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Manipulation of starch granule size distribution in potato tubers by modulation of plastid division

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Summary

Starch granule size is an important parameter for starch applications in industry. Starch granules are formed in amyloplasts, which are, like chloroplasts, derived from proplastids. Division processes and associated machinery are likely to be similar for all plastids. Essential roles for FtsZ proteins in plastid division in land plants have been revealed. FtsZ forms the so-called Z ring which, together with inner and outer plastid division rings, brings about constriction of the plastid. It has been shown that modulation of the expression level of FtsZ may result in altered chloroplast size and number. To test whether FtsZ is also involved in amyloplast division and whether this, in turn, may affect the starch granule size in crop plants, FtsZ protein levels were either reduced or increased in potato. As shown previously in other plant species, decreased StFtsZ1 protein levels in leaves resulted in a decrease in the number of chloroplasts in guard cells. More interestingly, plants with increased StFtsZ1 protein levels in tubers resulted in less, but larger, starch granules. This suggests that the stoichiometry between StFtsZ1 and other components of the plastid division machinery is important for its function. Starch from these tubers also had altered pasting properties and phosphate content. The importance of our results for the starch industry is discussed.

Introduction

Starch is the most abundant storage reserve carbohydrate in plants and is an important raw material for food and industrial applications (Röper, 2002). The suitability of starch for specific applications is determined by its granule size, physicochemical properties and the presence of non-starch components, such as protein and lipid (Ellis *et al*., 1998). Starches can be subjected to different kinds of chemical derivatization procedures to improve their properties for specific applications (Röper, 2002). Granule size is an important factor for many applications. Depending on the biological source, starch granule size may vary from less than 1 μm to more than 100 μm (Ellis *et al*., 1998). In potato, starch granules range from 5 to 100 μm. One way to obtain starch with a specific granule size is fractionation. However, this requires an additional step in the processing. It would be an advantage if starches with altered granule size distributions could be tailored *in planta*.

In recent years, our understanding of starch structure has increased greatly. Many of the genes encoding starch biosynthetic enzymes have been cloned and the function of many of these genes can be elucidated by over-expression and down-regulation in plants (Ball and Morell, 2003; Tetlow *et al*., 2004). A good example of this is the down-regulation of the granule-bound starch synthase (GBSS) in potato, resulting in plants with amylose-free starch (Visser *et al*., 1991).

The mechanism that produces the huge variation in the size of starch granules between plant species is not well understood. Recently, it has been shown that heteromultimeric isoamylase, a starch debranching enzyme, is an important factor in controlling starch granule initiation (Burton *et al*., 2002). Antisense suppression of two of the three isoamylases present in potato leads to the accumulation of large numbers of small granules not seen in normal tubers (Bustos *et al*., 2004). Likewise, the down-regulation of the barley limit dextrinase inhibitor, an inhibitor of the starch debranching

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enzyme limit dextrinase, leads to changes in granule size distribution (Stahl *et al*., 2004). The antisense transgenic barley plants showed decreased numbers of small starch granules, decreased amylose levels and a change in chain length distribution of the amylopectin. Surprisingly, numerous enzyme activities, such as α- and β-amylases and starch synthases, were affected by the down-regulation of limit dextrinase inhibitor. Ji *et al*. (2004) demonstrated, in a different way, the decrease in starch granule size by the expression of a tandem starch-binding domain derived from *Bacillus* cyclodextrin glycosyltransferase in potato. The mechanism by which this occurs is largely unknown.

In this study, we investigated the possibility of whether the size of starch granules could be modulated by affecting the size of the amyloplasts in which they are formed. It is well established that the number of divisions that plastids undergo influences their final size. Plastid division is mechanistically related to prokaryotic cell division (for reviews, see Osteryoung and McAndrew, 2001; Miyagishima *et al*., 2003; Osteryoung and Nunnari, 2003). Prokaryotic cell division requires FtsZ that assembles into filaments in a guanosine triphosphate (GTP)-dependent manner and is evolutionarily related to eukaryotic tubulins (Bramhill, 1997). FtsZ forms a ring structure, called the Z ring, on the inner face of the cytoplasmic membrane at the division site. The FtsZ ring, which constricts as division progresses, probably serves as a scaffold for the recruitment of additional cell division proteins.

It has been shown that homologues of FtsZ are also key structural components of the chloroplast division machinery in photosynthetic eukaryotes (Miyagishima *et al*., 2001). Chloroplastic FtsZ proteins of land plants are nuclear encoded and fall into two groups, FtsZ1 and FtsZ2 (Stokes and Osteryoung, 2003), with different biochemical properties and subplastidial localizations (El-Kafafi *et al*., 2005). Functional analysis has shown that members of both families are required for chloroplast division (Osteryoung *et al*., 1998).

Essential roles for FtsZ1, FtsZ2 (Osteryoung *et al*., 1998), ARC6 (Vitha *et al*., 2003), ARTHEMIS (Fulgosi *et al*., 2002), MinD (Colletti *et al*., 2000), MinE (Itoh *et al*., 2001), CRL (Asano *et al*., 2004), GC1 (Maple *et al*., 2004) and SulA (Raynoud *et al*., 2004) in the division of chloroplasts of land plants have been revealed. As in prokaryotes, the plant FtsZ proteins are components of the Z ring, whereas ARC6 stabilizes Z ring formation and MinD/E regulates its positioning. ARTHEMIS is localized at the inner envelope and may facilitate the assembly of the division apparatus and regulation of chloroplast division. The functions of CRL, GC1 and SulA are not yet known. It has been shown that the size of chloroplasts can be manipulated by over-expression or antisense repression of *FtsZ* (Osteryoung *et al*., 1998; Stokes *et al*., 2000; McAndrew *et al*., 2001) and *MinE* (Itoh *et al*., 2001; Reddy *et al*., 2002). Over-expression (Stokes *et al*., 2000; McAndrew *et al*., 2001) or antisense repression (Osteryoung *et al*., 1998) of *AtFtsZ1* or *AtFtsZ2* in *Arabidopsis* resulted in one or few large chloroplasts per cell, whereas wild-type plants typically contained 80–100 chloroplasts. A threefold increase in the AtFtsZ1-1 protein level inhibited chloroplast division (Stokes *et al*., 2000). Higher AtFtsZ1-1 protein levels resulted in more severe phenotypes. Plastid division defects resulting from AtFtsZ1-1 overproduction possibly reflect the stoichiometric imbalance in plastid division components.

Starch in higher plants is produced in specialized plastids, the amyloplasts. Like chloroplasts, they develop from proplastids, and ultrastructural, molecular and genetic data suggest that the components required for the division process are similar for all plastid types (Osteryoung and McAndrew, 2001). Therefore, it may be possible to manipulate starch granule size in crop plants by changing the expression level of *FtsZ1*, and thereby promoting or inhibiting amyloplast division. In this report, the potato *FtsZ1* cDNA was isolated and either down-regulated or over-expressed in potato, using the cauliflower mosaic virus (CaMV) 35S or GBSS promoter. We show that the chloroplast number in guard cells could be modulated. More importantly, an increased StFtsZ1 protein level resulted in a substantial decrease in the number of starch granules and an increase in the size of the starch granules in tubers.

Results

Cloning of potato *FtsZ1* and plant transformation

A full-length potato (*Solanum tuberosum*) *FtsZ1* cDNA (GenBank accession number AY601110) was cloned by rapid amplification of cDNA ends (RACE) polymerase chain reaction (PCR) based on the sequence homology between a potato expressed sequence tag (EST) sequence (dbEST Id 5349838) and the *Arabidopsis FtsZ1* cDNA. The deduced protein sequence was 73% identical (79% similarity) to AtFtsZ1-1 from *Arabidopsis* (Figure 1) and was therefore proposed to be a potato homologue of AtFtsZ1-1 and named StFtsZ1. The homology of StFtsZ1 with AtFtsZ2-2 and AtFtsZ2-1 was 50% and 48%, respectively.

The StFtsZ1 coding region was cloned in sense and antisense orientation under control of the CaMV 35S promoter or the potato promoter of *GBSS* in binary vectors. The resulting constructs were introduced into potato plants. For each construct, 20–28 independent transformants were obtained.

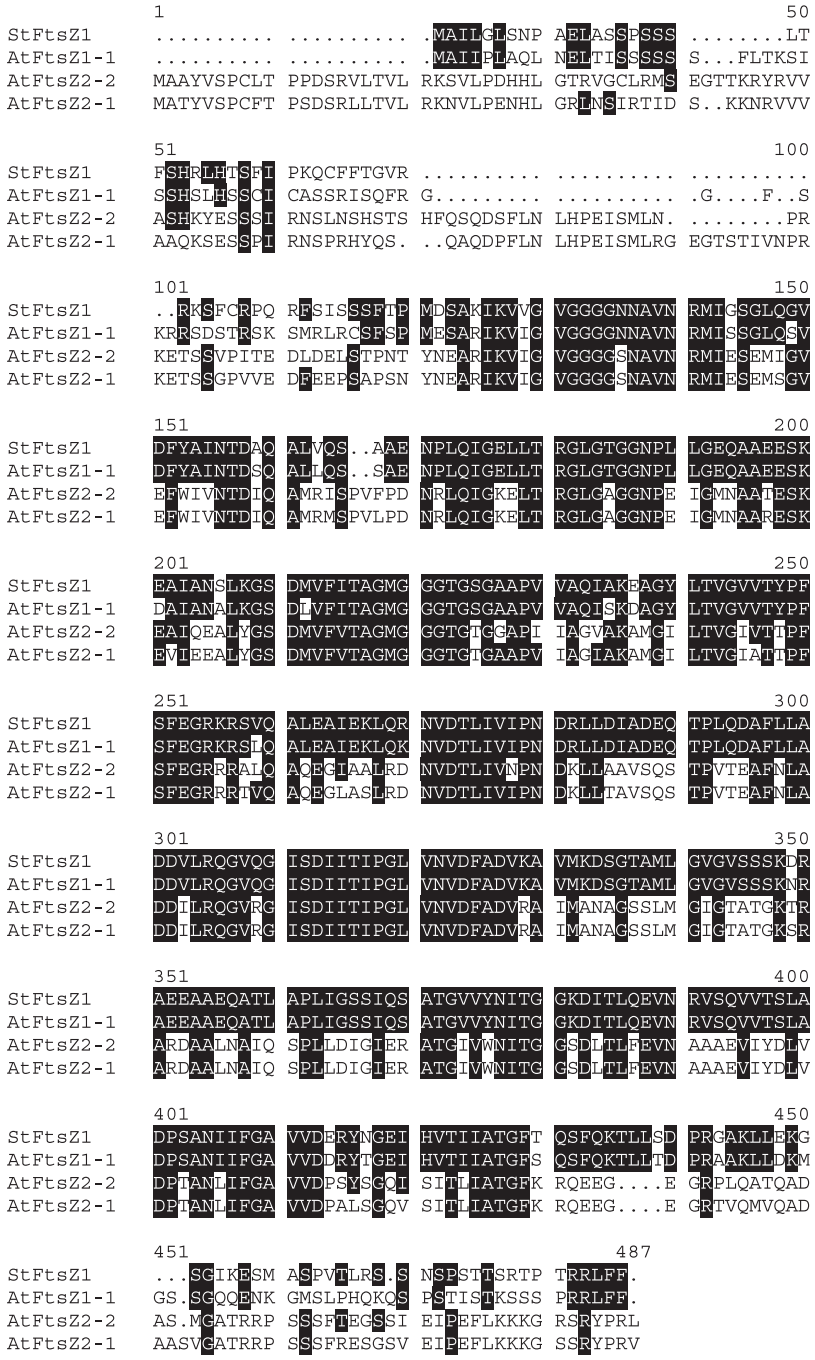

Figure 1 Amino acid sequence alignment of FtsZ from potato (StFtsZ1, accession number AY601110) and *Arabidopsis* (AtFtsZ1-1, accession number U39877; AtFtsZ2-2, accession number AF384167; AtFtsZ2-1, accession number AF089738). Amino acids identical in StFtsZ1 and at least one of the AtFtsZ proteins are indicated in black boxes.

Chloroplast number is affected by *StFtsZ1* expression

The plants with the *StFtsZ1* gene (sense or antisense) under control of the CaMV 35S promoter were analysed by confocal scanning laser microscopy (CSLM) to study the number and size of chloroplasts in guard cells, as a change in FtsZ1 is expected to influence the division of plastids. About 33% of the transformed plants (four of 20 35S-sense plants and 12 of 28 35S-antisense plants) had fewer chloroplasts in their guard cells (results not shown). Detailed analysis of one of these transformants (35S-AS15) showed that the number of chloroplasts per pair of guard cells was decreased by more than 30% (14.1 ± 1.5 in 35S-AS15 vs. 20.5 ± 1.6 in wild-type potato). In Figure 2, representative regions of the lower leaf epidermis are shown of untransformed potato and transformant 35S-AS15. We did not observe a significantly different chloroplast size in the guard cells of this transformant.

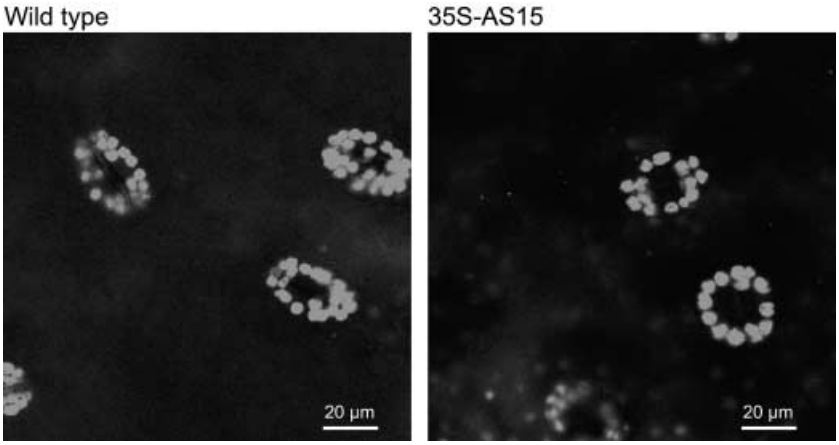

Figure 2 Chloroplasts in guard cells of the lower leaf epidermis from wild-type potato and a 35S-antisense *StFtsZ1* transformant (35S-AS15) visualized by confocal laser scanning microscopy (CSLM). The scale bars are 20 µm.

The FtsZ1 protein level in leaves of 35S-AS15 was analysed using FtsZ1 peptide antibodies (Stokes *et al*., 2000). In addition to some minor background bands of cross-reactive material, a band of about 40 kDa was visible in wild-type leaves, representing StFtsZ1. The amount of FtsZ1 protein was below the detection level in the transformant (Figure 3). These results show that StFtsZ1 is a functional homologue of AtFtsZ1, and that our approach to manipulate plastid division in potato was successful.

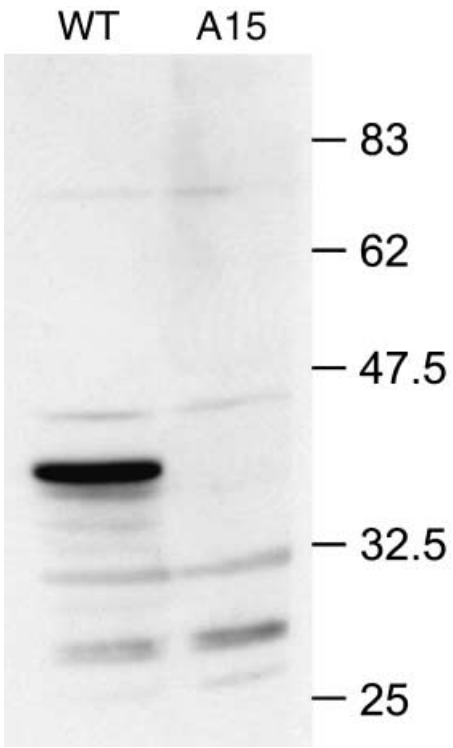

Figure 3 Western blot showing StFtsZ1 protein levels in leaf tissue from wild-type potato (WT) and the 35S-AS15 transformant (A15). Sizes of marker proteins are indicated.

Starch granule size is affected by *StFtsZ1* expression

The CaMV 35S promoter is not very active in tubers (Kuipers *et al*., 1995). Therefore, potato was transformed with *StFtsZ1* under control of the GBSS promoter for the high expression of sense and antisense constructs in tubers. All plants (25 sense and 24 antisense plants) were potted in soil and tuber starch was isolated and analysed. Statistical analysis was performed for the mean diameter and granule size distribution (results not shown). A number of plants of this first generation of potato plants over-expressing *StFtsZ1* exhibited starch granule sizes between 42 and 54 µm, whereas the starch granule size of control plants was around 28 µm (Table 1). No transformant with a granule size smaller than that of the control was detected.

Three transformants were selected with the largest mean granule size, as well as four untransformed control lines. For each line, five plants were grown in large pots under glasshouse conditions. Starch was isolated from harvested tubers

Table 1 Mean diameter (µm) of granules in starch samples measured by laser diffraction of starch isolated from untransformed potato plants (controls) and three transgenic lines (one measurement per line, per condition)

Sample	Small pots	Large pots	Field grown
Control 1	26.0	35.6	46.6
Control 2	29.3	39.7	49.5
Control 3	28.4	40.5	49.1
Control 4	29.1	40.8	45.3
Average control (SD)	28.2 (± 1.5)	39.1 (± 2.4)	47.6 (± 2.0)
GBSS-S04	49.0	59.0	62.4
GBSS-S12	54.0	56.3	64.1
GBSS-S25	42.4	49.3*	54.8*
Average GBSS-FtsZ (SD)	48.4 (± 5.9)	54.9 (± 5.0)	60.4 (± 5.0)

*Not significantly different from control.

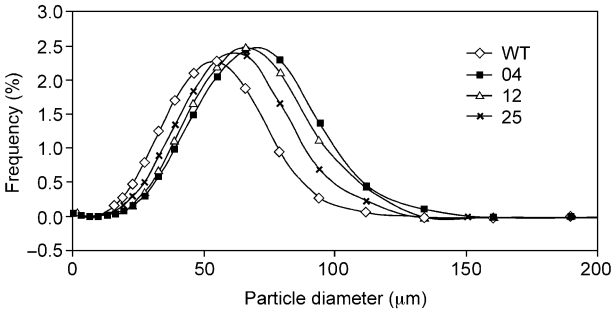

Figure 4 Particle size distribution of starches isolated from wild-type tubers (WT) and tubers from transgenic lines GBSS-S04 (04), GBSS-S12 (12) and GBSS-S25 (25). GBSS, granule-bound starch synthase.

of roughly equal size (tuber weight of between 20 and 40 g) and analysed for particle size distribution (Table 1). The four control clones had an average mean diameter of 39.1 μm, with values varying between 35.6 and 40.8 μm. The mean diameter of starch from two of the GBSS sense plants (GBSS-S04 and GBSS-S12) was significantly larger than that of the control, with values of 59.0 and 56.3 μm, respectively. Compared with the average of the control, the increase in mean diameter of GBSS-S04 was 51% and of GBSS-S12 was 44%.

Subsequently, the same lines were grown under field conditions and the starch granule sizes were analysed again. The particle size distributions of the field-grown starch batches of each line are shown in Figure 4 and the mean diameters are given in Table 1. In general, the size of the starch granules of the field-grown plants was substantially larger than that of the plants grown in large pots. As shown for the plants grown in large pots, transformants GBSS-S04 and GBSS-S12 had significantly larger starch granules than the control plants under field conditions, although the relative increase was less pronounced (31% and 35% for GBSS-S04 and GBSS-S12, respectively). The differences observed for the plant lines grown under different conditions may be due to the more optimal growth conditions in the field, resulting in larger tubers with larger starch granules, masking the effect of expression of the transgene. When transformant GBSS-S25 was grown in large pots and under field conditions the increase in starch granule size was not significant ($P < 0.05$).

Tuber slices of fourth generation plants grown in large pots were analysed by scanning electron microscopy (SEM) to determine the number and size of starch granules *in situ*. Potato amyloplasts usually consist of only one starch granule surrounded by a thin layer of stroma (Kram *et al*., 1993). Therefore, the number and size of starch granules represent the number and size of amyloplasts. Slices were used from similar regions of tubers of comparable age and size. The sections in Figure 5 show that, in wild-type tubers, 12.6 ± 2.8 granules are visible per cell. This number does not represent the total number of granules per cell. In the transformants, the number of granules per cell was decreased, whereas the size was increased. The numbers for GBSS-S04 and GBSS-S25 were 6.0 ± 1.2 and 6.0 ± 1.6, respectively. The decrease was most severe in transformant GBSS-S12 with only 3.8 ± 0.8 granules per cell.

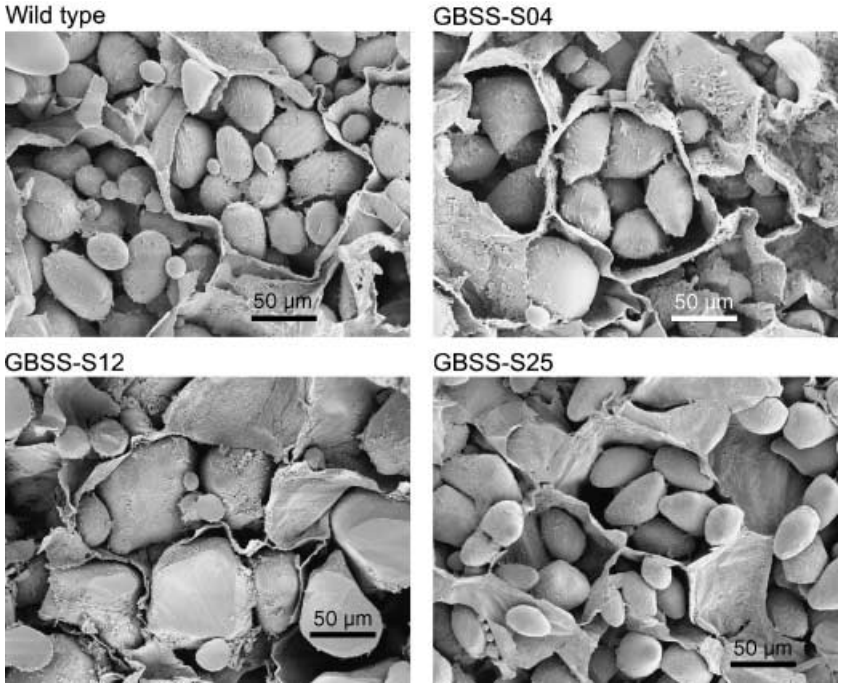

Figure 5 Scanning electron micrographs of tubers from wild-type potato and transgenic lines GBSS-S04, GBSS-S12 and GBSS-S25. The scale bars are 50 μm. GBSS, granule-bound starch synthase.

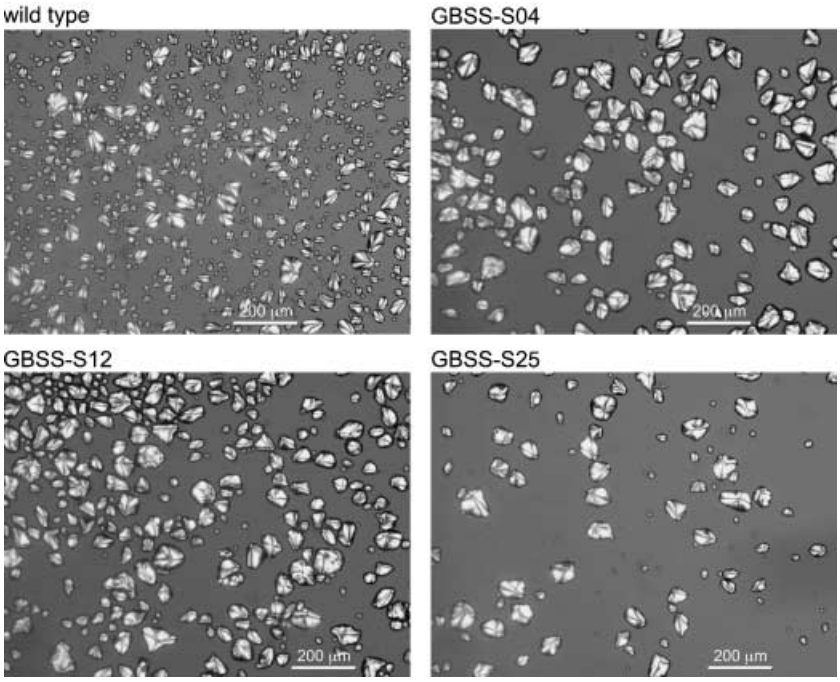

Figure 6 Starch granules isolated from wild-type potato tubers and from tubers from three transgenic lines GBSS-S04, GBSS-S12 and GBSS-S25 analysed by light microscopy. The scale bars are 200 µm. GBSS, granule-bound starch synthase.

Isolated starch granules were also analysed by light microscopy (Figure 6). The differences in size are clearly visible. The overall morphology of the granules in these starch samples was not significantly different. The starch content of the selected StFtsZ1 transformants (196 ± 4 mg/g fresh weight) was not significantly different from that of the control lines (202 ± 7 mg/g fresh weight). Together, these results show that the transformants have less, but larger, starch granules, resulting in a similar total amount of starch. No other obvious phenotypic differences were observed.

Characterization of starch properties

The impact of the large granule size on the physical properties was measured by Brabender viscometric analysis, which measures the change in viscosity of the starch as it is heated and then cooled under constant stirring, in a manner similar to that of preparing a sauce or pudding. As shown in Table 2, the transformants have a significantly higher T_o and T_{top}, suggesting that starch of the transformants starts to swell and is completely swollen at higher temperatures compared with the control. The peak viscosity, which is the viscosity when the granules are fully swollen and before the granule structure breaks down, was not significantly different between the control and transformants, whereas the viscosity of the starch pastes after cooling down (end viscosity) was significantly higher in starch of the transformants. To determine whether the difference in pasting properties is simply a result of the change in granule size or is affected by the composition of the starch, amylose and phosphate contents were

Table 2 Starch pasting properties using Brabender viscometric analysis (T_o, T_{top}, peak viscosity and end viscosity), apparent amylose content (three independent measurements) and phosphate content (two independent measurements) of untransformed potato plants (controls) and three transgenic lines. Peak and end viscosity are given in Brabender units (BU)

Sample	T_o (°C)	T_{top} (°C)	Peak viscosity (BU)	End viscosity (BU)	Amylose (%)	Phosphate (nmol/mg starch)
Control 1	62.6	67.9	2679	1550	24.5 (± 0.6)	24.0 (± 0.8)
Control 2	63.2	68.1	2765	1535	21.4 (± 0.7)	23.5 (± 0.4)
Control 3	63.6	69.2	2739	1577	21.1 (± 0.1)	23.5 (± 0.4)
Average control (SD)	63.1 (± 0.5)	68.4 (± 0.7)	2728 (± 44)	1554 (± 21)	22.3 (± 1.7)	23.7 (± 0.5)
GBSS-S04	64.1	70.3	2759	1729	25.1 (± 0.2)	29.4 (± 0.9)
GBSS-S12	64.7	70.7	2692	1764	22.9 (± 0.5)	29.2 (± 1.1)
GBSS-S25	64.9	70.5	2666	1739	23.8 (± 0.4)	27.3 (± 3.9)
Average GBSS-FtsZ (SD)	64.6 (± 0.4)	70.5 (± 0.2)	2705 (± 48)	1734 (± 18)	23.9 (± 1.0)	28.6 (± 2.1)

analysed. As can be seen in Table 2, the amylose content did not differ significantly between the transformants and the controls. In contrast, the phosphate content was significantly higher in the transformants.

FtsZ1 mRNA and protein level in transgenic potato

Subsequently, we analysed the *StFtsZ1* mRNA and StFtsZ1 protein level in untransformed tubers and tubers from GBSS-S04, GBSS-S12 and GBSS-S25. Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was used to determine the steady-state *StFtsZ1* mRNA level in tubers (Figure 7A) with the *GBSS* mRNA level as a control (Figure 7B). In the three transformants, more *StFtsZ1* PCR product was obtained, with the highest level in GBSS-S12. Western blot analysis (Figure 7C) was performed on the same tuber tissue using peptide antibodies against AtFtsZ1 (Stokes *et al*., 2000). The GBSS-S12 tuber contained 19.7 times more StFtsZ1 than wild-type tubers. This plant also exhibited the most severe effect on the number and size of starch granules (Figure 5; Table 1). GBSS-S04 and GBSS-S25 contained 6.6 and 2.9 times more StFtsZ1, respectively. On the basis of starch granule number, these plants showed an intermediate phenotype.

Discussion

FtsZ1 is a key structural component of the chloroplast division machinery. It forms a so-called Z ring which, together with the inner plastid division ring, outer plastid division ring and dynamin ring, constricts the plastid. As all plastids develop from undifferentiated proplastids, it is likely that differentiated plastids divide according to the same mechanism. Therefore, we hypothesized that the division of amyloplasts could be manipulated in the same way as shown for chloroplasts, namely by altering the expression level of *FtsZ*. As the size of starch granules formed in amyloplasts correlates with the size of amyloplasts (Kram *et al*., 1993), manipulation of *FtsZ* expression levels may result in an altered size distribution of starch granules.

Potato plants were produced expressing potato *FtsZ1* in sense or antisense orientation under control of the 35S promoter or the GBSS promoter. Analysis of the chloroplasts in guard cells of one of the 35S antisense transformants by CSLM revealed a decrease in the number of plastids and StFtsZ1 protein level. A similar decrease in the FtsZ1 protein level in *Arabidopsis* gave a more severe phenotype (Osteryoung *et al*., 1998). This apparent difference is probably the result of the presence of another *FtsZ1* gene, as an EST clone (CK257090) has been isolated with about 80% homology to the *StFtsZ1* cDNA isolated here. In tubers of over-expression lines, protein levels of threefold and sixfold above control levels were measured in plants with an intermediate phenotype (twofold less starch granules; Figure 5). The plant line with the more severe phenotype (threefold less starch granules) exhibited a 19.7-fold increase in StFtsZ1. The observed changes in StFtsZ1 protein levels in tubers resulted in less severe phenotypes than those found for leaves in over-expression *Arabidopsis* lines (Stokes *et al*., 2000), in which increased AtFtsZ1-1 levels, ranging from 13- to 26-fold, resulted in severe phenotypes of one or two chloroplasts per cell. The total starch content of the tubers was not affected:

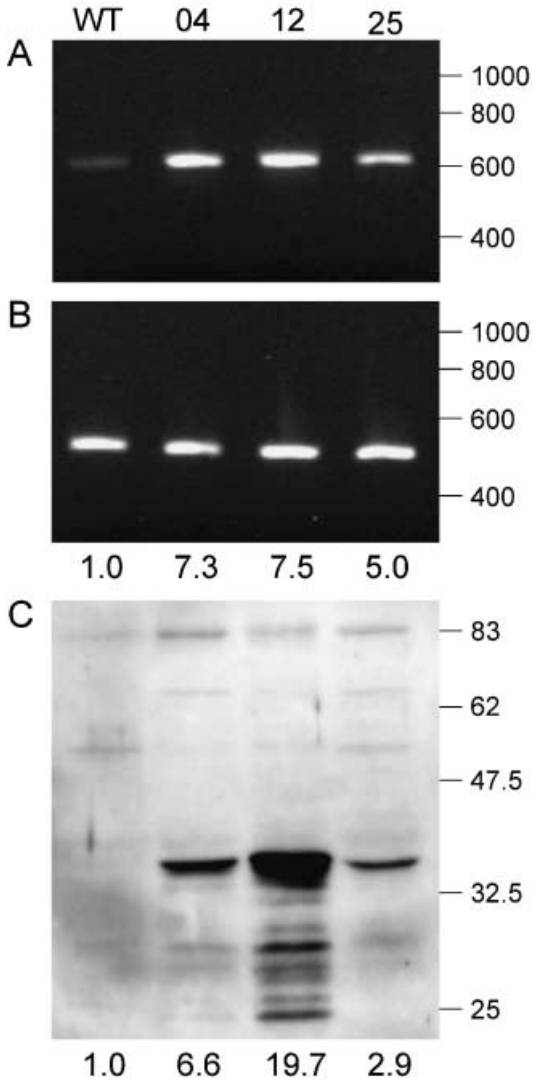

Figure 7 Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) showing amplified *StFtsZ1* cDNA (A) and *GBSS* cDNA (B), and Western blotting showing StFtsZ1 protein (C), in tubers from wild-type (WT) potato and three transgenic potato lines GBSS-S04 (04), GBSS-S12 (12) and GBSS-S25 (25). PCR products were normalized for the amount of GBSS product. The Western blot contains 10 μg of soluble protein per lane. Relative amounts of *StFtsZ1* mRNA and StFtsZ1 protein are shown. Size markers are indicated. GBSS, granule-bound starch synthase.

inhibition of plastid division resulted in less, but larger, starch granules.

Increased levels of StFtsZ1 resulted in a (partial) inhibition rather than an increase in plastid division. A similar result was obtained in *Arabidopsis* (Stokes *et al*., 2000). This suggests that the ratio between FtsZ1 and other components of the plastid division machinery is important, and that a stoichiometric imbalance results in the inhibition of division. Analysis of more transformants with slightly elevated FtsZ1 levels, or fine tuning of levels of different components of the plastid division machinery, may prevent a drastic change in the stoichiometry, and may result in more efficient plastid division and thus smaller starch granules.

A number of starch samples isolated from transgenic tubers showed a change in starch granule size distribution. A selection of these plants were grown again in large pots and under field conditions. They all clearly had larger starch granules compared with the first experiment, including the wild-type plants. This difference is probably due to the conditions under which the plants were grown, which resulted in larger tubers and, consequently, larger granule sizes (Noda *et al*., 2004). Under more optimal growth conditions, the effect of the altered FtsZ1 protein level on the starch granule size distribution was less pronounced.

In our studies, we observed an increase in the gelatinization temperature and end viscosity of the larger sized potato starches. This finding is in agreement with that of Goering and De Haas (1972) and Zheng and Sosulski (1997), showing that large granules in general tend to have a higher pasting temperature than smaller granules. In the bimodal wheat and barley starches, the smaller B-granules paste at a higher temperature than the large A-type starch granules (Myllärinen *et al*., 1998; Chiotelli and Le Meste, 2002). This difference in paste temperature may be explained by the difference in the apparent crystallinity of the starches.

Some reports have claimed that the amylose concentration is higher in the large granules of bimodal starches (Peng *et al*., 1999; Takeda *et al*., 1999), whereas others have found no difference in amylose concentration between the two starch sources (Myllärinen *et al*., 1998). We did not observe a relationship between granule size and amylose content. In contrast, the phosphate content seemed to be higher in starch of the transformants than in that of the controls. Several reports have shown a correlation between phosphate content and other physicochemical properties: phosphate and peak viscosity (Viksø-Nielsen *et al*., 2001); phosphate, peak viscosity, end viscosity and amylose (negative correlation) (Blennow *et al*., 2003); and phosphate, peak viscosity and granule size (Noda *et al*., 2005). We found an increase in pasting temperatures (T_o and T_{top}) and end viscosity, in addition to the higher phosphate content. A similar correlation has been described in a variety of genetically modified potato starches (Wischmann *et al*., 2005).

The starch industry has considerable interest in starches with diverse functional properties (Jobling, 2004). Granule size is one of the most important characteristics when using starch for industrial applications. Small granule starches are used, for example, as fat replacers in food applications, as carrier material in cosmetics and in biodegradable films (for a review, see Lindeboom *et al*., 2004). For maize and cassava starch, increased starch granule size is often desirable, because this improves the wet-milling efficiency and thus the starch yield (Gutiérrez *et al*., 2002). There are several methods of obtaining starch with a selected size distribution. Separation techniques, such as wind sifting, dry or wet sieving or separation by hydrocyclones, can be used to produce either small or large granular starch. The disadvantages of all of these fractionation techniques are the cost of the extra processing step and the relatively low efficiency of recovering either the small or large granules. Genetic modification, however, offers the possibility to produce starch with altered granule size *in planta*. Recently, it has been reported that the granule size was decreased in potato by antisense repression of isoamylase (Bustos *et al*., 2004) or by expression of an engineered starch binding domain (Ji *et al*., 2004). Stahl *et al*. (2004) showed that the granule size of barley could be increased by the antisense repression of a limit dextrinase inhibitor. In these plants, the activity of a number of enzymes was also changed, probably resulting from unpredicted pleiotropic effects. We have performed semi-quantitative RT-PCR analysis of genes encoding isoamylase, soluble starch synthase, GBSS, starch branching enzyme, α- and β-amylases and tubulin (results not shown). The expression levels of these genes were similar in wild-type tubers and in tubers of the transgenic lines, ruling out pleiotropic effects or somaclonal variation on starch metabolism in general. Here, we show that genetic modification of the plastid division machinery may result in altered starch granule size. As plastid division is a very complex biological process and additional structural components and regulatory elements are being discovered, new possibilities to fine tune granule size formation may become available.

Experimental procedures

Cloning of potato *FtsZ*

Potato (*S. tuberosum*) *FtsZ1* cDNA was cloned using the Clontech (Palo Alto, CA) SMART™ RACE cDNA amplification kit

according to the manufacturer's recommendations. A potato EST sequence (dbEST Id 5349838) was used to design the 5′ primer SP107 (TTCACTAGTGATTCCATGGCGATTTTAGGG). To obtain visible PCR fragments on gels, touchdown PCR was performed with 30 cycles. The PCR products were cloned in pGEM®-T easy (Promega, Madison, WI). Clones were tested for the presence of *StFtsZ1* by nested PCR using SP102 (GCGCCTCGAGCTTCTATGCTATAAACACGG) and SP103 (GGCCGAGCTCCGTCCTTCAAAGCTGAAAGG). Both strands of one clone were sequenced (BaseClear, Leiden, the Netherlands).

Vector construction

To obtain convenient restriction sites at the ends of the *StFtsZ1* coding region, the pGEM®-T easy plasmid containing the PCR fragment was digested with *Eco*RI, blunted by Klenow treatment and cloned in the *Sma*I site of pBluescript SK+ in both orientations. To generate the 35S-*StFtsZ1* sense construct, the green fluorescent protein (GFP) gene in pMP2164 (35S promoter-GFP) was replaced by *StFtsZ1* using *Nco*I and *Pst*I restriction sites. For construction of the antisense orientation, pMP2164 was digested with *Nco*I and *Not*I and blunted by Klenow treatment. Blunt *StFtsZ1* fragment was obtained by *Eco*RI digestion and Klenow incubation. The orientation after blunt ligation was checked by restriction analysis. The *Eco*RI fragments of the resulting plasmids were cloned in the plant vector pMOG402 (Jongedijk *et al*., 1995).

For the construction of GBSS promoter-*StFtsZ1* fusions, the plant vector pPGB121S (Kuipers *et al*., 1995) was used. *StFtsZ1* was cloned as *Bam*HI-*Sal*I fragments in both orientations.

All plant vectors were introduced into *Agrobacterium tumefaciens* strain MOG101 (Hood *et al*., 1993) by triparental conjugation.

Potato transformation

In vitro-grown potato plants (cv. Kardal) were used to produce transgenic plants via the stem transformation method of Visser (1991). Plants were propagated *in vitro* in Murashige–Skoog (MS) medium containing 3% sucrose and 50 mM kanamycin in 10-cm-high containers. Before potting in soil, *in vitro* plants were grown in medium without kanamycin. First generation plants were grown for 19 weeks in 9-cm pots in a glasshouse and were used for CSLM and to determine granule size. Second generation plants were grown for 16 weeks in 16-cm pots in a glasshouse and were used to determine granule size. Third generation plants were grown for 22 weeks under field conditions and were used to determine granule size and physicochemical properties. Fourth generation plants were grown for 21 weeks in 16-cm pots in a glasshouse and were used for CSLM, SEM, RT-PCR and Western blotting.

DNA isolation and PCR analysis

Plants growing on selection medium containing kanamycin were tested for the presence of the *StFtsZ1* constructs by PCR analysis. Fresh leaf tissue (1–2 cm^2) was ground in 200 μL of CTAB extraction buffer [2% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (CTAB), 1.4 M NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA), 100 mM Tris/HCl, pH 8.0]. After washing the potter equipment with 300 μL of CTAB and combining both the sample and the washing solution, the sample was incubated with RNase (0.2 mg/mL) for 10 min at 37 °C. Subsequently, the sample was incubated for 15 min with 100 μL of chloroform at 65 °C and twice extracted with phenol/chloroform/isoamylalcohol (24/24/1) and once with chloroform. The DNA was precipitated with 10 μL of 3 M NaAC (pH 4.8) and 1 mL of ethanol. The DNA was washed, dried and dissolved in 50–200 μL of 10 mM Tris/HCl, 1 mM EDTA (pH 8.0). One microlitre was used for PCRs in a final volume of 25 μL. The sense primer was as-1b (CCACTGACGTAAGGGATGAC) for 35S promoter constructs and GBSS1 (GAGGGAGTTGGTTTAGTTTTTAGA) for GBSS promoter constructs. For sense constructs, SP103 (GGCCGAGCTCCGTCCTTCAAAGCTGAAAGG) was used as antisense primer and, for antisense constructs, SP108 (GGAGCAAAGCTACTTGAGAAGGGC) was used as antisense primer.

Chloroplast visualization

Chloroplasts in guard cells of the lower leaf epidermis were examined by CSLM (Bio-Rad [Hercules, CA] MRC1024ESo; excitation at 488 nm/588 nm and emission at 585 nm for EFLP or excitation at 647 nm and emission at 680 nm for DF 32, depending on leaf age). To determine the number of chloroplasts in wild-type potato and 35S-AS15, 75 pairs of guard cells of comparable leaf regions derived from different leaves were examined from plants of the same age. One plant per construct was used. The average number and standard deviation are given.

Scanning electron microscopy

Tuber slices were frozen by fast immersion in liquid N_2 and dehydrated in acetone/methanol (1/1) at –80 °C for 4 days with three changes of solution. Air-dried slices were mounted

on stubs with carbon tabs and cleaved, sputtered with gold and analysed in a scanning electron microscope (Jeol SEM 6400, Tokyo, Japan). The average numbers and standard deviation of visible starch granules from five cells were determined.

Starch isolation and analysis

Tubers of roughly equal weight derived from glasshouse-grown or field-grown plants were used for starch isolation; 100 or 5000 g of tubers, respectively, was homogenized for 1 min in a Waring blender with half volume of water. To prevent brown colouring, 1000 p.p.m. of sodium bisulphite was added. The slurry was filtered on a sieve (150 μm) to remove fibre material and washed with five volumes of water. The starch was allowed to settle for 2 h at room temperature. The supernatant was removed and the starch was washed again with five volumes of water. After settling for 2 h, the supernatant was decanted and the starch was dried overnight at 30 °C. The starch content was determined by incubating 50 mg of tuber material in 0.5 mL of 25% HCl and 2 mL of dimethylsulphoxide (DMSO) for 1 h at 60 °C. After neutralization with 5 M NaOH, the solution was diluted with 0.1 M citrate buffer (pH 4.6) to a final volume of 10 mL. The glucose content in an aliquot of the hydrolysed starch sample was measured spectrophotometrically using the coupled glucose oxidase-catalysed reduction of nicotinamide adenine dinucleotide (NAD^+) (Boehringer, Mannheim, Germany). The measurements were repeated three times.

The granule size distributions of the isolated starches were estimated by laser diffraction spectroscopy (Sympatec HELOS H1140, Clausthal-Zellerfeld, Germany). The measurement duration was 10 s with a cycle time of 1000 m/s. The particle size distribution was registered between 0.5 and 350 μm. For each starch sample, one measurement was performed.

The viscosity measurements were carried out using a Brabender Viskograph E (Brabender OHG, Duisberg, Germany) on a 5% starch suspension in distilled water. The heating profile used was as follows: from 30 to 90 °C at a rate of 1.5 °C/min; heating at 90 °C for 20 min; cooling from 90 to 30 °C at a rate of 1.5 °C/min. A plot of the paste viscosity in arbitrary Brabender units (BU) was used to determine the pasting temperature and temperature at maximal viscosity, maximal (peak) viscosity and viscosity at the end of cooling.

The apparent amylose content of the starches was determined according to the method described by Hovenkamp-Hermelink *et al*. (1988). The total phosphate content of starch was determined colorimetrically according to Morrison (1964).

Reverse transcriptase-polymerase chain reaction

Tissue was disrupted to a powder under liquid N_2 in a TissueLyser (Qiagen, Valencia, CA). RNA was isolated using an RNeasy® Plant Mini Kit (Qiagen). Residual DNA was removed with DNA-*free*™ (Ambion, Austin, TX). After annealing with oligo-dT for 5 min at 70 °C and chilling on ice, cDNA was produced on 0.5 μg RNA for 1 h at 42 °C with MMLV (Molony Murine Leukemia Virus) reverse transcriptase (Promega), in the presence of 2 mM deoxynucleoside triphosphates (dNTPs) and 10 U Rnasin, in a total volume of 12.5 μL. PCR was performed on 0.25 μL cDNA with sense primer SP174 (CATATCCTTTCAGCTTTGAAG) and antisense primer SP175 (GCCCTTCTCAAGTAGCTTTGC) for detection of a fragment of *StFtsZ1* cDNA (30 cycles), and sense primer SP152 (CTTGGGATACTAGCGTTGCGGTTGAG) and antisense primer SP153 (CCAGTTGATTTTCCTACCCTTAACAGGC) for detection of a fragment of *GBSS* cDNA (25 cycles).

Protein isolation and Western blotting

Tissue was disrupted to a powder under liquid N_2 in a TissueLyser (Retch). For leaf tissue, 1 mL of sample buffer [60 mM Tris/HCl, pH 8.0, 2% sodium dodecylsulphate (SDS), 100 mM dithiothreitol (DTT), 14.3 mM β-mercaptoethanol, 10% glycerol, 0.02% BFB (Bromophenol Blue), supplemented with protease inhibitor cocktail complete EDTA-free, Roche Diagnostics, Almere, the Netherlands] was added to 100 mg of powder and proteins were extracted by incubation for 15 min at 70 °C. Prior to gel loading, the samples were centrifuged to remove particulate material. For tuber tissue, 100 μL of extraction buffer (50 mM Tris/HCl, pH 8.0, 2 mM EDTA, 10 mM DTT, supplemented with protease inhibitor cocktail complete EDTA-free, Roche) was added to 200 mg of powder and soluble proteins were isolated by centrifugation at 4 °C. The protein concentration was determined using Biorad protein assay reagent.

Ten microlitres of leaf protein or 10 μg of tuber protein was loaded on to a 10% SDS-polyacrylamide gel and semidry blotted on to nitrocellulose. Equal loading of the gels and the quality of protein preparations were checked by staining extra sets of gels with Coomassie Brilliant Blue R250 (not shown). Blots were blocked for 3 h in 5% non-fat dry milk in PBST (10 mM $NaPO_4$, pH 7.4, 120 mM NaCl, 2.7 mM KCl, 0.05% v/v Tween 20) and incubated overnight at 4 °C with FtsZ1-1 peptide antibodies (1 : 10 000) (Stokes *et al*., 2000) in the same buffer. The blots were washed four times with PBST and incubated for 3 h with anti-rabbit horseradish peroxidase (HRP) antibodies (1 : 7500) (Promega). The blots were washed

four times with PBST and detection was performed using a LumiGLO™ chemiluminescence detection kit (Cell Signalling Technology, Beverley, MA).

Relative amounts of *StFtsZ1* mRNA and FtsZ1 protein were determined by scanning the UV photographs and autoradiogram, and analysis of the bands was performed with the software program Quantity One (Bio-Rad).

Statistical analysis

Average values and standard deviations (SD) are given. To determine significant differences, *t*-tests and chi-squared tests (for single values of the mean diameter) were performed. Values were considered to be significantly different when $P < 0.05$.

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