This effect was specific for the gene encoding the B-subunit. No significant changes were observed when the expression of four other genes encoding enzymes of starch biosynthesis was analysed. It is therefore unlikely that the expression of the set of genes coding for the different enzymes of starch metabolism is positively controlled by a metabolite that either succeeds ADP-glucose, or is ADP-glucose itself. This result is in agreement with the situation in AGPase deficient pea and *Arabidopsis* mutants where no significant changes in the activity of enzymes apart from AGPase were detected (Smith *et al.*, 1989; Lin *et al.*, 1988b). We have previously observed that the RNA levels of several genes, including the AGPase S-subunit, BE and starch phosphorylase, increase in detached leaves in the dark if the leaves are fed with sucrose through the cut petiole (Müller-Röber *et al.*, 1990; Kossmann *et al.*, 1991; and unpublished results), suggesting that sucrose, or a metabolite of sucrose, might be directly involved in inducing the expression of these genes. The results described here for the starchless, high sucrose containing tubers are in agreement with this hypothesis. In contrast to the unchanged expression of genes encoding starch biosynthetic enzymes, a strong increase is observed in the RNA level of the sucrose phosphate synthase gene. This observation, together with the unchanged level of expression of the sucrose synthase gene, indicates that the sucrose accumulating in the starchless potato tubers is probably not derived directly from the sucrose present in the phloem. Rather, sucrose after being unloaded from the phloem into the tuber parenchyma cells is probably first cleaved via the action of sucrose synthase and then resynthesized via the sucrose phosphate synthase. Inhibiting the AGPase activity seems to strongly induce a futile cycle of sucrose synthesis and degradation in potato tubers. The existence of such a futile cycle as a means of regulating partitioning between sucrose, starch and respiration was recently postulated by Geigenberger and Stitt (1991) for *Ricinus communis* seedlings.

Inhibition of AGPase has a profound effect on the expression of the main protein species present in potato tubers

Tuberization is accompanied by a variety of biochemical changes, the two most prominent ones being the massive accumulation of starch and the dramatic change in protein pattern leading to the appearance of new highly abundant protein species (see e.g. Park, 1983). The three major protein species are represented by a family of immunologically related glycoproteins with a molecular weight of ~40 kDa

which have been given the trivial name patatin, a family of proteins with a molecular weight of ~22 kDa and the proteinase inhibitor I and II proteins. As described in the results section, the starchless tubers have dramatically different protein patterns, with new protein species appearing or increasing and others decreasing in their relative concentrations. These effects are most pronounced with respect to the storage protein species mentioned above which significantly decrease in their concentration. As this decrease is also reflected at the RNA level, this strongly suggests that the conversion of a starch-storing into a sucrose-storing organ directly affects the expression of these genes at either the transcriptional level or the RNA stability level.

This result is similar to the situation found in pea and maize mutants that have a reduced starch content and an increased level of sucrose. In several mutants of maize, such as *brittle-2* or *shrunk-2*, which contain a lowered starch level in the endosperm, a strongly reduced expression of the major storage proteins, i.e. zein, is observed (Tsai, 1983; Tsai *et al.*, 1978). Similarly in the pea *r* mutant showing a reduced starch content in developing embryos (due to the complete absence of one isoform of starch branching enzyme; Smith, 1988) the amount of one of the major storage proteins, legumin, is halved (Davies, 1980). By measuring transcriptional activity in isolated nuclei evidence for post-transcriptional control of legumin RNA accumulation was obtained and sucrose was suggested to have a direct effect on the stability of the legumin RNA (Turner *et al.*, 1990).

The reduction in the amounts of the major protein species in starchless potato tubers high in soluble sugar content strongly suggests that such a complex and interlinked control between the expression of storage proteins and carbohydrate composition in storage sinks of higher plants is a general phenomenon. A decrease in starch, a concomitant increase in sucrose and glucose resulting in a dramatic change of the osmotic conditions of the cell, is common to all three cases where a change in the expression of major protein species as a result of changes of the carbohydrate content was observed. We strongly feel that future research on control of gene expression in higher plants should take these factors, i.e. metabolites of very general pathways as well as physical parameters, into more consideration. Finally the data described clearly disprove the hypothesis that the expression of patatin and the proteinase inhibitor II is induced by sucrose as previously suggested by several groups including ourselves (Rocha-Sosa *et al.*, 1989; Wenzler *et al.*, 1989; Johnson and Ryan, 1990; Jefferson *et al.*, 1990). This hypothesis was based on experiments where exogenous sucrose was fed to detached leaves or petioles. These contradictory results strongly suggest that experiments where changes in gene expression are related to the addition of exogenously applied substances have to be repeated on the whole plant level by creating plants changed *in vivo* with respect to the parameters supposed to be important for the regulation of gene expression. Transgenic plants obviously represent a useful tool in this respect.

Materials and methods

Plants, bacterial strains and growth conditions

Solanum tuberosum L. cv. Désirée was obtained through 'Vereinigte Saatuchten eG' (3112 Ebstorf, FRG). Plants in tissue culture were maintained under a 16 h light–8 h dark regime on MS medium (Murashige and Skoog, 1962) containing 2% sucrose. Plants in the greenhouse were cultivated in soil under a light–dark regime of 16 h light (250 $\mu\text{E}/\text{m}^2/\text{s}$;

22°C) and 8 h dark (15°C). Each plant was grown in an individual pot (200 cm², 15 cm deep) and was watered daily, except for the last two weeks prior to collection of the tubers. Tubers were harvested 4 months after transfer to tissue culture plants to the greenhouse. Tubers with a fresh weight of 9–15 g were used for biochemical analysis.

Escherichia coli strain DH5 α (Bethesda Research Laboratories, Gaithersburg, USA) was cultivated using standard techniques (Sambrook *et al.*, 1989). *Agrobacterium tumefaciens* strain C58C1 containing plasmid pGV2260 (Deblaere *et al.*, 1985) was cultivated in YEB medium (Vervliet *et al.*, 1975).

Enzymes and chemicals

DNA restriction and modification enzymes were purchased from Boehringer Mannheim (Ingelheim, FRG) and New England Biolabs (Danvers, USA). Reagents for SDS–PAGE were obtained from BioRad (St Louis, USA). Enzymes for activity tests and carbohydrate determinations were obtained from Boehringer Mannheim (Ingelheim, FRG). Chemicals were purchased from Sigma Chemical Co. (St Louis, USA) or Merck (Darmstadt, FRG).

DNA manipulations

DNA manipulations were done essentially as described by Sambrook *et al.* (1989). For construction of the AGPase antisense gene, the 1.6 kb *EcoRI* cDNA insert of plasmid B22-1 (Müller-Röber *et al.*, 1990), coding for potato AGPase B, was blunt ended by a fill-in reaction with T4 polymerase and introduced in its reverse orientation into the *SmaI* site of a plant expression vector, containing the CaMV 35S promoter and the polyadenylation signal of the T-DNA octopine synthase gene in pUC18 (von Schaeuwen, 1989), to create pAnti-AGP-1. The *EcoRI*–*SspI* antisense gene insert of pAnti-AGP-1 was ligated between the *EcoRI* and *SmaI* sites of the plant transformation vector Bin19, resulting in the vector pBin-AntiAGP-1.

Potato transformation

Vector pBin-AntiAGP-1 was used to transform *S. tuberosum* L. cv. Désirée via *A. tumefaciens* strain C58C1:pGV2260 as described (Rocha-Sosa *et al.*, 1989).

RNA extraction and Northern blot experiments

Total RNA was extracted from frozen plant materials as described (Logemann *et al.*, 1987). RNA was denatured in 40% formamide, subjected to agarose gel electrophoresis (1.5% agarose, 15% formaldehyde) and blotted onto nylon membranes (Hybond N, Amersham, UK). Membranes were hybridized in buffers containing polyethylene glycol and formamide as described (Amasino, 1986). Radioactive labelling of DNA probes was performed using a multiprime labelling kit (Amersham, UK). Filters were washed twice in 1.5 \times SSC, 0.5% SDS for 30 min at 68°C, and once in 0.1 \times SSC, 0.5% SDS for 30 min at 68°C. The following cDNA clones were used for labelling reactions: AGPase B, *EcoRI* insert of plasmid B22-1 (Müller-Röber *et al.*, 1990); AGPase S, *EcoRI* insert of plasmid S25-1 (Müller-Röber *et al.*, 1990); granule bound starch synthase, a 1.4 kb long cDNA in pUC18, obtained from U. Wienand, MPI Köln, FRG; branching enzyme, *EcoRI* fragments of plasmid BE7 (Kossmann *et al.*, 1991); starch phosphorylase, cDNA insert of plasmid pSTP3 (Brisson *et al.*, 1989); sucrose synthase, a 1.3 kb *EcoRI* fragment of clone λ 10a, corresponding to the C-terminus of the potato sucrose synthase (Salanoubat and Belliard, 1987); patatin, *PstI* insert of plasmid pcT58 (Rosahl *et al.*, 1986); proteinase inhibitor II, cDNA 1 as described in Sanchez-Serrano *et al.* (1986). The cDNA probe representing potato sucrose phosphate synthase was obtained by immunoscreening a potato expression library (U. Sonnewald, unpublished results). The DNA probe corresponding to the 22 kDa protein complex was obtained in our laboratory by differential screening of a potato tuber cDNA library (H. Hesse, personal communication). Partial DNA sequence analysis of the isolated clone revealed >95% homology to clone p34021 encoding at least one of the polypeptides of the 22 kDa protein complex (Stiekema *et al.*, 1988; Suh *et al.*, 1990).

SDS–PAGE and Western blot experiments

Proteins were extracted from frozen tuber slices using 25 mM sodium phosphate buffer (pH 7.0) containing 2 mM sodium bisulphite (Racusen and Foote, 1980) and separated on 10% SDS–polyacrylamide gels (Laemmli, 1970). For Western blot analysis, proteins were transferred onto nitrocellulose membranes using a semi-dry electroblotting apparatus (Multiphor II; LKB Bromma, Sweden). Immunodetection was performed using a commercial biotin–streptavidin/horseradish peroxidase system (Amersham, UK) according to the manufacturer's instructions. Anti-maize brittle-2 antiserum (provided by M. Giroux and L. C. Hannah) was used at a 1:1000 dilution in TBST-BSA (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.05% Tween-20, 0.5% BSA).

The specificity of the anti-maize brittle-2 antiserum in recognizing the

homologous potato protein was shown by screening a λ ZAP II potato tuber cDNA expression library (Kossmann *et al.*, 1991) with the antiserum. Positive plaques arising were purified. The phage DNAs strongly hybridized to the AGPase B cDNA insert of plasmid B22-1 (Müller-Röber *et al.*, 1990). Partial sequence analysis of the λ ZAP II cDNA inserts revealed complete identity to the published AGPase B sequence (Müller-Röber *et al.*, 1990; du Jardin and Berhin, 1991).

Determination of AGPase activity

Crude protein extracts were used for determination of AGPase enzyme activity. Frozen tuber slices (80–100 mg fresh weight) were homogenized in 100 μ l extraction buffer (Neuhaus and Stitt, 1990), centrifuged (5 min, 15 000 g) and the supernatant used for activity tests. Production of glucose-1-phosphate from ADP–glucose was determined in an NAD-linked glucose-6-phosphate dehydrogenase system (Plaxton and Preiss, 1987; Smith, 1990). The reaction assay contained 80 mM HEPES (pH 7.4), 10 mM MgCl₂, 1 mM ADP–glucose, 0.6 mM NAD, 10 μ M glucose-1,6-phosphate₂, 3 mM DTT, 0.02% BSA, 1 U phosphoglucosyltransferase, 2.5 U NAD-linked glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*) and tuber extract. The reaction was initiated by addition of sodium pyrophosphate (2 mM final concentration). NAD reduction was measured spectrophotometrically at 340 nm and 30°C. Protein concentrations were determined by the method of Bradford (1976).

Determination of starch and soluble sugars

Tuber slices (80–100 mg fresh weight) were extracted twice for 30 min each in 500 μ l 80% ethanol–50 mM HEPES, pH 7.5 at 80°C. The supernatant of the first extraction was used for enzymatic analysis of sucrose, glucose and fructose as described (Stitt *et al.*, 1983). For measurement of starch, extracted tuber slices were homogenized in 800 μ l 0.2 N KOH, incubated at 95°C and adjusted to pH 5.5 by addition of 140 μ l 1 N acetic acid (Lin *et al.*, 1988a). Starch was hydrolysed with amyloglucosidase and the released glucose enzymatically determined as described (Jones *et al.*, 1977).

For iodine staining of starch, tuber discs were incubated in Lugol's solution (2 g KI and 1 g I₂/300 ml) for 5 min and subsequently washed in distilled water for 30 s. Photographs were taken from freshly stained and washed samples.

Determination of tuber fresh and dry weight

Fresh weight of tubers was determined directly after harvest. For determination of dry weight, slices of randomly selected tubers were incubated at 80°C until no further decrease in weight could be observed.

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