Starch synthase 4 is essential for coordination of starch granule formation with chloroplast division during Arabidopsis leaf expansion

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Summary

- Arabidopsis thaliana mutants lacking the SS4 isoform of starch synthase have strongly reduced numbers of starch granules per chloroplast, suggesting that SS4 is necessary for the normal generation of starch granules. To establish whether it plays a direct role in this process, we investigated the circumstances in which granules are formed in ss4 mutants.
- Starch granule numbers and distribution and the accumulation of starch synthase substrates and products were investigated during ss4 leaf development, and in ss4 mutants carrying mutations or transgenes that affect starch turnover or chloroplast volume.
- We found that immature ss4 leaves have no starch granules, but accumulate high concentrations of the starch synthase substrate ADPglucose. Granule numbers are partially restored by elevating the capacity for glucan synthesis (via expression of bacterial glycogen synthase) or by increasing the volumes of individual chloroplasts (via introduction of arc mutations). However, these granules are abnormal in distribution, size and shape.
- SS4 is an essential component of a mechanism that coordinates granule formation with chloroplast division during leaf expansion and determines the abundance and the flattened, discoid shape of leaf starch granules.

Introduction

The process by which starch granules arise is not known. Suggestions range from largely physico-chemical mechanisms (Dow, 1965; Geddes & Greenwood, 1969; Ziegler et al., 2005) to the existence of specific protein primers analogous to the glycogenins of fungi and animals (e.g. Rothschild & Tandecarz, 1994; Singh et al., 1995; Langeveld et al., 2002; Chatterjee et al., 2005). Recent attention has focussed on the role of one isoform of soluble starch synthase, starch synthase 4 (SS4, At4g18240). Although SS4 contributes little to total starch synthase activity, ss4 mutants of Arabidopsis have at most one or two starch granules per chloroplast (Roldán et al., 2007) rather than the normal five or six (Crumpton-Taylor et al., 2012). No other starch synthase is individually necessary for normal granule numbers (Roldán et al., 2007), thus SS4 may have a specific function in granule formation. However, other isoforms of starch synthase may partially substitute for this function. The additional loss of SS3 further reduces starch granule numbers in the ss4 mutant background (Szydlowski et al., 2009; Mérida & D’Hulst, 2012).

The importance of SS4 for starch granule formation remains to be established. First, it is not known whether SS4 is required primarily for maintenance of starch granule numbers in mature leaves, or whether it also has a role in immature leaves where new granules arise in concert with chloroplast division (Crumpton-Taylor et al., 2012). Second, it is not clear whether the reduction in starch granule numbers in ss4 mutants is a direct or an indirect consequence of the loss of SS4. Mutants have several additional phenotypes including reduced growth rates, altered starch granule anatomy and morphology and a reduction in the extent of diel starch turnover (Roldán et al., 2007). It remains possible that the reduction in granule numbers in ss4 mutants is an indirect consequence of one of these alterations. Third, a recent study suggests that SS4 may be limiting for starch synthesis in wild-type plants. Its overexpression reportedly results in higher concentrations of starch at the end of the day and accelerated plant growth (Gámez-Arjona et al., 2011). These results have important implications for the control of starch turnover and are of biotechnological interest, but the relationship between starch concentrations and starch granule numbers and sizes in plants with elevated SS4 was not reported.

The aim of our work was to establish whether SS4 has a direct or an indirect role in starch granule formation, and to shed

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Key words: ADPglucose, Arabidopsis thaliana, chloroplast, leaf expansion, starch granule, starch synthase, starch synthesis.

These authors contributed equally to this work.

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Summary

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- Starch granule numbers and distribution and the accumulation of starch synthase substrates and products were investigated during ss4 leaf development, and in ss4 mutants carrying mutations or transgenes that affect starch turnover or chloroplast volume.
- We found that immature ss4 leaves have no starch granules, but accumulate high concentrations of the starch synthase substrate ADPglucose. Granule numbers are partially restored by elevating the capacity for glucan synthesis (via expression of bacterial glycogen synthase) or by increasing the volumes of individual chloroplasts (via introduction of arc mutations). However, these granules are abnormal in distribution, size and shape.
- SS4 is an essential component of a mechanism that coordinates granule formation with chloroplast division during leaf expansion and determines the abundance and the flattened, discoid shape of leaf starch granules.

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The aim of our work was to establish whether SS4 has a direct or an indirect role in starch granule formation, and to shed...
further light on where and when its actions are required for the establishment of normal granule numbers. To this end we examined the phenotype of the ss4 mutant through leaf development, and investigated the impact of loss of SS4 in mutant and transgenic backgrounds in which starch metabolism is altered or chloroplast volumes are abnormally large. Our results indicate that SS4 is directly and specifically required for the establishment of normal granule numbers during leaf expansion, and that it is also necessary for the normal flattened, discoid shape of leaf starch granules.

Materials and Methods

Plant material

*Arabidopsis thaliana* plants were grown in compost at 20°C with 12 h light (200 μmol photons m⁻² s⁻¹), 12 h dark and were used as mature, nonflowering rosettes unless otherwise stated. For examination of roots, plants were grown on Phytagel with nutrients (Haughn & Somerville, 1986). The ss4-1 mutant and the double mutant ss3ss4 were described in Roldán et al. (2007) and Szydlowski et al. (2009), respectively. The ss4-3 mutant (SALK_096130) was used unless otherwise stated. It carries a T-DNA insertion in intron 4 of the SS4 gene, + 2186 bp from the start codon. The sec1-8 (gwd) mutant (SALK_077211) was described in Ritte et al. (2006). It carries a T-DNA insertion in an intron of GWD, lacks detectable GWD protein, and is phenotypically indistinguishable from the null mutant sec1-3 (Yu et al., 2001). arc mutants were: arc3-2 (SALK_057144), arc5-2 (SAIL_71D11), arc6-5 (SAIL_693G04) and arc10-2 (SALK_073878) (Crumpont-Taylor et al., 2012). Double mutants were identified in F₂ populations derived from crosses by PCR on genomic DNA, using the oligonucleotide primers listed in Supporting Information Table S1.

Complementation of the ss4 mutant

A full-length SS4 cDNA was introduced into the destination vector pK7FWG2.0 (Karimi et al., 2002) by Gateway® LR clonase™ II (Invitrogen) recombination, under the control of a 35S promoter and upstream of eGFP present in the vector. The resulting binary vector was introduced into *Agrobacterium tumefaciens* strain GV3101 and used to transform ss4-3 plants. Transformants were selected on media containing kanamycin. Presence of the transgene was confirmed using the oligonucleotide primers shown in Table S1 and homozygous lines were developed.

Generation of lines expressing *Agrobacterium glgA*

The full-length coding sequence of glycogen synthase glgA was amplified from genomic DNA of *A. tumefaciens* strain GV3101 and cloned into pDONR 221 via Gateway® BP clonase™ II (Invitrogen) recombination. Using Gateway® LR clonase™ II recombination, glgA was transferred into the binary vector pB7WGY2, modified to contain a chloroplast targeted transit peptide (cTP) sequence (the first 53 amino acids of the Rubisco small subunit; Atg38430) upstream and in frame with the N-terminal YFP (Fig. S9a). The resulting GS-containing construct (YFP-GS) was transformed into ss3ss4 double mutant plants. Four independent GS-transformed plants (GS-5-3, GS-2-2, GS-2-3 and GS-2-4) were obtained by Basta® resistance screening. Transgene expression was confirmed by YFP fluorescence observation using a Zeiss LSM510 confocal fluorescence microscope.

Generation and analysis of lines with dexamethasone-inducible RNAi

Dexamethasone-inducible constructs were made with the pOpOff2(hyg) destination vector system (Wielopolska et al., 2005). The two targeted regions of the SS4 gene are shown in Fig. S8, and oligonucleotide primers are in Table S1. PCR products SS4A and SS4B were cloned into the GATEWAY-ready pCR8/GW/TOPO TA entry plasmid and transferred into the destination vector to create pOpOff2(hyg)::SS4A and pOpOff2(hyg)::SS4B. These plus an empty vector were separately transformed into plants. Transformants were selected on media containing hygromycin, and single-copy homozygous lines were produced.

Ten-day-old plants were sprayed with 30 μM dexamethasone daily, 10 h into the 12-h light period, for 10 d. For measurements of SS4 transcript and protein, plants were harvested immediately before spraying and 24 h after the final spraying. RNA was extracted using the RNeasyTM Plant Mini kit (Qiagen), digested with RQ1-DNAse (Promega), and used for cDNA synthesis. Semi-quantitative PCR was carried out with oligonucleotide primers listed in Table S1, using the TUBULIN gene as a control. For starch analysis, samples were taken at the end of the night and the day following the final spraying (14 h and 26 h afterwards, respectively).

Microscopy

Sample preparation for electron and light microscopy was as described by Crumpton-Taylor et al. (2012) and Delatte et al. (2005).

Starch analysis

Analysis of chain lengths was as described by Streb et al. (2008). Starch samples (100 mg) were boiled for 10 min in water, then debranched by incubation with isoamylase and pullulanase at pH 4.8 and 73°C for 2 h. Neutral glucans were separated by passage through sequential Dowex 50 and Dowex 1 micolumns, lyophilized, redissolved, then subjected to HPAEC-PAD analysis on a Dionex PA-200 column. The relative proportions of each chain length in the total population (from d.p. 3 to d.p. 50) are expressed as a percentage of the total number of chains.

Metabolite and enzyme assays

Starch, soluble glucans and sugars were extracted and assayed enzymatically (Critchley et al., 2001; Delatte et al., 2005). Other metabolites were measured by high pressure anion exchange
chromatography coupled to tandem mass spectrometry (HPAEC-MS/MS: Lunn et al., 2006). Leaves were frozen as rapidly as possible then extracted in chloroform/methanol. Where young and older leaves were harvested separately, rosettes were placed immediately after excision on a metal plate cooled to −80°C and the centre of the rosette was excised with a 1-cm diameter cork borer.

Starch synthase activity was assayed according to Jenner et al. (1994). To visualise glycogen and starch synthase activities, leaves were extracted with 100 mM MOPS, pH 7.2, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol (300 mg leaf ml\(^{-1}\)). Extracts (32-μl) were loaded onto non-denaturing polyacrylamide gels containing 0.3% (w/v) glycogen. After incubation for 16 h at 20°C in 100 mM HEPES-NaOH, pH 7.5, 2 mM DTT, 10% (v/v) glycerol, 0.5 mM EDTA, 0.5 M Na-citrate, 2 mM ADPglucose, starch and glycogen synthase activities were revealed by iodine staining.

Immunoblotting

An antiserum was raised commercially in rabbits using the synthetic peptide DIGHDDGKNLDNIT (present in SS4 but not other starch synthase isoforms). Antibodies were affinity-purified using this peptide. Leaf tissue was powdered in liquid nitrogen then extracted in chloroform/methanol. Where young and older leaves were harvested separately, rosettes were placed immediately after excision on a metal plate cooled to −80°C and the centre of the rosette was excised with a 1-cm diameter cork borer.

**Results**

**Immature leaves of ss4 plants contain almost no starch**

We extended the characterisation of ss4 mutants, using the ss4-1 mutant allele (Roldán et al., 2007) and a further T-DNA insertion line, ss4-3, which lacks SS4 protein (Fig. S1a).

Examination of whole rosettes and mature leaves largely confirmed previous reports of the ss4 phenotype (Roldán et al., 2007). Compared with wild-type plants, ss4 plants had slightly reduced soluble starch synthase activities, 40% less chlorophyll (Table S2), less degradation of starch during the night (measured as end-of-day minus end-of-night starch contents) and higher end-of-night starch contents (Fig. S1b), and slower growth rates under both long and short photoperiods (Fig. S1c).

As reported previously (Roldán et al., 2007), mature ss4 leaves appeared to have only one large, rounded starch granule per chloroplast (Figs 1a,b, S1d,e). Quantification of granule numbers (using Method 2 from Crompton-Taylor et al., 2012) revealed that mature, nonflowering rosettes of wild-type and ss4-3 plants had 5.54 ± 0.28 and 0.87 ± 0.14 granules per chloroplast, respectively (mean ± SE from eight chloroplast preparations in both cases).

We found a different situation in immature leaves. Whereas immature leaves of wild-type plants have more starch granules per chloroplast than mature leaves (Crompton-Taylor et al., 2012), no starch granules were visible by light or electron microscopy in immature leaves of ss4 plants (Fig. 1c,d). Consistent with this observation, the youngest leaves of ss4 rosettes did not stain with iodine at the end of the day (Fig. 2a), and quantitative measurements revealed very low starch contents and little diel starch turnover (Fig. 2c,d). Starch content and turnover increased with leaf age, but turnover was limited even in mature leaves. By contrast, in wild-type plants starch content and starch turnover were at their maximum in young leaves (leaves 5–8), and values were similar or somewhat lower in mature leaves (Fig. 2b,d). Starch was not replaced with soluble glucan in ss4 leaves. For both mature and immature leaves, ss4 soluble glucan contents were lower than or comparable with wild-type contents (Fig. 2e).

Thus, loss of SS4 dramatically reduces the rate of acquisition of glucan storage and turnover capacity during leaf development. Mutants fail to form starch granules until late in leaf development, and then only about one granule is formed per chloroplast.

We examined whether loss of SS4 affected starch content in the primary root cap, a region of high starch content in wild-type plants. Starch in the columnella cells is essential for the normal gravitropic response of the root (Kiss et al., 1989; Blancaflor et al., 1998; Kiss & Edelmann, 1999). There was wide variation in the amount and location of starch in root caps of ss4 seedlings grown on vertical agar plates. Some ss4 root caps were not distinguishable from those of wild-type plants, but in most cases starch content was reduced with some or all cells having no visible starch. Roots of ss4 seedlings tended to deviate from vertical growth, and the degree of deviation was broadly negatively correlated with starch content (Fig. S2).

**Starch synthesis in immature ss4 leaves is not restored by blocking starch degradation**

We considered the possibility that starch granules are formed in immature ss4 leaves, but are immediately degraded. To examine

![Fig. 1](image-url)
this possibility, we introduced into the ss4 background a mutation that blocks starch degradation in wild-type plants. The sex1 mutation affects glucan, water dikinase (GWD), a starch-phosphorylating enzyme that renders the surface of the starch granule accessible to starch degrading enzymes at night (Stitt & Zeeman, 2012). Mutants lacking GWD have a strongly reduced rate of starch degradation at night, and accumulate very high concentrations of starch in all leaves (Zeeman & ap Rees, 1999; Yu et al., 2001).

The ss4sex1 double mutants grew more slowly than ss4 mutants, at about the same rate as sex1 mutants (Fig. S3). Mature leaves had high starch contents at the end of both the day and the night. However, immature leaves contained very little starch and most chloroplasts appeared to contain no granules (Figs 3, S3).

Leaves of ss4 mutants accumulate very high concentrations of ADPglucose

The experiments described above show that neither starch granules nor soluble glucans accumulate in immature ss4 leaves. This observation suggests that the actions of starch synthase isoforms other than SS4 may be dependent on SS4. To test this idea, we measured the impact of the loss of SS4 on concentrations of the starch synthase substrate ADPglucose. Concentrations were 50–200 times higher in ss4-1 and ss4-3 rosettes than in wild-type rosettes, and were elevated in both mature and immature leaves (Tables 1, S3). This was a highly specific effect. Analysis of mutants lacking any of the other three isoforms of soluble starch
Table 1 Metabolite contents of Arabidopsis rosettes lacking starch synthase isoforms

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Amount (nmol g⁻¹ FW)</th>
<th>Wild-type Col</th>
<th>Wild-type Ws</th>
<th>ss1 (Ws)</th>
<th>ss2 (Col)</th>
<th>ss3 (Col)</th>
<th>ss4-3 (Col)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADPglucose</td>
<td>2.14 ± 0.88</td>
<td>2.32 ± 1.10</td>
<td>4.02 ± 1.16</td>
<td>1.78 ± 0.53</td>
<td>2.36 ± 0.40</td>
<td>124.2 ± 25.8</td>
<td></td>
</tr>
<tr>
<td>UDPglucose</td>
<td>105 ± 11</td>
<td>104 ± 14</td>
<td>111 ± 15</td>
<td>103 ± 11</td>
<td>107 ± 10</td>
<td>109 ± 4</td>
<td></td>
</tr>
<tr>
<td>Trehalose 6-P</td>
<td>0.23 ± 0.02</td>
<td>0.22 ± 0.03</td>
<td>0.27 ± 0.02</td>
<td>0.25 ± 0.02</td>
<td>0.31 ± 0.04</td>
<td>0.38 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Glucose 6-P</td>
<td>151 ± 43</td>
<td>175 ± 19</td>
<td>212 ± 17</td>
<td>167 ± 15</td>
<td>204 ± 12</td>
<td>265 ± 21</td>
<td></td>
</tr>
<tr>
<td>Glucose 1-P</td>
<td>51.0 ± 2.7</td>
<td>50.2 ± 5.2</td>
<td>51.8 ± 4.1</td>
<td>51.9 ± 3.8</td>
<td>54.0 ± 3.3</td>
<td>61.0 ± 3.9</td>
<td></td>
</tr>
<tr>
<td>Fructose 6-P</td>
<td>44 ± 15</td>
<td>46 ± 12</td>
<td>59 ± 8</td>
<td>48 ± 6</td>
<td>56 ± 7</td>
<td>69 ± 4</td>
<td></td>
</tr>
<tr>
<td>Fructose 1,6-bisphosphate</td>
<td>39 ± 6</td>
<td>35 ± 11</td>
<td>42 ± 7</td>
<td>41 ± 9</td>
<td>47 ± 7</td>
<td>59 ± 10</td>
<td></td>
</tr>
<tr>
<td>Glycerol 3-P</td>
<td>10.9 ± 1.0</td>
<td>9.7 ± 0.6</td>
<td>10.7 ± 1.4</td>
<td>10.7 ± 1.2</td>
<td>10.7 ± 1.3</td>
<td>8.5 ± 0.8</td>
<td></td>
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<tr>
<td>3-Phospho-glycerate</td>
<td>395 ± 86</td>
<td>407 ± 43</td>
<td>404 ± 60</td>
<td>334 ± 78</td>
<td>416 ± 55</td>
<td>386 ± 83</td>
<td></td>
</tr>
<tr>
<td>Phosphoenol-pyruvate</td>
<td>29 ± 6</td>
<td>27 ± 4</td>
<td>33 ± 8</td>
<td>26 ± 7</td>
<td>31 ± 8</td>
<td>34 ± 7</td>
<td></td>
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<tr>
<td>Pyruvate</td>
<td>101 ± 7</td>
<td>111 ± 10</td>
<td>122 ± 9</td>
<td>112 ± 9</td>
<td>92 ± 7</td>
<td>75 ± 10</td>
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<tr>
<td>Malate</td>
<td>1207 ± 671</td>
<td>1004 ± 271</td>
<td>1684 ± 441</td>
<td>1293 ± 234</td>
<td>1352 ± 267</td>
<td>1015 ± 250</td>
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</tr>
<tr>
<td>Fumarate</td>
<td>1734 ± 616</td>
<td>1826 ± 357</td>
<td>2329 ± 289</td>
<td>2000 ± 223</td>
<td>1948 ± 365</td>
<td>1680 ± 218</td>
<td></td>
</tr>
<tr>
<td>Aconitate</td>
<td>91 ± 13</td>
<td>90 ± 8</td>
<td>95 ± 8</td>
<td>87 ± 9</td>
<td>93 ± 11</td>
<td>83 ± 6</td>
<td></td>
</tr>
<tr>
<td>2-Oxo-glutarate</td>
<td>84 ± 30</td>
<td>85 ± 13</td>
<td>105 ± 14</td>
<td>120 ± 10</td>
<td>102 ± 6</td>
<td>64 ± 6</td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>6103 ± 2438</td>
<td>5594 ± 1429</td>
<td>6441 ± 503</td>
<td>5957 ± 562</td>
<td>6299 ± 700</td>
<td>5710 ± 323</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>92 ± 71</td>
<td>47 ± 42</td>
<td>143 ± 41</td>
<td>97 ± 39</td>
<td>100 ± 53</td>
<td>104 ± 45</td>
<td></td>
</tr>
<tr>
<td>Shikimate</td>
<td>21 ± 2</td>
<td>21 ± 3</td>
<td>21 ± 1</td>
<td>23 ± 2</td>
<td>22 ± 1</td>
<td>32 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

Plants were grown together in identical conditions. Rosettes were excised c. 4 h into the 12 h photoperiod, and very rapidly frozen in liquid nitrogen. Metabolites were extracted in chloroform/methanol and assayed by anion exchange chromatography linked to tandem mass spectrometry. Values are means of measurements on six rosettes ± SD.

synthase (the ss1, ss2 and ss3 mutants) revealed less than two-fold differences between their ADPglucose concentrations and those of wild-type plants (Table 1). In the ss4 mutant there were no large alterations with respect to wild-type plants in concentrations of soluble glucans (as previously shown, Fig. 2e) or other intermediates of starch and sucrose synthesis, glycolysis and the Calvin–Benson cycle, and the Krebs cycle (Table 1).

As expected of any mutant with a reduced capacity to generate sugars from starch at night (e.g. pgm1, Gibon et al., 2004; mex1, Niittyla et al., 2004; lsf1, Comparot-Moss et al., 2010), sucrose concentrations were elevated in ss4 plants during the day, and sucrose and hexose concentrations were lower in ss4 than in wild-type plants during most of the night (Fig. S1b; see also Roldán et al., 2007).

Starch synthesis in an ss4 background is partially but not fully restored by expression of Agrobacterium glycogen synthase

The results above imply that SS4 directly or indirectly provides a ‘primer’ required for the actions of other starch synthases. To investigate this possibility, we overexpressed glycogen synthase (GS) from A. tumefaciens in an ss4 background. We chose this enzyme because it is reported both to elongate glucan chains and to initiate chains de novo via an autoglycosylation mechanism (Ugalde et al., 2003), using ADPglucose as its substrate. Thus it might replace the function of SS4 by providing glucosylated proteins as primers for other starch synthases, resulting in the formation of starch granules. We introduced the GS into plants lacking both SS4 and SS3 (the ss3ss4 double mutant), because these mutants contain even fewer starch granules than the ss4 mutant (Szydlowski et al., 2009; Mérida & D’Hulst, 2012). Indeed, in our growth conditions, (12 h : 12 h, light : dark), ss3ss4 plants were pale, slow growing and contained very little starch (Figs 4, S4).

Agrobacterium GS was introduced into ss3ss4 plants as a fusion protein consisting of an N-terminal Rubisco small subunit chloroplast transit peptide, then a yellow fluorescent protein (YFP), then GS (Fig. S4). Examination of four independent GS-transformed ss3ss4 plants with confocal fluorescence microscopy confirmed that the protein was targeted to chloroplasts. Analysis of crude extracts of leaves by nonequilibrium PAGE on gels containing glycogen revealed abundant GS activity in these plants (Fig. S4).

All GS-expressing lines had higher growth rates and more starch than ss3ss4 mutants (Figs 4, S4). However, complementation was quantitatively and qualitatively incomplete. Although ADPglucose was reduced from 423 ± 113 nmol g⁻¹ FW in ss3ss4 plants to 40 ± 19 nmol g⁻¹ FW in transformed line G-5-3 (see Figs 4, S4), this concentration was still many times greater than that of wild-type plants grown under the same conditions (1.29 ± 0.23 nmol g⁻¹ FW: means ± SD of measurements on three to five plants per line). Importantly, both the distribution and the nature of starch granules in the GS-expressing plants were very different from those of wild-type plants. In leaf sections, starch granules were visible in some chloroplasts but not in others. Furthermore, chloroplasts of a single cell appeared to contain a huge range of granule morphologies and sizes (Fig. 4). As expected of amylopectin, polymers from starch granules in GS-expressing lines had a polydispersed distribution of chain lengths. However, the pattern of distribution was not the same as that of amylopectin from wild-type leaves (Fig. S4). Thus, either the additional glucan-synthesizing capacity or the
autoglucosylation activity provided by Agrobacterium GS can promote starch granule formation in the ss3ss4 background, but neither of these functions of GS can fully restore normal starch synthesis.

Starch synthesis in immature ss4 leaves is partially restored by increased chloroplast volumes

The results above show that formation of new granules occurs much less frequently during leaf development in ss4 leaves than in wild-type leaves. We showed previously that in wild-type plants the number of starch granules present in a chloroplast is a function of its volume, and that the number per unit volume is relatively constant for a given leaf developmental stage (Crumpton-Taylor et al., 2012). Based on these observations, we speculated that the unit volume required per granule formation event might be much larger in ss4 than in wild-type leaves. Average chloroplast volumes double over the course of leaf expansion (Crumpton-Taylor et al., 2012), thus the delayed formation of granules in ss4 leaves might reflect that fact that chloroplast volumes are larger in mature than in immature leaves.

In order to test this idea, we examined the impact on granule formation in the ss4 background of the introduction of mutations that dramatically increase chloroplast volumes. The accumulation and replication of chloroplast (arc) mutants have few, giant chloroplasts per mesophyll cell, but the same number of starch granules per unit chloroplast volume as wild-type plants (Crumpton-Taylor et al., 2012). Chloroplast sizes and numbers in arc3ss4, arc5ss4, arc6ss4 and arc10ss4 double mutants were similar to those in the respective arc parent (between one (arc6) and 25 (arc10) chloroplasts per cell, compared with ~100 chloroplasts per wild-type cell, not shown). Nondenaturing PAGE analysis revealed no differences in activities of starch synthase isoforms other than SS4 between the parental and double mutant lines (Fig. S5).

The double mutants differed from the ss4 parent in that starch granules were visible in some chloroplasts in sections of immature leaves (Fig. S5) and starch turnover was comparable with that of the arc parent and wild-type plants (Fig. S5 h, i). However, starch synthesis in double mutants was different in several respects from that of arc and wild-type plants. Although some chloroplasts in immature ss4 leaves contained granules, others apparently contained none (Fig. S5f, g). Granule numbers per unit chloroplast volume appeared to be lower than in wild-type plants throughout leaf expansion. In both immature and mature leaves, the range of granule sizes was larger in double mutants than in ss4 or arc mutants, or wild-type plants (Figs 5c, S5). The granules of double mutants were rounded rather than flattened, similar to those...
of ss4 mutants (Fig S5). Thus, although granule formation in the absence of SS4 started earlier during leaf expansion development in arc mutant backgrounds than in a wild-type background, it was highly sporadic and qualitatively different from that in wild-type plants.

Loss of SS4 from established plants affects granule numbers in immature but not mature leaves

Taken together, the observations above suggest that the primary effect of loss of SS4 is a strongly delayed and infrequent formation of new starch granules during leaf development. The alterations in granule number and starch turnover in mature leaves may be secondary consequences of this effect. To distinguish more clearly between primary and secondary effects, we reduced SS4 concentrations in mature rosettes by inducing expression of an RNAi hairpin construct targeted at the SS4 gene, and examined the consequences for granule numbers and starch turnover.

Wild-type plants were transformed with constructs allowing expression of two hairpin RNAs based on different parts of the SS4 gene sequence, under the control of a dexamethasone-inducible promoter (Figs S6, S7). Transgenic plants were treated with dexamethasone daily for 10 d. For each construct, one line showing strong reductions in SS4 protein was selected for further study. Before dexamethasone treatment, SS4 transcript and protein concentrations were the same in transgenic lines and wild-type plants. SS4 protein concentrations were unaffected by dexamethasone treatment in wild-type controls (Fig. S6b and not shown). In transgenic lines, SS4 transcript levels declined to very low values within 24–48 h of the first dexamethasone treatment and remained low. Concentrations of SS4 protein fell slowly to undetectable levels over the first 7 d of dexamethasone treatment (Fig. 6a).

After 10 d of dexamethasone treatment, whole rosettes of transgenic lines had higher end-of-night starch contents than control plants (although lower than in ss4 plants), but diel starch turnover was at least 94% of that in wild-type plants and more than twice that in ss4 plants. There was little or no difference between transgenic and control plants in whole-rosette starch content at the end of the day, average granule radius (Fig. 6b) and granule size distribution (Fig. S6c). In sections of mature leaves, the chloroplast area per starch granule was the same in transformed and control plants. However, in immature leaves that had emerged during the dexamethasone treatment (leaves 1–4) the chloroplast area per starch granule was c. 50% greater in transgenic than in control plants (Fig. 6c,d). In summary, reduced SS4 concentrations strongly reduced starch granule numbers in young leaves, but had little effect in mature leaves.

SS4 concentrations can be substantially reduced without affecting starch granule formation

We investigated whether the amount of SS4 in a wild-type leaf limits starch granule formation. This possibility is suggested by a report that transgenic Arabidopsis plants with elevated concentrations of SS4 protein have increased rates of starch synthesis.
(Gámez-Arziona et al., 2011). First, we examined starch turnover in leaves of ss4 plants expressing the Arabidopsis SS4 cDNA from the CaMV 35S promoter. Starch content and turnover were restored to near wild-type levels in both immature and mature leaves of several transgenic lines that had much lower concentrations of SS4 protein than wild-type plants (Fig. S8).

Second, we examined growth and starch metabolism of heterozygous (SS4ss4-3) plants. As expected, both mature and immature leaves had approximately half the SS4 protein content of wild-type plants (Figs 7, S9). Heterozygous plants were statistically significantly different from ss4 mutant plants but not from wild-type plants with respect to FW, starch granule size distribution, granule volume, starch content and turnover in mature and immature leaves, and mean stromal area per starch granule (measured on light micrographs of leaf sections) (Figs 7, S9). Thus, loss of half of the SS4 protein does not affect granule formation.

**Discussion**

SS4 is necessary for granule formation during leaf expansion

Our results show that SS4 is essential for the coordinated formation of starch granules that occurs during leaf expansion. In wild-type plants, chloroplasts have multiple starch granules from a very early stage of leaf development (Sakamoto et al., 2009; Crumpton-Taylor et al., 2012). Although chloroplasts undergo
about three rounds of division as leaf cells expand, resulting in an eight-fold increase in chloroplast numbers (Marrison et al., 1999), the numbers of starch granules per chloroplast remain relatively constant (a 1.5-fold decrease between early expansion and maturity: Crumpton-Taylor et al., 2012). Thus, large numbers of new granules must be produced in association with rounds of chloroplast division. By contrast, no starch granules are visible in chloroplasts of immature leaves of ss4 mutant plants. Granules appear only at a later stage of leaf expansion, and granule numbers per chloroplast are much lower in mature leaves than in wild-type plants.

These observations suggest that the low numbers of starch granules in chloroplasts of mature ss4 leaves are a consequence of the major defect in granule formation during leaf expansion in this mutant. Support for this view comes from our analysis of plants in which concentrations of SS4 protein were progressively reduced by induction of RNAs specifically targeted at the SS4 gene. Loss of SS4 protein was accompanied by reduced granule numbers in newly formed leaves but not in mature leaves, showing that SS4 is required for normal granule formation in immature leaves but not for maintenance of granule numbers in mature leaves. This conclusion is consistent with a specific role for SS4 in granule formation: although new granules arise frequently during leaf expansion this is likely to be a rare event once leaves reach maturity (Crumpton-Taylor et al., 2012).

SS4 also appears to be necessary for a normal frequency of granule formation following cell division in root caps. The root caps of ss4 plants generally contained less starch in fewer cells than wild-type plants, and there was a much higher degree of plant-to-plant variation in root cap starch content.

SS4 plays a direct role in the formation of new granules

Loss of SS4 has several major effects on the Arabidopsis plant in addition to reduced starch granule numbers. These include reduced growth rates, altered patterns of starch turnover and concentrations of sugars, altered starch granule morphology, and reduced starch content and gravitropic response in roots. It is conceivable that failure of starch granule formation during leaf expansion is an indirect consequence of one or a combination of these effects. However, taking our results as a whole, we propose that SS4 is involved directly in the formation of granules, and that the other effects of its loss are secondary consequences of reduced granule numbers. The basis for this conclusion is as follows.

First, the absence of starch granules from immature leaves of SS4 mutants is likely to be caused directly by failure of granule formation rather than indirectly by alterations in other aspects of sugar or glucan metabolism. Immature ss4 leaves did not accumulate abnormal concentrations of soluble glucans, but contained exceptionally high concentrations of ADPglucose, suggesting that in the absence of SS4 the remaining soluble starch synthases are largely inactive. The fact that introduction of the sex1 mutation failed to restore either starch or glucan accumulation is also consistent with the idea that no starch is made. These observations point to a direct role for SS4 in a mechanism that generates a ‘primer’ that is acted on by other starch synthases, resulting in the formation of new granules.

Second, introduction of additional capacity for self-priming glucan synthesis in ss4 leaves complemented some of the effects of loss of SS4 but did not restore normal starch synthesis. Expression of glycogen synthase in the highly compromised ss4ss3 mutant reduced ADPglucose concentrations, increased starch concentrations, and restored wild-type appearance and growth rates. However, although starch granules were formed earlier in leaf expansion in plants expressing glycogen synthase, new granules arose sporadically and there were highly variable numbers per chloroplast. We conclude that glycogen synthase could catalyse the synthesis of glucans from which granules could be formed, but that it could not replace a specific and direct requirement for SS4 in a mechanism that controls the timing of granule formation and its coordination with chloroplast volume.

Third, in ss4 mutants with increased chloroplast volume (arc × ss4 mutants) some granules were present in immature leaves and starch turnover was comparable with that of wild-type plants, but granule distribution, shape and size remained highly variable. Thus, large chloroplast volumes permit more frequent formation of granules and higher starch turnover in the absence of SS4, but do not restore the coordinated production of typical transitory starch granules seen in wild-type leaves.

Fourth, comparison of the ss4 phenotype with those of mutants lacking other components of the pathways of starch synthesis and degradation suggests that SS4 has an exclusive and direct role in granule formation. Several features of the ss4 phenotype are also seen in other starch mutants. For example the starch synthesis mutant pgm1 and the starch degradation mutant sex1 – lacking plastidial phosphoglucomutase and glucan, water dikinase, respectively – have reduced growth rates, elevated daytime concentrations of sugars (Caspar et al., 1985, 1991) and defective gravitropism (Caspar & Pickard, 1989; Vitha et al., 2007). Other starch degradation mutants with reduced growth rates, reduced starch turnover and elevated daytime concentrations of sugars include mex1, pwd1 and dpe1, lacking the chloroplast maltose transporter, phosphogluconater, water dikinase and the plastidial disproportionating enzyme, respectively (Critchley et al., 2001; Niittylä et al., 2004; Baunsgaard et al., 2005; Köting et al., 2005). However, none of these mutants is reported to have reduced numbers of starch granules per chloroplast, or starch in mature but not immature leaves. Similarly, mutants lacking combinations of isoforms of starch synthase other than SS4 also exhibit reduced starch and elevated sugar contents and reduced growth rates, but are not reported to have reduced numbers of starch granules per chloroplast (ss2ss3 mutants, Zhang et al., 2008; ss1ss2ss3 mutants, Szylowski et al., 2009). The impact of the ss4 mutation on granule formation is thus likely to reflect a primary role for SS4 in this process, rather than a general secondary effect of perturbations in growth, starch and sugar metabolism, or gravitropism.

We suggest that most of the effects of loss of SS4 may be consequences of a primary failure of granule formation in immature leaves. As discussed above, the exceptionally high concentrations
SS4 is required for coordination of granule formation with chloroplast volume and for determination of granule morphology

ss4 mutants are defective in the relationship between starch granule initiation and chloroplast volume, and in starch granule morphology. In wild-type plants, there is a tight relationship between stromal volume and numbers of starch granules. At a given stage of leaf expansion the number of granules per unit volume of stroma is remarkably constant, regardless of the total volume of the chloroplast. Granule sizes are also relatively uniform within and between chloroplasts (Crumpton-Taylor et al., 2012). These relationships are absent in ss4 mutants, and are not restored by manipulations that increase the frequency of granule initiation. Although immature leaves of ss4 mutant plants expressing glycogen synthase had more starch granules than those of ss4 mutants, these granules were highly variable in distribution and apparent size. Whereas a few chloroplasts appeared to have large numbers of granules, many others had none. Transfer of the ss4 mutation into an arc mutant background, in which chloroplast volumes are greatly increased, had a similar effect. Granule numbers in immature leaves of arc × ss4 mutants were greater than in ss4 mutants. However, granule distribution and size were highly variable, and there was no obvious relationship between starch granule number and chloroplast volume. We conclude that SS4 is an essential component of a mechanism that links granule initiation to chloroplast volume. In its absence, granule initiation appears to be stochastic.

Starch granules in leaves of ss4 mutants are different in shape from those in wild-type plants. Rather than being flattened and discoid, they are rounded with an electron-transparent core (Roldán et al., 2007; Fig. S1d). Manipulations of glucan-synthesizing capacity (by expression of Agrobacterium glucan synthase) and chloroplast volume (by introduction of arc mutations) did not restore normal granule morphology even though they increased the frequency of granule formation in immature leaves.

Thus, the mechanism of granule formation in leaves in the presence of SS4 appears to be qualitatively different from that in its absence. It is interesting to note that flattened, discoid granules are found in leaves but not in other plant organs. Starch granules in nonphotosynthetic organs are generally rounded with a distinct core or hilum, resembling the leaf starch granules of ss4 mutants. However, SS4 appears to be necessary for normal granule initiation in root caps as well as leaves, suggesting that it interacts with different granule-initiation mechanisms in the two organs.

What is the function of SS4 in granule initiation? As discussed above, it seems to be an essential part of a mechanism that generates a specific ‘primer’ on which other starch synthases can act, leading to granule formation. The primer might simply be small malto-oligosaccharides, perhaps elaborated by SS4 from ADPglucose alone or from maltose, which is produced during photosynthesis (Linden et al., 1975; Szecowka et al., 2013). This explanation seems unlikely, however, because starch synthases other than SS4 are reported to synthesize malto-oligosaccharides from ADPglucose or maltose. Szydlowski et al. (2009) showed that recombinant SS3 can form glucans from ADPglucose alone. Recombinant SS1, SS2 and SS3 from kidney bean (Phaseolus vulgaris) can elongate both maltose and maltotriose (Senoura et al., 2004, 2007). It is also difficult to explain why SS4 is required for normal granule shape, and why it cannot be fully replaced by Agrobacterium glucan synthase, if its sole function is to provide small oligosaccharides on which other starch synthases can act. An alternative explanation (Szydlowski et al., 2009; Mérida & D’Hulst, 2012) is that SS4 proteins adopt a quaternary structure – perhaps as part of a complex with other proteins – which serves as a synthetic and/or nucleation centre for specific glucans required for granule formation. This centre could also determine the subsequent direction of granule growth, and hence granule shape. Alone among the starch synthases, SS4 possesses coiled-coil domains in the noncatalytic N-terminal part of the protein. Coiled-coil domains can mediate protein–protein interactions (Mason & Arndt, 2004). Further work is required to establish precisely the nature of the primer made in the presence of SS4, and how it allows initiation of appropriate numbers of uniform granules of defined shape as chloroplasts expand and divide.
SS4 is not limiting for starch granule formation in wild-type leaves

Although SS4 is required for the formation of normal transitory starch granules, it is unlikely that its concentration exerts significant control over the numbers of granules initiated in wild-type plants. First, granule numbers in SS4ss4 heterozygotes with half the wild-type concentrations of SS4 protein were indistinguishable from those of wild-type plants. Second, normal starch metabolism was restored in ss4 mutant plants by transgenic expression of SS4 at concentrations far below those in wild-type plants. These results imply that the tight relationship between stromal volume and numbers of starch granules is not simply a function of numbers of SS4 protein molecules, but is determined by a complex mechanism of which SS4 is an essential but non-limiting component. In this light, it is interesting that overexpression of SS4 in wild-type Arabidopsis plants results in increased rates of leaf starch synthesis and up to 50% more starch at the end of the day (Gámez-Arjona et al., 2011). Important new information about the role of SS4 may be gained from analysis of the total starch synthase activity and the size, number and distribution of starch granules in these overexpressing plants.

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**Supporting Information**

Additional supporting information may be found in the online version of this article.

**Fig. S1** Characterization of ss4 mutants.

**Fig. S2** Growth of ss4 roots.

**Fig. S3** Phenotypes of ss4sex1 mutants.

**Fig. S4** Expression of *Agrobacterium glgA* in ss3ss4 mutants.

**Fig. S5** Starch granules in arc x ss4 mutants.

**Fig. S6** Effects of inducing RNAi targeted at the SS4 gene.

**Fig. S7** SS4 coding sequence showing regions targeted by RNAi.

**Fig. S8** Transgenic ss4 plants expressing SS4.

**Fig. S9** Further characterization of heterozygous (SS4ss4) plants.

**Table S1** Oligonucleotide primers used in this study

**Table S2** Starch synthase activities and chlorophyll contents of ss4 mutants

**Table S3** ADPglucose contents of mature and immature leaves of ss4 mutants

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