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Enhancing the expression of starch synthase class IV results in increased levels of both transitory and long-term storage starch

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Summary

Starch is an important renewable raw material with an increasing number of applications. Several attempts have been made to obtain plants that produce modified versions of starch or higher starch yield. Most of the approaches designed to increase the levels of starch have focused on the increment of the amount of ADP-glucose or ATP available for starch biosynthesis. In this work, we show that the overexpression of starch synthase class IV (SSIV) increases the levels of starch accumulated in the leaves of *Arabidopsis* by 30%–40%. In addition, *SSIV*-overexpressing lines display a higher rate of growth. The increase in starch content as a consequence of enhanced *SSIV* expression is also observed in long-term storage starch organs such as potato tubers. Overexpression of *SSIV* in potato leads to increased tuber starch content on a dry weight basis and to increased yield of starch production in terms of tons of starch/hectare. These results identify SSIV as one of the regulatory steps involved in the control of the amount of starch accumulated in plastids.

Introduction

Starch is the main storage carbohydrate in plants. Its abundance as a naturally occurring compound is surpassed only by cellulose, and it represents the most important carbohydrate in human nutrition. Because of its unique physicochemical properties, starch has multiple applications in the paper, cardboard and corrugating industries, textiles, cosmetics, pharmaceuticals, construction and in the production of first-generation biofuels (Somerville, 2007). Therefore, identifying and understanding all the factors involved in the starch biosynthetic process is critically important for the rational design of experimental traits required to improve yields in agriculture, generate starches that might lead to new uses and produce more elaborated polymers to meet industrial needs.

Besides these industrial applications, starch is essential in the physiology of plants, and modifications in its levels of accumulation lead to alteration in growth rate, flowering time or seed yield (Schulze *et al.*, 1991, 1994; Corbesier *et al.*, 1998). In photosynthetically competent organs such as leaves, starch is transitorily stored in chloroplasts during the day and subsequently mobilized during the night to provide skeletons and energy to support plant growth. In heterotrophic organs such as tubers and seed endosperms, starch is accumulated over long periods to provide carbon skeletons and energy for plant sprouting or germination. The synthesis of starch requires the participation of different activities: glucosyl-transferase activity carried out by starch synthases (SS), which transfer the glucosyl moiety of the activated donor, ADP-glucose, to the free non-reducing end of an elongating α-(1 → 4)-D-glucan chain. The branching activity is performed by the starch branching enzyme (BE), which establishes chain branches through α-(1 → 6) linkages, while the debranching activity, conducted by debranching enzymes (isoamylases), consists of the removal of excess branches that would otherwise prevent the formation of the semi-crystalline structure of the starch granule.

Different biotechnological approaches have been followed to increase the production of starch in heterotrophic organs of plants. They are based on the increment in the levels of ADP-glucose or the amount of ATP available for the synthesis of starch. One strategy for enhancing ADP-glucose linked to starch biosynthesis consisted of increasing the activity of ADP-glucose pyrophosphorylase (AGPase). Transgenic potato plants expressing in the plastid the product of *glgC-16* (a mutant form of the *Escherichia coli* AGPase that shows reduced response to allosteric effectors) showed an average of 35% more tuber starch than the untransformed control (Stark *et al.*, 1992), although there was no correlation between the levels of starch accumulated and the AGPase activity in the different lines. An attempt to reproduce this result in another potato cultivar did not render tubers with increased levels of starch and ADP-glucose, although they showed increased levels of AGPase activity (Sweetlove *et al.*, 1996). Expression of allosterically insensitive AGPase has also been used in other crops such as rice (Sakulsingharoj *et al.*, 2004), maize (Wang *et al.*, 2007), wheat (Smidansky *et al.*, 2002) or cassava (Ihemere *et al.*, 2006). Enhanced

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AGPase activity in heterotrophic organs of cassava and rice resulted in enhanced biomass, but there was no increase in the starch content on a fresh weight (FW) basis (Smidansky *et al.*, 2003; Ihemere *et al.*, 2006). Other attempts at achieving increments in the starch accumulated in cereal endosperms have used modified variants of the large subunit of the AGPase (ApL), which is responsible for the allosteric activation of the enzyme by 3-phosphoglycerate and its inhibition by inorganic phosphate. Giroux *et al.* (1996) obtained maize lines with a modified version of ApL that was less sensitive to inhibition by orthophosphate. The absolute starch content of these lines was higher than in control lines. However, there was no increase in starch as a percentage of seed weight. Instead, these lines had significantly heavier seeds (Giroux *et al.*, 1996). Most recently, Li *et al.* (2011) have shown that overexpression of either the *BT2* or *SH2* genes, encoding for the small and large subunit of maize AGPase, respectively, results in enhanced seed weight and starch content.

An increase in the transport activity in transgenic tuber expressing a plastidic ATP/ADP transporter from *Arabidopsis* resulted in 16%–36% more starch per gram FW (Tjaden *et al.*, 1998; Geigenberger *et al.*, 2001), although more recent studies have not confirmed this observation (Zhang *et al.*, 2008). Downregulation of the plastidial adenylate kinase, an enzyme that catalyses the interconversion of ATP and AMP into ADP, in transgenic potato plants led to a substantial increase in the levels of adenylates and a concomitant increase in the levels of tuber starch on a per plant basis that was as high as twofold in field trials (Regierer *et al.*, 2002). More recently, a successful strategy for increasing ADP-glucose linked to starch biosynthesis has consisted in enhancing sucrose synthase (SuSy) expression. Tubers of transgenic potato plants constitutively expressing SuSy showed a substantial increase in ADP-glucose and starch content and yield when compared with untransformed controls under both green house and open-field conditions (Muñoz *et al.*, 2005; Baroja-Fernández *et al.*, 2009).

The manipulation of the expression of *SS* genes has hardly ever been used to increase the accumulation of starch. This is mainly because of the presence of different classes of SSs in plants, which seems to interact with each other in a protein complex (Hennen-Bierwagen *et al.*, 2008; Tetlow *et al.*, 2008) and display a specific role in the synthesis of the final architecture of the starch granule. Therefore, changes in the expression of an *SS* gene may lead to complex effects. This is well exemplified by the results obtained using transgenic potato plants expressing a bacterial glycogen synthase, whose tubers accumulated less starch with altered structural properties (Shewmaker *et al.*, 1994).

We have recently shown that SS class IV (SSIV) is involved in the initiation of the starch granule and seems to control the number of starch granule in chloroplasts of *Arabidopsis* leaves (Szydlowski *et al.*, 2009). The elimination of this protein determines a reduction in the amount of starch in leaves, which is accumulated in one (two in some cases) huge starch granule per chloroplast (Roldán *et al.*, 2007). On the other hand, accumulation of exceptionally high levels of starch in potato leaves exposed to microbial volatile emissions is accompanied by a dramatic increase in the expression levels of SSIV (Ezquer *et al.*, 2010). These results suggested that the levels of expression of *SSIV* could affect the accumulation of starch. In this work, we have investigated the effect of overexpressing *SSIV* in the amount of starch accumulated in both *Arabidopsis* leaves and potato (*Solanum tuberosum* L.) tubers. We show that *SSIV* overexpression increases the accumulation of starch in both photosynthetic and sink organs. Taking into account all the limitations inherent in basing conclusions on genetically engineered plants, the overall data show that enhancement of *SSIV* expression represents a useful biotechnological strategy for increasing starch accumulation in both autotrophic and heterotrophic organs.

Results and discussion

Overexpression of *SSIV* induces the accumulation of starch in *Arabidopsis*

To address the effect of *SSIV* overexpression on the starch metabolism in *Arabidopsis* leaves, *SSIV* overexpressing lines were generated (see Experimental procedures). Nine independent T2 transgenic lines were selected and the levels of *SSIV* mRNA (Figure 1a) and starch accumulated in leaves (Figure 1b) in all lines were determined. A positive correlation between the levels of *SSIV* mRNA and starch accumulation was observed, so that higher levels of *SSIV* expression corresponded to increments of accumulated starch (lines 2, 3, 5, 6 or 7 for instance). In contrast, those lines with reduced levels of *SSIV* mRNA displayed lower levels of accumulated starch than wild-type (WT) control plants (lines 8 or 9). This correlation was confirmed by the quantitation of SSIV protein levels in transgenic lines. Figure 1c shows that lines with low levels of SSIV protein (lines 8 and 9) displayed low levels of starch, whereas lines with high levels of SSIV (lines 3 and 7) accumulated more starch than WT plants. Immunoblots displaying SSIV protein in the different *Arabidopsis* lines are shown in Figure S1a,b. Lines 3 and 7 were selected for further studies, and their stable T3 transgenic plants were subsequently used.

To characterize the accumulation of starch in the selected lines, we determined the levels of starch over a day/night cycle in leaves of plants grown under a photoregime of 16-h day/8-h night. Both transgenic lines displayed a higher rate of starch accumulation during the day, reaching by the end of the light period around 40%–45% more starch than WT plants (Figure 2). The starch degradation rate of both lines was also higher than in control plants, and by the end of the night period, leaf starch levels were similar in transgenic and WT plants. This behaviour is the opposite to that found in *Arabidopsis ssIV* mutant lines, where the elimination of the SSIV protein led to plants with lower levels of starch than WT and with reduced rates of accumulation and degradation of starch during the day and night, respectively (Roldán *et al.*, 2007). These results indicate that SSIV may represent a rate-limiting step in the biosynthesis of starch and identify SSIV as a target for modifying the levels of accumulated starch in plants.

Overexpression of *SSIV* promotes the growth of *Arabidopsis* plants

Plants overexpressing *SSIV* seemed to be bigger than WT control plants as illustrated in Figure 3a. To characterize the effect of overexpression of *SSIV* on the growth rate of *Arabidopsis* plants, we followed the growth of the aerial part of transgenic and WT plants in a time-course experiment until the bolting phase. Both transgenic lines grew faster than WT plants (Figure 3b), and they had more biomass by the end of the vegetative growth phase (1.3- and 1.7-fold more biomass than WT

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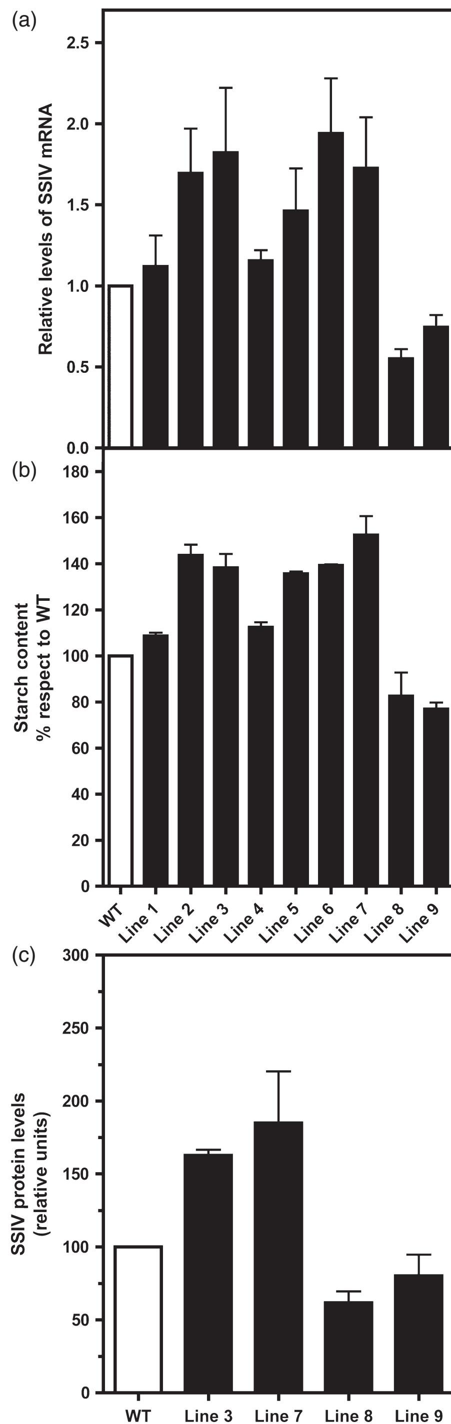

Figure 1 Expression levels of starch synthase class IV (SSIV) and starch accumulation in *Arabidopsis* transgenic plants. Leaves of the different lines of SSIV-overexpressing transgenic plants were harvested by the end of the day period and used for the following determination: (a) Levels of *SSIV* mRNA determined by quantitative real-time RT–PCR. *SSIV* mRNA levels were normalized against the *Ubiquitin10* control and relative expression calculated by setting the expression in wild-type (WT) to 1. Values are the mean ± SD of three independent experiments. (b) Levels of accumulated starch. Values represent the mean ± SD of four independent experiments. (c) SSIV protein in WT and transgenic *Arabidopsis* lines. Levels of SSIV protein were determined by immunoblot as described in Experimental procedures. Values are the mean ± SD of three independent experiments.

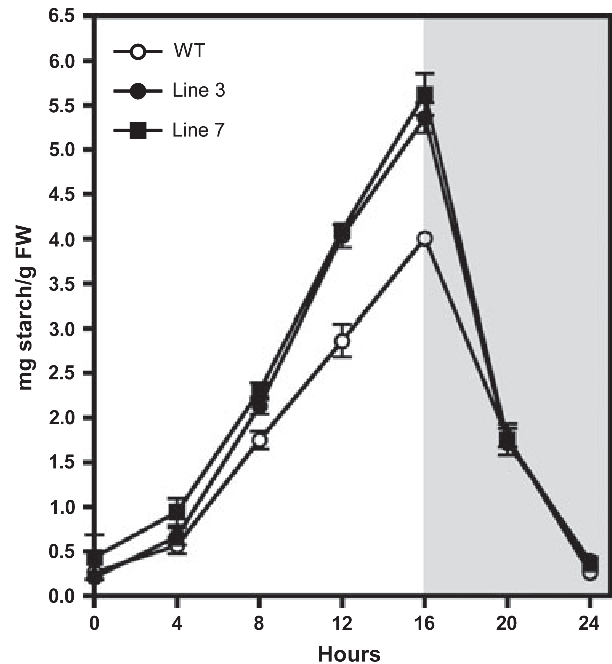

Figure 2 Time-course of starch content over a day in *Arabidopsis* wild-type and *SSIV* overexpressing transgenic lines. Plants were cultivated in growth cabinets under a 16 h-light/8-h dark photoperiod, and full-expanded leaves were collected, at times indicated, to determine the levels of starch accumulated as described in Experimental procedures. The grey zone indicates the night period. Values represent the mean ± SD of three independent experiments.

plants for lines 3 and 7, respectively). Transgenic lines developed the same number of leaves than WT plants at bolting, and they accumulated the same number of seeds per silique as control plants. The rate of starch degradation has been correlated with the rate of plant growth, so that plants with reduced levels of starch degradation during the night displayed low rates of growth (Gibon *et al.*, 2009). This statement coincides with our observation that the elimination of SSIV decreases the rate of starch degradation during the dark period and leads to a reduction in plant growth (Roldán *et al.*, 2007). Our results with *SSIV* transgenic lines indicate that this correlation is also valid with increased levels of starch. Thus, the overexpression of *SSIV* increments the levels of starch accumulated by the end of the day, which is completely mobilized during the night, leading to an increment in the rate of starch degradation. This increase in the carbon skeletons and energy available during the night would determine a higher rate of growth in the transgenic lines.

Effect of overexpression of *SSIV* on other components of starch metabolism

The increment of starch levels observed in the transgenic lines could be a secondary effect of the overexpression of *SSIV* exerted over the expression of other genes of the starch pathway or over the activity of the enzymes encoded by these genes. To address this point, we determined the mRNA levels of key genes as well as the enzymatic activities of their encoded proteins of both starch synthesis and degradative pathways in leaves of *SSIV* overexpressor transgenic lines. Most of the genes analysed remained unaffected and we only noticed a slight

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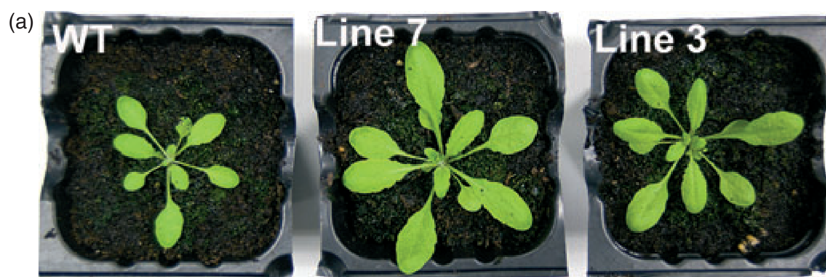

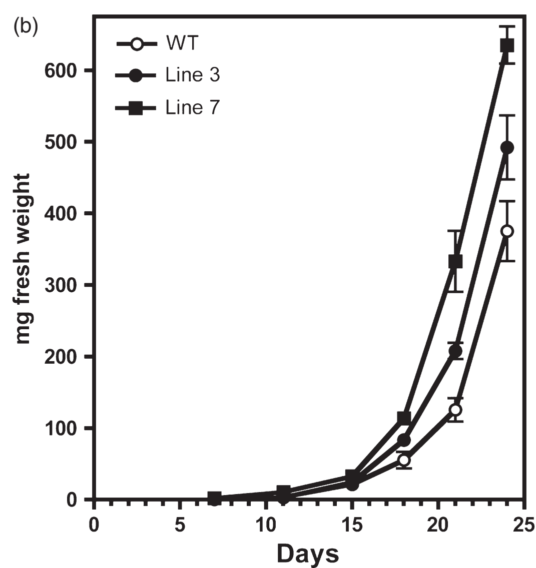

Figure 3 Growth of *Arabidopsis SSIV* overexpressing transgenic lines. Plants were cultivated in growth cabinets under a 16-h light/8-h dark photoregime, and the growth of wild-type (WT) plants and transgenic lines 3 and 7 was documented. (a) Picture was taken 15 days after sowing. (b) Time-course of growth of WT and Lines 3 and 7 transgenic plants. To determine the weight of plants at different times of growth, the aerial parts of five plants were collected. The values are the average of three independent determinations.

increment in the expression of *Aps1* and a decrease in the expression of *SSIII* (Figure 4). These changes were not reflected in the AGPase or total SS activities (Figure 5). In contrast, a clear decrease in β-amylase activity was detected in both transgenic lines, with levels of activity of 54 ± 16% and 68 ± 9% in relation to WT activity for lines 3 and 7, respectively. The expression of the *BAM3* gene, which encodes the isoform that accounts, together with BAM1, for most of the β-amylase activity in chloroplasts of *Arabidopsis* leaves (Fulton *et al.*, 2008), was not affected in the transgenic lines (Figure 4) nor was the rate of starch degradation altered during the night (Figure 2). These data suggest that the reduction in activity detected in the transgenic lines may be the consequence of a pleiotropic effect of *SSIV* overexpression over β-amylases with an extra-chloroplastidial location.

Production of potato plants expressing *AtSSIV* in the plastidial compartment of tubers

The increment of starch observed in the leaves of *Arabidopsis* plants overexpressing *SSIV* raises the question of whether a similar result could be obtained in long-term storage starch organs, such as potato tubers. To address this point, potato plants were transformed by means of *Agrobacterium tumefaciens* using the pK2GW7.0-AtSSIV binary vector (see Experimental procedures). As illustrated in Figure S1c, immunoblot analyses using polyclonal antiserum specifically raised against AtSSIV (Roldán *et al.*, 2007) revealed that, unlike WT tubers, tubers of potato plants expressing *AtSSIV* accumulated one peptide recognized by AtSSIV antiserum with a mass of approximately 112 kDa, which matches the size of the expected mature AtSSIV. In addition, *AtSSIV*-expressing tubers accumulated two smaller peptides that were clearly recognized by the AtSSIV antiserum. The presence of these bands is ascribed to post-translational modification of AtSSIV (Roldán *et al.*, 2007).

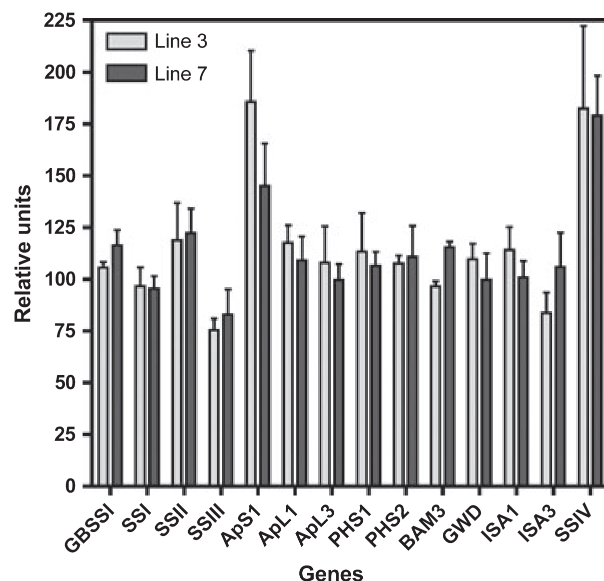

Figure 4 mRNA levels of genes of starch metabolism in *Arabidopsis* Lines 3 and 7. Total RNA of leaves collected at mid-day was extracted, and the mRNA levels of genes indicated were determined by quantitative real-time RT–PCR using oligonucleotides specific for each gene. Levels are referred to as the percentage of expression occurring in wild-type plants. Values represent the mean ± SD of three independent experiments.

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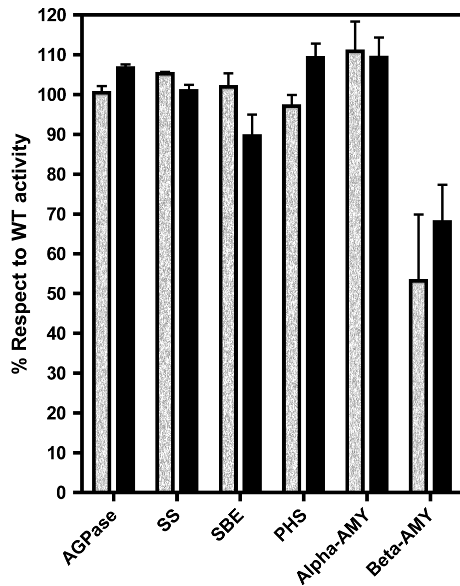

Figure 5 Activity of different enzymes of the starch metabolism in *Arabidopsis* Lines 3 and 7. Full-expanded leaves of 21-day-old plants were collected at mid-day, and the different activities were determined as described in Experimental procedures. Values are expressed as percentages in relation to levels determined in wild-type plants and represent the mean ± SD of four independent experiments. Grey columns: Line 3. Black columns: Line 7.

Szydlowski *et al.* (2009) have previously shown that AtSSIV locates in specific regions associated with the edges of the starch granule. To confirm the plastidial localization of AtSSIV in the transgenic potato plants, we performed confocal fluorescence microscopy analyses of potato plants expressing full-length SSIV protein fused with green fluorescent protein (GFP). These plants constitutively express the translationally fused *SSIV-GFP*-encoding gene under the control of the 35S promoter (Szydlowski *et al.*, 2009). Plants constitutively expressing potato adenosine diphosphate sugar pyrophosphatase fused with GFP (*StASPP-GFP*) (Muñoz *et al.*, 2008) and plants expressing *Arabidopsis* granule-bound SS (*GBSS*) fused with *GFP* (*GBSS-GFP*) (Szydlowski *et al.*, 2009) were used as controls for stromal and starch granule localizations, respectively. Analyses of *GBSS-GFP*-expressing plants showed uniform GFP fluorescence distribution within starch granules occurring in amyloplasts of tuber parenchyma cells (Figure 6a) and in isolated amyloplasts (Figure 6b), whereas fluorescence of the stromal marker StASPP-GFP was present in the starch-free peripheral part of the intact amyloplast (Figure 6c), but not in the isolated starch granules (Figure 6d). Fluorescence in *SSIV-GFP*-expressing cells was not uniformly distributed within the stroma, but was mainly located in one pole of the amyloplast, in specific regions associated with the edges of the starch granule (Figure 6e) (Figure S2). This association seems to be weak, as immunoblot analyses of *AtSSIV*-expressing potato tubers revealed that AtSSIV was found in the stroma containing soluble fractions, whereas no signal was detected in starch granules containing pellets (data not shown). Furthermore, no fluorescence could be detected in the poles of starch granules isolated from *SSIV-GFP*-expressing potato tubers (Figure 6f).

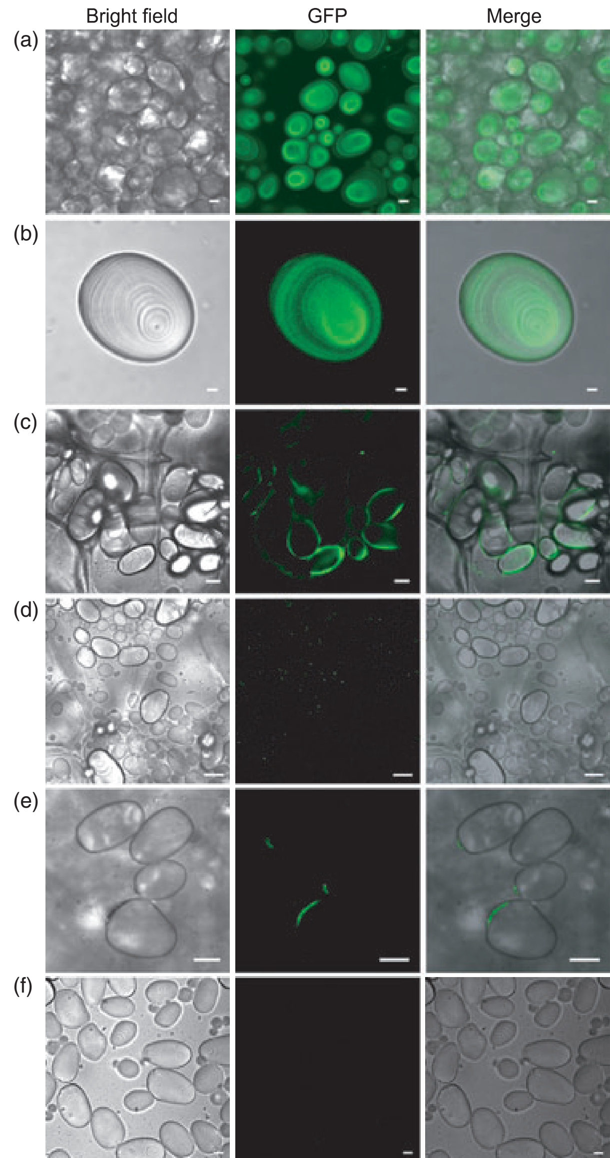

Figure 6 AtSSIV locates in specific regions associated with the edges of the starch granule of potato tuber amyloplasts. Granule-bound SS (GBSS)-green fluorescent protein (GFP) fluorescence was localized within the starch granule of intact amyloplasts (a). Isolated starch granules of *GBSS-GFP*-expressing tubers were GFP-positive (b), as expected of a protein strongly associated with the starch granule. Potato adenosine diphosphate sugar pyrophosphatase fused with GFP (StASPP-GFP) fluorescence was localized in the periphery of intact amyloplasts, between the starch granule and the plastid envelope (c). Isolated starch granules of *StASPP-GFP*-expressing tubers were GFP-negative (d). AtSSIV-GFP fluorescence was not uniformly distributed within the stroma, but was mainly located in one pole of the amyloplast, in specific regions associated with the edges of the starch granule (e) (further details are shown in Figure S2). Isolated starch granules of *AtSSIV-GFP*-expressing tubers were GFP-negative (f), indicating that AtSSIV association with the starch granule is weak. Bar = 20 μm.

Enhancing *SSIV* expression results in increased starch content in tubers of transgenic potato plants when grown in both greenhouse and open-field conditions

Once confirmed that AtSSIV exclusively locates in the plastidial compartment of *AtSSIV*-expressing potato plants, four lines of

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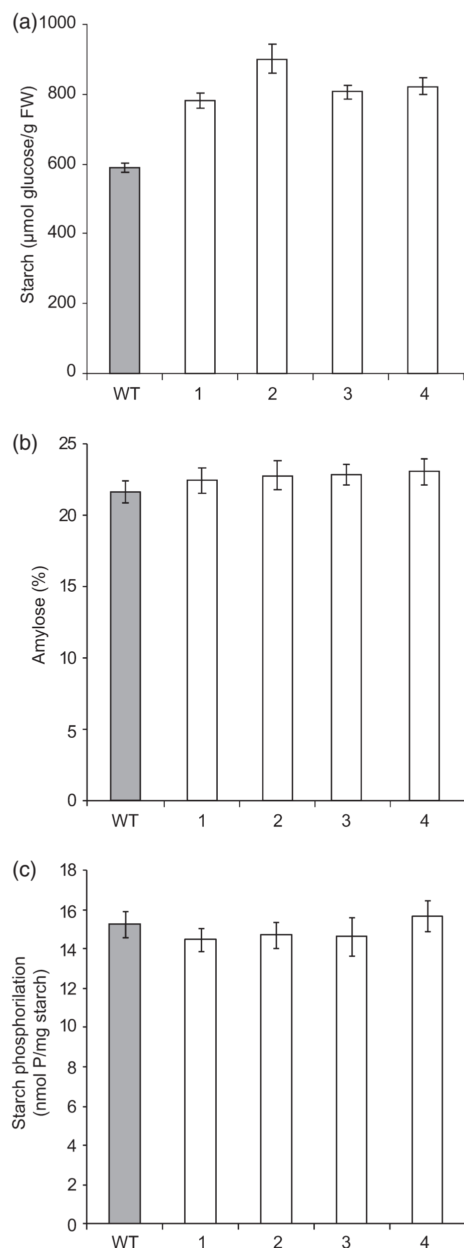

Figure 7 Enhancing *SSIV* expression results in increased starch content in tubers of transgenic potato plants and unaltered amylose/amylopectin ratio and phosphate content. (a) Starch content in *AtSSIV*-expressing plants cultured in greenhouse. (b) Amylose/amylopectin ratio. (c) Phosphate content in *AtSSIV*-expressing plants cultured in open-field conditions (May–September 2010). In (b) and (c), essentially the same results were obtained using potato tubers from 2009 field trials (not shown). The results are the mean ± SE of three independent experiments.

AtSSIV-expressing plants were grown in greenhouse, and their tubers were characterized for starch content. Because starch metabolism under greenhouse conditions may be directly influenced by factors such as pot size and by temperature, light and humidity regimes, we also characterized *AtSSIV*-expressing potato tubers from plants grown in the field (trials 2009 and 2010). As experience has shown that biochemical analyses are prone to considerable variation, we analysed ten plants per line to ensure we obtained reliable data. No changes were observed in the morphology, size, biomass and photosynthetic

Table 1 Fresh weight (FW), dry weight (DW) and starch content and yield of tubers of control and *AtSSIV*-expressing plants grown under field conditions (May–September 2009)

	WT	SS4-2	SS4-3	SS4-4
Tuber DW (% FW)	20.1 ± 0.9	19.1 ± 1.8	<u>18.3 ± 0.4</u>	19.6 ± 0.5
Tuber starch (% FW)	11.3 ± 0.3	**15.3 ± 0.9**	**13.3 ± 0.3**	**13.7 ± 0.5**
Tuber starch (% DW)	56.1 ± 1.6	**80.2 ± 4.5**	**72.5 ± 1.8**	**70.0 ± 2.6**
Tuber FW (g/plant)	839.8 ± 78.8	895.2 ± 15.7	<u>743.4 ± 37.9</u>	756.9 ± 95.4
Tuber DW (g/plant)	168.8 ± 7.5	170.9 ± 16.1	<u>136.1 ± 2.7</u>	148.4 ± 13.9
Tuber starch (g/plant)	94.6 ± 2.2	**137.1 ± 2.9**	98.6 ± 2.4	**103.9 ± 2.0**
Tuber FW (Kg/hectare)	37750 ± 3540	39786 ± 591	<u>33042 ± 1664</u>	33640 ± 7957
Tuber DW (Kg/hectare)	7593 ± 711	7612 ± 113	<u>6042 ± 304</u>	6610 ± 305
Tuber starch (Kg/hectare)	4254 ± 112	**6091 ± 108**	4381 ± 160	**4619 ± 197**

The results are the mean ± SE of extracts from 90 independent plants per line. Values that are significantly higher than those of control tubers are marked in bold. Values that are significantly lower than those of control tubers are underlined.
WT, wild-type.

parameters of the aerial part of the *AtSSIV*-expressing plants when compared with WT plants (Figure S3). Noteworthily, analyses of starch content revealed that *AtSSIV*-expressing tubers contained 30%–45% and 15%–30% more starch than control tubers when plants were grown in greenhouse and open-field conditions, respectively (Figure 7a, Tables 1 and 2). Analyses of physico-chemical parameters of starch from transgenic lines did not reveal significant changes with respect to WT starch. Thus, both the amylose/amylopectin ratio (Figure 7b) and the phosphate content (Figure 7c) remained unchanged. Furthermore, no differences were observed between the size and morphology of the starch granule and *AtSSIV*-expressing tubers (Figure S4). Tuber dry weight (DW) (referred to as percentage of tuber FW) of *AtSSIV*-expressing tubers was normal when compared with WT tubers of plants grown in field conditions (Tables 1 and 2). Therefore, tuber starch content to DW ratio was higher in the AtSSIV lines than in control plants. These data indicate that other cellular components, such cell wall or proteins, should be diminished in the transgenic tubers. In addition, starch yield per plant was significantly higher in three of four *AtSSIV*-expressing plants than in the control plants (Tables 1 and 2), although tuber FW and DW per plant were normal in *AtSSIV*-expressing plants. Plants from three of four *AtSSIV*-expressing lines produced more starch than WT plants in terms of productivity per land surface unit (tons of starch/hectare) (Tables 1 and 2).

These results indicate that the role of SSIV in photosynthetic tissues such as *Arabidopsis* leaves would be conserved in sink, storage organs such as potato tubers. In addition, and taking into account all the limitations inherent in basing conclusions on genetically engineered plants, the overall data would

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Table 2 Fresh weight (FW), dry weight (DW) and starch content and yield of tubers of control and *AtSSIV*-expressing plants grown under field conditions (May–September 2010)

	WT	SS4-1	SS4-2	SS4-3	SS4-4
Tuber DW (% FW)	19.7 ± 0.3	19.2 ± 0.3	19.5 ± 0.2	19.2 ± 0.3	20.0 ± 0.1
Tuber starch (% FW)	9.0 ± 0.3	**10.5 ± 0.4**	**12.5 ± 0.4**	**10.5 ± 0.4**	**11.9 ± 0.6**
Tuber starch (% DW)	45.5 ± 2.5	**54.7 ± 3.0**	**63.9 ± 4.1**	**54.6 ± 2.1**	**59.6 ± 2.9**
Tuber FW (g/plant)	761.7 ± 78.9	722.5 ± 79.5	649.4 ± 54.1	624.5 ± 74.6	721.1 ± 21.0
Tuber DW (g/plant)	150.1 ± 15.4	138.7 ± 24.9	122.6 ± 10.6	119.9 ± 14.3	144.2 ± 4.2
Tuber starch (g/plant)	68.2 ± 2.2	**75.9 ± 2.7**	**80.9 ± 2.7**	65.5 ± 2.4	**86.0 ± 4.1**
Tuber FW (Kg/hectare)	33 851 ± 3465	32 112 ± 5754	29 860 ± 2405	29 755 ± 3316	32 050 ± 934
Tuber DW (Kg/hectare)	6669 ± 632	6165 ± 1104	5823 ± 468	5713 ± 636	6410 ± 186
Tuber starch (Kg/hectare)	3033 ± 99	**3372 ± 120**	**3720 ± 386**	3219 ± 185	**3823 ± 790**

The results are the mean ± SE of extracts from 90 independent plants per line. Values that are significantly higher than those of control tubers are marked in bold. Values that are significantly lower than those of control tubers are underlined.
WT, wild-type.

indicate that enhancement of SSIV expression represents a useful biotechnological strategy for increasing tuber starch accumulation and total yield in potato plants.

Expression of *AtSSIV* in potato tubers is accompanied by pleiotropic changes in AGPase and SuSy and enhancement of ADP-glucose content

Regulation of starch metabolism involves a complex network wherein transcriptional and post-transcriptional regulation of starch enzymes play a crucial role. Furthermore, the occurrence of a reverse correlation between expression levels of SuSy and acid invertase in genetically engineered potato plants with altered SuSy activity (Zrenner *et al.*, 1995; Baroja-Fernández *et al.*, 2009) strongly indicated that sucrose and starch metabolism enzymes are co-ordinately regulated in response to identical cues. Analyses of enzymes closely associated with starch and sucrose metabolism revealed that tubers of *AtSSIV*-expressing plants grown in open-field conditions display normal alkaline pyrophosphatase, alkaline invertase, sucrose-phosphate synthase, starch phosphorylase and total amylolytic activities when compared to control tubers (not shown). Noteworthily, acid invertase activity in tubers of *AtSSIV* lines was markedly lower than in control tubers (Figure 8a). Furthermore, AGPase and SuSy activities in the *AtSSIV*-expressing tubers were significantly higher than in control tubers (Figure 8b,c, respectively). These two enzymes produce ADP-glucose, and previous studies have shown that SuSy is a major determinant of intracellular ADP-glucose content in potato tubers (Baroja-Fernández *et al.*, 2003, 2009). Furthermore, SuSy is a major determinant of UDP-glucose in potato tubers (Baroja-Fernández *et al.*, 2009). Consistently, ADP-glucose and UDP-glucose contents were significantly higher in the tubers of the *AtSSIV* lines than in WT tubers (Figure 9). The overall results would indicate that the increase in ADP-glucose, UDP-glucose and starch content in *AtSSIV*-expressing tubers can be ascribed to enhanced SSIV activity, pleiotropic upregulation of both AGPase and SuSy and downregulation of acid invertase. These changes in *AtSSIV*-expressing tubers were accompanied by enhanced glucose and sucrose content, whereas the levels of ATP, ADP, AMP and hexose phosphates were comparable to those observed in WT tubers (Figure 9). Previous reports have shown that changes in ADP-glucose content are accompanied by concomitant changes in the amylose content (Clarke *et al.*, 1999). This is because of the high Km for ADP-glucose of the amylose synthesizing GBSSI in comparison with the soluble SS isoforms. The fact that *AtSSIV*-expressing tubers accumulate high ADP-glucose (Figure 9) but did not show a significant change in the amylose content (Figure 7b) could be ascribed to the fact that a sizable pool of ADP-glucose accumulating in potato tubers has a cytosolic localization (Baroja-Fernández *et al.*, 2009).

Overexpression of *SSIV* results in both reduction in the total tuber number per plant and slight increase in the tuber size

The onset of the tuberization process is accompanied by a massive increase in starch synthesis. Previous studies have shown that inhibition of starch biosynthesis in *AGPase* antisensed potato tubers leads to increase in total number per plant and a dramatic decrease in the tuber size (Müller-Röber *et al.*, 1992). These developmental changes were initially ascribed to inhibition of the starch biosynthetic process and indicated that the sink strength of growing potato tubers is inversely correlated with the tuber number per plant. However, Zrenner *et al.* (1995) showed that the number and size of tubers in the starch-deficient SuSy antisensed potato plants were normal when compared with WT plants, demonstrating that a reduction in the accumulation of starch does not necessarily correlate with changes in the tuberization process.

As shown previously, overexpression of *SSIV* resulted in enhanced AGPase activity. It was therefore of interest to analyse whether the overexpression of *SSIV* would lead to changes in the number and size of tubers. As shown in Figure 10a, the number of tubers produced per plant was significantly lower in *AtSSIV*-expressing plants than in WT plants. Furthermore, the percentage of tubers with a diameter shorter than 30 mm was lower in *AtSSIV*-expressing plants (15%–31%) than in WT plants (34%) (Figure 10b). In addition, the percentage of tubers with a diameter ranging between 30 and 50 mm in WT plants (39%) was lower than in *AtSSIV*-expressing plants (45%–60%). The overall data are consistent with those of Müller-Röber *et al.* (1992) showing an effect of *AGPase* expression in the tuberization process and indicate that changes in *SSIV* expression are accompanied not only by pleiotropic changes in sucrose and starch metabolic enzymes (cf. Figure 9) but also by developmental changes in the plant. Further research will be necessary to understand this phenomenon.

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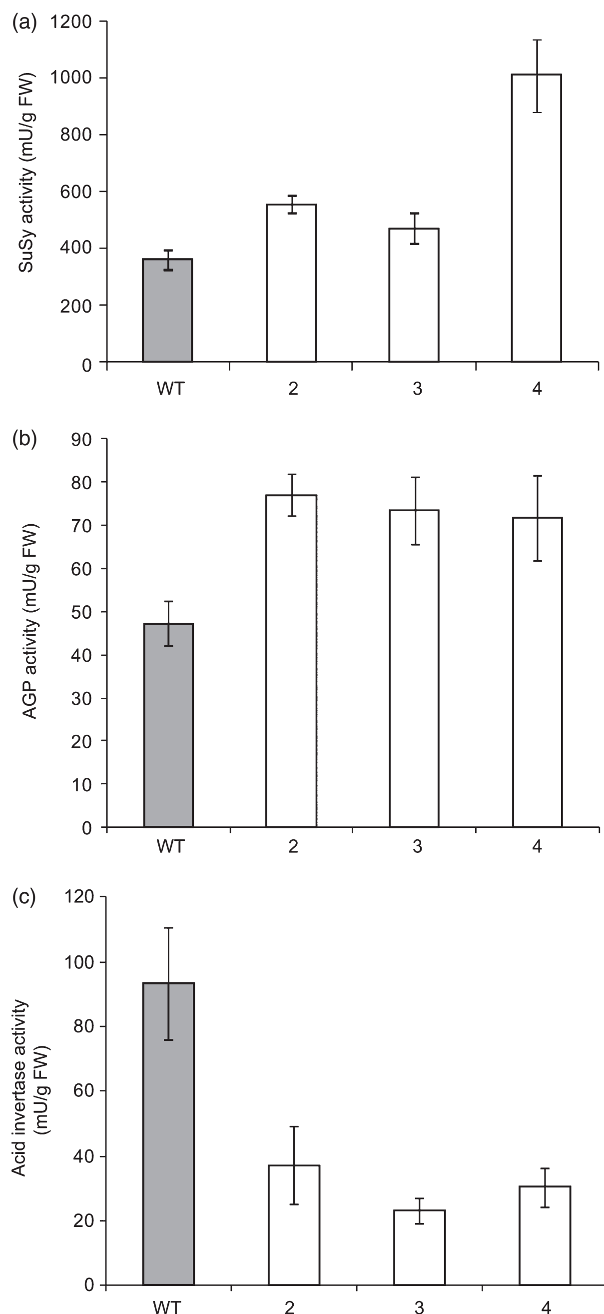

Figure 8 Expression of *AtSSIV* is accompanied by the upregulation of (a) Sucrose synthase and (b) ADP-glucose pyrophosphorylase, and (c) downregulation of acid invertase in potato tubers. Plants were cultured in open-field conditions (May–September 2009). Essentially the same results were obtained using potato tubers from 2010 field trials (not shown). The results are the mean ± SE of three independent experiments.

Experimental procedures

Plant material and growth conditions

Arabidopsis thaliana WT (ecotype Columbia-0) and transgenic lines were grown in growth cabinets under a 16-h light/8-h dark photoregime at 23 °C (day)/20 °C (night), 70% humidity and a light at the plant levels of 120 $\mu E/m^2/s^1$ supplied by white fluorescent lamps. Seeds were sown in soil and irrigated with 0.5× MS medium (Murashige and Skoog, 1962).

Potato (*S. tuberosum* L. cv. Désirée) plants were grown in both greenhouse and field conditions. Large-scale greenhouse trials were performed with plants individually grown in 5-L pots (ten plants/line) under a light regime with a minimum of 250 μmol photons/m^2 at 22 °C. Field trials were conducted in the vicinity of Sartaguda (Navarre, Spain) between May and September of 2009 and 2010, using 90 plants per line. Plants from each line were randomly distributed in three 50 m^2 parcels, each containing 30 plants per line. The distance between plants of the same row was 35 cm, and the distance between rows was 90 cm. Analyses of enzyme activities and measurement of sugar content were carried out using mature tubers of 30–50 g FW immediately after harvest. The term 'mature tubers' refers to tubers harvested from senescent plants. Tissue cylinders were obtained from harvested mature tubers using a stainless steel cork borer, nominally 19.1 mm in diameter. Tissue samples were taken from the central part of cylinders cut perpendicular to the long axis of the tuber, to avoid the large textural and composition differences occurring between the cortex and pith tissues. Three samples were taken from each tuber, immediately freeze-clamped and finally ground to a fine powder in liquid nitrogen with a pestle and mortar.

Generation of SSIV overexpressing plants

We used the *SSIV* full-length cDNA cloned into the binary vector pCTAPi (Rohila *et al.*, 2004) previously described (Roldán *et al.*, 2007). The transformation vector was mobilized to *A. tumefaciens* strain C58 and introduced into WT *Arabidopsis* using the floral dip method (Clough and Bent, 1998). Putative transformant plants expressing *SSIV* under the 35S promoter were selected by resistance to BASTA and verified by PCR using the oligonucleotides SA397 (5′-GTTGTTCAATACCTTCAAATTCCC-3′) and SA398 (5′-CATTGTAACAACCGTGTCCCC-3′), which amplify a fragment of 667 bp from the genomic copy of *SSIV* and a fragment of 413 bp of the *SSIV* cDNA.

Agrobacterium tumefaciens-mediated transformations of potato (*S. tuberosum* L. cv. Désirée) plants were conducted as described by Rocha-Sosa *et al.* (1989). *SSIV-GFP*- and *GBSS-GFP*-expressing plants were produced using the pAtSS4-GFP and pGBSSI-GFP expression vectors, respectively (Szydlowski *et al.*, 2009). *StASPP-GFP*-expressing plants were produced using the pStASPP-GFP plasmid (Muñoz *et al.*, 2008). We also used potato plants expressing *AtSSIV*, which were produced using the pK2GW7,0-AtSSIV plasmid (Figure S5). Transgenic potato plants were selected on kanamycin-containing medium. The presence of recombinant *AtSSIV* was confirmed by Southern blot analyses.

Analytical procedures

Starch content in *Arabidopsis* leaves was determined using the method described in Szydlowski *et al.* (2009). Measurement of starch content in potato tubers was carried out as described by Baroja-Fernández *et al.* (2009). The amylose content of the starch was measured iodometrically according to Andersson *et al.* (2006). The phosphate content of the starch was measured using the enzyme glucose-6-phosphate dehydrogenase after acid hydrolysis of the starch essentially as described by Nielsen *et al.* (1994). Photosynthetic parameters of the first fully expanded (fifth) leaves attached to the plants were determined

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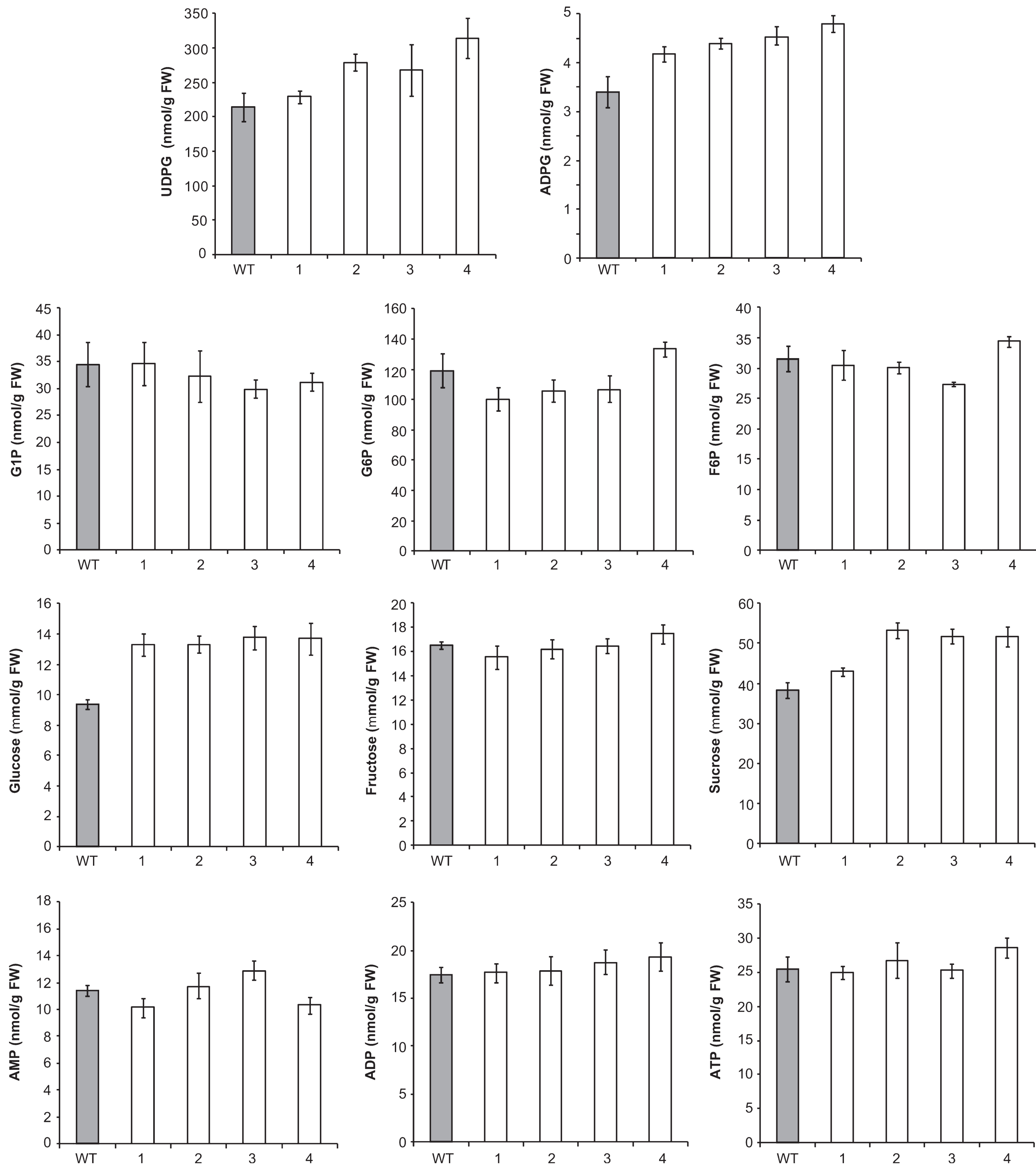

Figure 9 Expression of *AtSSIV* results in increased tuber ADP-glucose, UDP-glucose, sucrose and glucose content, whereas the levels of adenylates and hexose phosphates are comparable to those observed in wild-type plants. Plants were cultured in open-field conditions (May–September 2010). The results are the mean ± SE of three independent experiments.

under growth conditions by means of a Lci portable photosynthesis system (ADV BioScientific Ltd., Hoddesdon, UK) in the same chamber where the leaves were grown. For measurement of ADP-glucose content, 0.5-g aliquot of the frozen powdered tissue was resuspended in 0.4 mL of 1.4 M $HClO_4$, left at 4 °C for 2 h and centrifuged at 10 000 ***g*** for 5 min. The supernatant was neutralized with K_2CO_3, centrifuged at 10 000 ***g*** and subjected to measurement analyses of ADP-glucose by HPLC on a system obtained from P. E. Waters and Associates fitted with a Partisil-10-SAX column (Muñoz *et al.*, 2005).

In vitro assays of starch metabolism enzymes

Arabidopsis AGPase was assayed in the synthesis direction according to the procedure described by Crevillén *et al.* (2003). Soluble SS activity, using glycogen as primer, was performed as described in Szydlowski *et al.* (2009). α-Glucan phosphorylase

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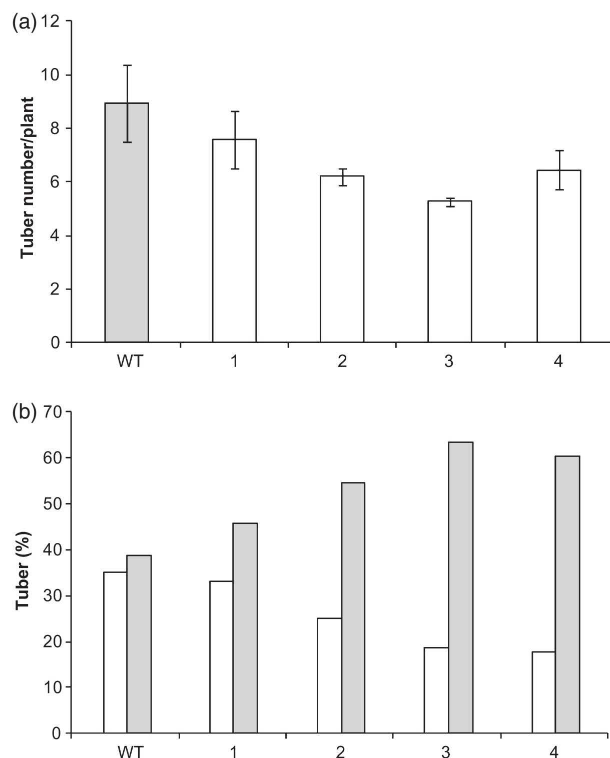

Figure 10 Overexpression of starch synthase class IV (SSIV) results in both reduction in the total tuber number per plant and a slight increase in the tuber size. (a) Number of tubers produced per plant. The results are the mean ± SE of 90 independent plants per line. (b) Percentage of tubers with a diameter smaller than 30 mm (white bars) and with a diameter between 30 and 50 mm (grey bars). Plants were cultured in open-field conditions (May–September 2010). Essentially the same results were obtained using potato tubers from 2009 field trials (not shown).

and BE activities were determined according to the procedure described by Zeeman *et al.* (1998). Endoamylase (α-amylase) and β-amylase activity were determined using the Ceralpha and Betamyl Kits (Megazyme), respectively, according to the manufacturer's instructions. Potato tuber SuSy, AGPase, acid invertase, starch phosphorylase, alkaline pyrophosphatase, alkaline invertase, sucrose-phosphate synthase and total amylolytic activities were assayed as described by Baroja-Fernández *et al.* (2009). One unit (U) is defined as the amount of enzyme that catalyses the production of 1 μmol of product per min.

Immunoblot analyses

For immunoblot analyses, protein samples (30 μg protein/lane) were separated on 10% SDS–PAGE, transferred to nitrocellulose filters and immunodetected by using antisera raised against AtSSIV as primary antibody (Roldán *et al.*, 2007) and a goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma-Aldrich Química S.A., Madrid, Spain) as secondary antibody. To quantify SSIV protein levels, protein samples (15 μg/lane) were separated on 12% SDS–PAGE, transferred to nitrocellulose filters and immunodetected by using anti-SSIV and a goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad, Hercules, CA) as secondary antibody. Immunodetection was performed using the ECL Advance Western Blotting Detection kit (GE Healthcare, Uppsala, Sweden), and the chemiluminescence was quantified using a Chemidoc XRS (Bio-Rad) and the software Quantity One (Bio-Rad).

Real-time RT–PCR analysis

Isolation of total RNA from *Arabidopsis* leaves was performed using the TRIsure reagents (Bioline Ltd., London, UK) following the manufacturer's instructions. First-stranded cDNA was synthesized from total RNA using the Quantitec Reverse Transcription kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Real-time quantitative PCR assays were achieved using an iCycler instrument (Bio-Rad). The PCR mixture contained in a total volume of 25, 5 μL of cDNA, 0.2 mM of dNTPs, 2.5 mM $MgCl_2$, a 1 : 10 0000 dilution of SYBR® Green I nucleic gel staining (Molecular Probes, Eugene, OR)/Fluorescein calibration dye (Bio-Rad), 0.3 units of Taq polymerase, 2.5 μL 10× Taq polymerase buffer and 0.2 μM of each primer. Specific oligonucleotides used were SA419 (5′-CGTGACTTAAGGGCTTTGGA-3′) and SA420 (5′-GCAGCTCGGCTAAAATACGA-3′) for *SSIV*; SA198 (5′–3′ TGATGAGAAGAGGAATGACCCGAAA-3′) and SA199 (5′-CCATAGATTTTCG ATAGCCGA-3′) for *SSI*; SA126 (5′- GGAACCATTCCGGTGGTCCATGCCG-3′) and SA127 (5′-CTCACCAATGATACTTAGCAGCAACAAG-3′) for *SSII*; SA200 (5′- GTGCAAGACGGTGATGGAGCAA-3′) and SA201 (5′- CAC-GTTTTTTATA TTGCTTTGGGAA-3′) for *SSIII*; SA 124 (5′- GGA-TCCAGATGATGTGATAGCAACAGC-3′) and A125 (5′-CCTCAA-CCTTCCCTACTCCTAACTTCCC-3′) for *GBSSI*; SA253 (5′-GA-AAAACCAAAAGGGGAGCA-3′) and SA254 (5′-CCTCAGTC-CAAGGGAAGTGG-3′) for *ApS1*: SA257 (5′-TCCCCACAGC-AAACGACTT-3′) and SA258 (5′-GGTGGCAGGTTTCTCCTTGA-3′) for *ApL1*; SA261 (5′-AGATCGGGAAAAACGTGGTG-3′) and SA262 (5′-CTCTTTTTAACTTCCGGCCAAAC-3′) for *ApL3*; ISA1_F (5′- ACGGCGATGAAGCAGTATAGG-3′) and ISA1_R (5′- AG-TGGGCATATCTGAAGCCG-3′) for *ISA1*; ISA3_F (5′- ATGAC-GGTATAGGTGGCCGTG-3′) and ISA3_R (5′- TTATTTTTCCTC-TACCGTGCG-3′) for *ISA3*; BAM3_F (5′-CTGCCAATAGCTAA-GATGTTCAA-3′) and BAM3_R (5′-GCGGTTAACCCATTT-CCAGAATCTG-3′) for *BAM3*; SA546 (5′-TGGAAGGAAAC-GAAGGCTTTG-3′) and SA547 (5′-TGTCTTTGGCGTATTCGT-GGA-3′) for *AtPHS1*; SA548 (5′-ACAGGTTTTGGACGTGGTG-ATT-3′) and (5′-ACAGGACAAGCCTCAATGTTCCA-3′) for *At-PHS2*; GWD_F (5′-TTCAAGCTTGAAGGCAGTGC-3′) and GWD_R (5′-CAACAGCACCACGTACTGG-3′) for *GWD*; UBQF (5′-GATCTTTGCCGGAAAACAATTGGAGGATGGT-3′) and UBQR (5′-CGACTTGTCATTAGAAAGAAAGAGATAACAG-3′) for *UBQ10*.

Thermal cycling consisted of 94 °C for 3 min, followed by 40 cycles of 10 s at 94 °C, 15 s at 61 °C and 15 s at 72 °C. After that, a melting curve was generated to check the specificity of the amplified fragment. The efficiency of all the primers in the above-mentioned conditions was between 75% and 110% in all the tested samples, and product identity was confirmed by sequence analysis. *Arabidopsis Ubiquitin 10* (Sun and Callis, 1997) was used as house-keeping gene in the expression analysis. Relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method according to Livak and Schmittgen (2001). Threshold cycle values were determined using ICYCLER Q software (Bio-Rad), normalized against the ubiquitin control, and relative expression was calculated by setting the expression in WT plants to 1.

Statistical analysis

Statistical analysis was determined by *t*-test analysis using EXCEL (Microsoft Corporation, Redmond, WA) considering $P < 0.01$ as a significance value.

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Microscopy

Subcellular localization of SSIV-GFP was performed using D-Eclipse C1 confocal microscope (Nikon, Tokyo, Japan) equipped with standard Ar 488 laser excitation, BA515/30 filter for green emission and transmitted light detector for bright field images. For analysis of the starch granule size and morphology, samples of purified starch were suspended in water and viewed with the transmitted light in a confocal microscope.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 AtSSIV protein in Arabidopsis and potato transgenic lines.

Figure S2 Location of AtSSIV in potato tubers amyloplasts.

Figure S3 Over-expression of SSIV does not affect the biomass of the aerial part of the plant (a), and photosynthetic parameters such as rates of O_2 production (b), transpiration (c), CO_2 stomatal conductance (d) and substomatal CO_2 concentration (e).

Figure S4 Over-expression of SSIV is not accompanied by changes in the size and morphology of the starch granules in potato tubers.

Figure S5 Stages to construct the pK2GW7,0-AtSSIV plasmid necessary to produce potato plants expressing *AtSSIV.*

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