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Starch synthase 4 is located in the thylakoid membrane and interacts with plastoglobule-associated proteins in *Arabidopsis*

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SUMMARY

Starch synthesis requires the formation of a primer that can be subsequently elongated and branched. How this primer is produced, however, remains unknown. The control of the number of starch granules produced per chloroplast is also a matter of debate. We previously showed starch synthase 4 (SS4) to be involved in both processes, although the mechanisms involved are yet to be fully characterised. The present work shows that SS4 displays a specific localization different from other starch synthases. Thus, this protein is located in specific areas of the thylakoid membrane and interacts with the proteins fibrillin 1a (FBN1a) and 1b (FBN1b), which are mainly located in plastoglobules. SS4 would seem to be associated with plastoglobules attached to the thylakoids (or to that portion of the thylakoids where plastoglobules have originated), forming a complex that includes the FBN1s and other as-yet unidentified proteins. The present results also indicate that the localization pattern of SS4, and its interactions with the FBN1 proteins, are mediated through its N-terminal region, which contains two long coiled-coil motifs. The localization of SS4 in specific areas of the thylakoid membrane suggests that starch granules are originated at specific regions of the chloroplast.

INTRODUCTION

Starch produced in the chloroplasts is known as transitory starch since it accumulates during daylight hours and is mobilized at night to provide carbon skeletons and energy. Long-term storage starch is found in specialized plastids known as amyloplasts, which are located in the roots, tubers and/or seed endosperm, depending on the species. Although most of the starch biosynthesis pathway has been elucidated, involving the action of starch synthases as well as starch branching and debranching enzymes (isoamylases) (Stitt and Zeeman, 2012), the initiation of starch granule production remains a mystery. The mechanism described for the synthesis of glycogen in yeast and animals, which involves the synthesis of a primer by the protein glycogenin, does not seem to operate in starch synthesis (D'Hulst and Mérida, 2012; D'Hulst and Mérida, 2010).

Five different classes of starch synthases are found in all plants: granule-bound starch synthase (GBSSI), which is responsible for the synthesis of amylose (the minor component of starch), and soluble starch synthase (SS) classes 1, 2, 3 and 4, which have been proposed to take part in the synthesis of amylopectin, the major component of starch (Ball and Morell, 2003). Our group has shown that starch synthase class 4 (SS4) controls the number of starch granules (5-7) produced in *Arabidopsis* chloroplasts and that SS4 is involved in the initiation of their production (Roldán et al., 2007). The role of SS4 cannot be performed by other SS enzymes, and its elimination in *Arabidopsis* determines the accumulation of just 1-2 starch granules per chloroplast (Roldán et al., 2007). The hilum (or core) of granules thus produced has a structure not seen in normal granules, thereby suggesting that the initiation of starch granule production in the absence of SS4 is different to that which occurs in wild-type plants (Roldán et al., 2007). The *Arabidopsis* triple mutant

ss1/ss2/ss3, which lacks SS1, SS2 and SS3 activities, produces the correct number of starch granules in the chloroplast, thus indicating that the function of SS4 in the absence of other starch synthases results in the normal number of granules per chloroplast. This finding supports the idea that SS4 plays a central role in the process of starch granule initiation (Szydlowski et al., 2009).

All classes of SS contain a highly conserved C-terminal region in which the catalytic and substrate binding sites are found (Leterrier et al., 2008). The N-terminal part of these proteins is, however, exclusive to each class, differing in size and amino acid sequence (Leterrier et al., 2008). This suggests that the N-terminal region is responsible for the specificity of function of each SS class. The N-terminal region in SS4 contains two long coiled-coil domains (Rose et al., 2004), extending from amino acids 194 to 407 and from amino acids 438 to 465, respectively (Szydlowski et al., 2009). Such motifs are found in a number of structurally different proteins and are often involved in the formation of protein complexes, the attachment of functional protein complexes to larger cellular structures, and in regulating protein positioning in the cell through their action as anchors (Rose and Meier, 2004). This region is highly conserved in the different SS4s sequenced to date, thereby suggesting that its function has been preserved across the plant species.

We have previously shown that SS4 is located within specific areas of the *Arabidopsis* chloroplast rather than being uniformly distributed within the stroma, thereby suggesting that SS4 participates in the formation of a protein complex that might be needed for it to perform its function (Szydlowski et al., 2009). The formation of a complex during starch synthesis, involving starch synthases and starch branching enzymes, has been reported in wheat (Tetlow et al., 2008) and maize amyloplasts (Hennen-Bierwagen et al., 2008).

The present work reports the localization of SS4 in the thylakoid membrane fraction of *Arabidopsis* chloroplasts. This localization pattern is specific to this enzyme and completely different from the patterns exhibited by the other starch synthases. We also show that SS4 interacts with the proteins fibrillin 1a and 1b (FBN1a and FBN1b respectively), which are mainly located in the plastoglobules (PGs), *in vivo*. The N-terminal part of SS4 is shown to be responsible for both this pattern of localization and interaction of the enzyme with these fibrillins.

RESULTS

SS4 localization in the chloroplast. SS4 distribution in the *Arabidopsis* chloroplast stroma and membrane fractions was first examined by immunoblotting, using antibodies specific to markers of each compartment. Chloroplasts isolated from *Arabidopsis* leaves harvested at the end of the day were disrupted, and the soluble, stromal fraction separated from the plastid envelopes and thylakoid membrane~~s~~ by ultracentrifugation (see Experimental Procedures). Figure 1a shows no signal for the stromal marker phosphoribulokinase (PRK) in the thylakoid lane (T), thus indicating the absence of any contamination of this fraction by soluble proteins. Similarly, the soluble fraction was not contaminated by membrane-associated proteins, as indicated by the absence of PsbA (a protein from PSII) in the stromal fraction (lane S, Fig. 1a). The lane corresponding to the chloroplast envelopes (E) showed slight contamination of proteins from both the stroma and the thylakoid fractions. SS4 was detected in the thylakoid fraction but no signal was detected in either the stroma or chloroplast envelope fractions (Fig. 1a), thus indicating that SS4 is entirely associated with the thylakoid membrane. Note that, as described by Roldán et al. (Roldán et al., 2007), SS4 appears as a double band. As mentioned above, SS4 is

involved in the initiation of starch granule production, thereby raising the possibility that it is associated with a glucan polymer that precipitates after ultracentrifugation, which would explain the presence of SS4 in the resulting pellet together with the thylakoid membrane. To examine this further, the chloroplast extract was treated with amyloglucosidase prior to separation of the different fractions by ultracentrifugation (Experimental Procedures). No differences were observed between the control and the sample treated with amyloglucosidase, with SS4 being detected in the thylakoid fraction in both cases (Fig. 1b).

The N-terminal region of SS4 is responsible for the localization pattern of the protein in the chloroplast. The presence of two long coiled-coil domains in the N-terminal region of SS4 suggests that this part of the protein is responsible for the localization pattern of SS4 described above. We analysed the contribution of each part of the SS4 protein to its localization pattern by translational fusion of each polypeptide (full-length protein, the N-terminal half comprising residues 1 to 542, and the C-terminal half comprising residues 544 to 1040) with green fluorescent protein (GFP). In the case of the C-terminal part polypeptide, the SS4 chloroplast transit peptide was fused to the construct to lead the protein to the chloroplast. The different constructs were transformed into *Agrobacterium* and agroinfiltrated into *Nicotiana benthamiana* leaves. Transient expression of the proteins was monitored by confocal microscopy. Full-length SS4 and the N-terminal portion of SS4 showed the same pattern of discrete spots in the chloroplast (Fig. 2), which agrees with the pattern previously described for *Arabidopsis* (Szydlowski et al., 2009). However, this was not seen for the C-terminal portion of SS4 fused to GFP. Rather, a single dot was seen at one end of the chloroplast, thus suggesting mistargeting or protein aggregation

(Fig. 2). These results indicate that the N-terminal part of SS4 determines the pattern of localization (discrete spots) of this protein in the chloroplast.

Localization of SS1, SS2 and SS3 in chloroplasts. The precise localization of SS4 in some areas of the chloroplast raises the question as to whether the other starch synthases (SS1, SS2 and SS3) are also located in the same regions, forming part of a multisubunit complex responsible for the synthesis of starch or, in contrast, they show distinctive and specific localization in the chloroplast. To address this question, we fused the cDNAs coding for the full-length SS1, SS2 and SS3 proteins to GFP. The plasmids were *Agrobacterium*-mediated transformed into *Nicotiana benthamiana* leaves and transient expression of the different constructs was monitored by confocal microscopy. Figure 3 shows that each SS displays a specific localization pattern in the chloroplast, with these patterns differing from those shown by SS4. SS1 is spread throughout the stroma, whereas SS2 has a dual localization: it is found in specific dots in the chloroplast and also in the stroma (see the stromules in Panels d and f of Figure 3). Finally, SS3 seems to be located in those stromal regions surrounding the starch granule (Figure 3. Panels g-l), which appear as black areas without autofluorescence (Szydlowski et al., 2009). However, further studies will be necessary to confirm this localization. A complementary analysis of the distribution of the starch synthases in the different plastidial compartments was performed in *Nicotiana* plants agro-infiltrated with His_5-tagged SS1, SS2, SS3, and the N- and C-terminal regions of SS4 constructs. Infiltrated leaves were disrupted in liquid nitrogen with a pestle and mortar and the extracts fractionated into soluble, membrane and starch fractions. The presence of the different starch synthases in these fractions was detected by immunoblot using anti-His_5. Figure 4 shows that

SS1 and SS3 are soluble in the plastidial stroma, whereas SS2 is distributed in all fractions and, although the major population of SS2 can be found in the stroma, it is also associated with membranes and bound to the starch granules. Finally, the N-terminal part of SS4 shows the same distribution pattern as the full-length SS4 protein (Figure 1), being found exclusively in the membrane fractions. In contrast, the C-terminal part of SS4 is only detected in the soluble fraction.

Searching for proteins that interact with SS4 using the yeast two-hybrid (Y2H) system. Long coiled-coil motifs, like those present in SS4, are known to take part in protein-protein interactions (Rose and Meier, 2004). Such interactions might be involved in determining the localization pattern of SS4 or in modulating its activity. To identify proteins that interact with SS4, an *Arabidopsis* library was screened using the Y2H system, employing different fragments of SS4 as bait. cDNA fragments coding for the full-length SS4 protein, its N-terminal region (amino acids 43 to 502) (both without the chloroplast transit peptide [CTP]), and C-terminal region (amino acids 543 to 1040), were fused to the DNA-binding domain of the yeast GAL4 transcription factor by cloning into the expression vector pGBKT7 and subsequent transformation into the yeast strain Y2HGOLD (Experimental Procedures). Each fragment was used to screen a normalized, commercial *Arabidopsis* cDNA library in the pGADT7-Rec yeast expression vector (Clontech). No positive clones were obtained with the full-length or C-terminal fragments. However, seven positive clones were found when using the N-terminal part of SS4 as bait: At3g47080 (a tetratricopeptide repeat-containing protein), At1g05450 (a lipid transfer family protein), At3g21190 (an O-fucosyltransferase family protein), At3g46940 (a dUTP-pyrophosphatase-like protein), At3g10910 (a RING/U box superfamily protein),

At4g22240 (FBN1b, a plastid associated protein), and At1g70730 (phosphoglucomutase 2). Two of these proteins (At3g47080 and At4g22240) are located in the chloroplast, whereas the rest are predicted to be found in other cell compartments. All interactions of these non-chloroplast proteins with SS4 therefore most likely lack physiological significance in the present context. At3g47080 and At4g22240 were thus selected for further study.

Analysis of SS4-interacting proteins by bimolecular complementation of fluorescence. The interactions found using the Y2H system were confirmed by bimolecular complementation of fluorescence (BiFC) during transient expression of the selected proteins in *N. benthamiana* leaves. Thus, full-length cDNA sequences of At3g47080 and At4g22240 (FBN1b) were cloned into the binary vector pXCGW (-*c*CFP) and subjected to *Agrobacterium*-mediated co-transformation in *N. benthamiana* together with the SS4 cDNA fragment coding for the N-terminal region of the protein (Nt-SS4) cloned into pXNGW (-*n*YFP). Confocal microscopy indicated that At3g47080 did not interact with Nt-SS4 in *N. benthamiana*, therefore it was studied no further. FBN1b, however, did interact with Nt-SS4. In agreement with the pattern previously established for the SS4-GFP fusion protein (Fig. 2), the signal was seen in discrete areas of the chloroplast rather than being uniformly distributed across the stroma (Figure 5, Panels d-f). The full-length SS4 protein also interacted with FBN1b. The same localization pattern as that obtained with Nt-SS4 was seen, although the signal intensity was lower (Figure 5, Panels a-c). The C-terminal part of SS4 did not interact with FBN1b (Figure 5, Panels g-i). The interaction observed *in vivo* between SS4 and FBN1b was corroborated by way of an *in vitro* pull-down assay (Figure S2) using *E. coli* cells expressing the same FBN1b clone obtained in

the Y2H screening and an N-terminal region of SS4 (amino acids 184 to 503) containing the coiled-coil domains. Figure S2 shows that this region of SS4 is sufficient to interact with FBN1b protein.

SS4 interacts with FBN1a. FBN1b belongs to a multigene family of 13 members in *Arabidopsis* (Singh and McNellis, 2011), and its nucleotide sequence shows 83.5% similarity to the sequence of FBN1a, including the region coding for the CTP. This high homology led us to analyse whether SS4 might also interact with FBN1a. A cDNA fragment coding for the full-length sequence of FBN1a was cloned into the vector pXCGW (-*c*CFP), transformed into *Agrobacterium*, and co-transformed in *N. benthamiana* leaves together with different constructs of SS4 cloned into pXNGW (-*n*YFP). The BiFC results indicated that, as was the case for FBN1b, FBN1a interacts with both the full-length SS4 protein and its N-terminal domain (Figure 6, a-f). No interaction with the C-terminal region of SS4 was seen (Figure 6, g-i).

Elimination of FBN1a and FBN1b does not change the pattern of SS4 localization. The present results suggest that SS4 is associated with PGs attached to the thylakoids, and that this localization is mediated by interaction with FBN1a and FBN1b. To examine this further, the localization pattern of SS4 in an *Arabidopsis* double mutant lacking both FBN1a and FBN1b was determined. Homozygous single mutants for T-DNA insertions were isolated, and the absence of FBN1a and FBN1b proteins determined by RT-PCR amplification of mRNA (*fbn1b* mutant) or immunoblot using anti-FBN1a antibody (*fbn1a* mutant) (see Figure S1c). Single mutants were crossed to obtain an *fbn1a/fbn1b* double mutant. Chloroplasts from *fbn1a/fbn1b* plants were isolated from leaves harvested at midday, and the membranes separated from the stroma by ultracentrifugation as previously

described. Immunoblot analysis using anti-SS4 indicated SS4 to be associated with the membranes in the *fbn1a/fbn1b* mutant, thereby suggesting that the pattern of SS4 localization is not exclusively mediated by its interaction with FBN1a and FBN1b (Figure 7). Starch metabolism was also analysed in the *fbn1a/fbn1b* mutant. The process of granule initiation did not seem to be affected since chloroplasts of the double mutant showed numbers of starch granules per chloroplast similar to those seen in wild-type chloroplasts (Figure 8). However, these mutant plants showed a small but reproducible increase in the amount of starch accumulated (Fig. S3). The expression of genes involved in starch metabolism, such as those coding for the different starch synthases, the small (APS1) and large (APL1) subunits of ADP-glucose pyrophosphorylase, isoamylase 1 and 3 (ISA1, ISA3), β-amylase 3 and 4 (BAM3, BAM4), and glucan phosphorylase 1 and 2 (PHS1, PHS2), were unaltered in the mutant plants. The only exception was SS3, whose mRNA levels experienced a small but statistically significant 1.5-fold increase with respect to WT plants (Fig. S4). Although the levels of SS4 mRNA were not affected in the double mutant plant (Figure S4A), we were unable to determine whether the glycosyltransferse activity of SS4 was affected as the SS4 activity and SS2 activity were not detected in a zymogram. No change in the glycosyltransferase activity of SS4 *in vitro* was seen after incubation with purified FBN1b. However, the assay conditions are considerably different to those found around the thylakoid membrane~~s~~ *in vivo*.

DISCUSSION

Starch synthases are divided into two groups depending on their localization in the chloroplast: those bound to starch granules, such as GBSSI, and those considered to be soluble in the stroma, including SS1, 2 and 3 and, at least until now, SS4.

Some SS2 is, in fact, tightly bound to the starch granule and has also been denominated as granule-bound starch synthase II (Smith, 1990), although it is not involved in the synthesis of amylose (Edwards et al., 1996). SS4 is involved in the initiation of starch granule production and control of the number of starch granules per chloroplast (Roldán et al., 2007). It does not seem to contribute to the synthesis of amylopectin since its elimination does not affect either the amylose/amylopectin ratio or the distribution of amylopectin chain-length (Roldán et al., 2007). The operating mechanisms via which SS4 participates in the initiation of starch granule production and granule number control remain unclear.

The present results indicate that SS4 is associated with the thylakoid membrane rather than being soluble in the stroma of *Arabidopsis* chloroplasts (Fig. 1), although it is not evenly distributed throughout them. In contrast, it appears to be located in specific areas (Fig. 2). This localization pattern is unique among the SSs, as the remaining SSs showed a different localization (Figures 3 and 4), thus pointing to a relationship between the specific function of SS4 as regards initiation of the starch granules and control of their numbers and the exclusive localization of the enzyme associated to specific areas of the thylakoids.

The localization of SS2 (Figures 3 and 4) agrees with data previously reported for this enzyme, which indicated that SS2 could be found soluble in the plastidial stroma and also associated with the starch granule (Edwards et al., 1996; Li et al., 2003; Grimaud et al., 2008). Our findings also indicate that part of the SS2 protein population is associated with membranes. SS2 is at the core of a protein complex formed together with SS1 and SBE2b in maize endosperm, and it has been proposed that SS2 plays a crucial role in trafficking SS1 and SBE2b into the granule matrix (Liu et al., 2012). It will therefore be interesting to characterize the

mechanisms that determine the distribution of SS2 in the different compartments. SS3 displays a characteristic localization pattern close to the surface of the starch granule. Note that starch granules are identified as black areas where chlorophyll autofluorescence is not detected (Szydlowski et al, 2009). It is tempting to speculate that the localization of SS3 close to the starch granule surface is mediated by the three starch binding domain (SBD) repeats present in the N-terminal part of the protein (Palopoli et al., 2006), although further studies will be necessary to confirm the localization of SS3 and to determine whether it differs from that shown by SS1.

The identification of proteins that interact with SS4 sheds some light on the specific location of this enzyme. Thus, SS4 was found to interact with FBNs 1a and 1b *in vivo* and *in vitro* (Figs. 5, 6, and S2), and these interactions showed the same localization pattern as found for the fusion protein SS4-GFP (Fig. 2). In addition, both the localization pattern for SS4, and SS4's interactions with the FBN1s, appear to be mediated by the N-terminal part of the protein (Figs. 2, 4, 5, and 6), thereby suggesting a role for the long coiled-coil structures in both phenomena. The FBN1s are preferentially located in the PGs, and are, in fact, the major protein-based component of these particles in *Arabidopsis* (Lundquist et al., 2012). PGs form a specific sub-organelle compartment in all types of plastid (Besagni and Kessler, 2013), which arise through a membrane-blistering mechanism along highly curved thylakoid margins, remaining attached to the thylakoids via a half-lipid bilayer that surrounds the globule contents and is continuous with the stroma-side leaflet of these membranes (Austin et al., 2006). It was believed for many years that PGs were just passive lipid deposition sites for the plastids. However, recent proteomic studies have shown them to contain proteins involved in the synthesis of tocopherol and carotenoids, along with enzymes involved in other chloroplast metabolic

pathways such as the Calvin cycle, and with regulatory proteins such as Absence of bc1 Complex Kinase (ABC1K) proteins (Lundquist et al., 2012). PGs therefore appear to have more functions than previously believed. FBN1s have been suggested to exercise a general function in the maintenance of plastid lipid body structure (Lundquist et al., 2012). However, evidence exists that they are involved in PG structural development, chromoplast pigment accumulation, hormonal responses, protection of the photosynthetic apparatus, and plant resistance to a range of biotic and abiotic stresses (Singh and McNellis, 2011).

Immune-gold electron microscopy has shown FBN1a to be located on the surface of chloroplast PGs (Austin et al., 2006). The interaction between SS4 and FBN1a and 1b, together with the membrane-associated localization of SS4, indicate that SS4 might also be associated with PGs. However, the elimination of both FBN1a and FBN1b does not change the localization pattern for SS4 (Figure 7), thus indicating that other proteins interact with SS4 to anchor it to the thylakoids. Complexes involving SS4, FBN1s and these as-yet unidentified proteins must therefore be formed. Nevertheless, we cannot discard that SS4 remains associated with the thylakoids in *fbn1a/fbn1b* mutants via interactions with other FBNs as a consequence of a functional redundancy among the members of this family.

The function performed by the interactions between SS4 and the FBN1s remains unclear, as does the influence of SS4's localization on the initiation of starch granule production. The number of starch granules produced per chloroplast is not altered in *fbn1a/fbn1b* plants (Figure 8), which only show a slight increase in the amount of starch accumulated at the end of the day. In addition, the expression of starch metabolism genes remained unaffected in these mutants, with the exception of a slight increase in SS3 mRNA levels (Fig. S4), which might be responsible for the

small increase of starch accumulation observed in *fbn1a/fbn1b* plants. Although FBN1b did not affect the glycosyltransferase activity of SS4 *in vitro*, the *in vitro* environment is rather different to that found around thylakoid membrane~~s~~ *in vivo*. The effects of FBN1s on SS4 activity in the living plant are therefore hard to predict. The function of the interaction of FBN1s with SS4 might be to contribute to the formation of protein complexes that also include as-yet unidentified proteins. These complexes would provide the environment necessary for SS4 to perform its function in the initiation of starch granule production. The localization of SS4 to specific areas of the thylakoidal membrane would restrict the formation of starch granules at these regions of the chloroplast. The restriction of the synthesis of a polymer as big as the starch granule at specific regions of the chloroplast might prevent interference with other metabolic processes of the organelle, such as photosynthesis reactions or the plastid division process. In this regard, it is interesting to note the work of Myers et al. (2011), which shows that altering thylakoid galactolipid composition apparently has an effect on starch granule initiation in maize endosperm. This is an independent line of evidence that thylakoid membrane activities can affect granule formation, and is therefore consistent with the study presented in this work.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions. All *Arabidopsis thaliana* (L) Heynh. mutants used in this work were of the Columbia-0 background. The *fbn1b* (Sail_384_a10) mutant was obtained from the Syngenta *Arabidopsis* Insertion Library (SAIL) (Sessions et al., 2002) and the *fbn1a* (Salk_024528) mutant from the Salk T-DNA Mutants Collection (Alonso et al., 2003). The *fbn1a* and *fbn1b* knock-out

mutants have a T-DNA insertion in exons 1 and 3 respectively. Homozygous knockout plants were selected using PCR-based genotyping (Figure S1a, b). Lines carrying double mutations were obtained by crossing and selecting homozygous double mutant plants from the segregating F_2 populations using PCR-based genotyping. Table S1 describes all the primers used.

Plants were grown in growth cabinets at 23ºC/20ºC day/night, 70% humidity, and under 120 µE m^{-2} s^{-1} irradiance supplied by white fluorescent lamps. All seeds were sown in soil and irrigated with 0.5X MS medium (Murashige and Skoog, 1962).

Plasmid construction. Full-length ORFs of *SS1*, *SS2*, *SS3*, *SS4*, *FBN1a*, *FBN1b*, and the tetratricopeptide repeat-containing protein, as well as the fragments coding for the N- (from 1 to 1695 bp) and C-terminal regions of SS4 (from 1699 to 3123 bp), were cloned (without the stop codon) into the pDONR221 entry vector (Invitrogen) using a BP reaction (Invitrogen). After sequence verification, the inserts were transferred into the binary vectors pEarleyGate103 (for fusion with GFP) (Earley et al., 2006) or pXNGW (-*n*YFP)/ pXCGW (-*c*CFP) (for the BiFC assay) (Yuan et al., 2013) using LR Clonase II (Invitrogen). This resulted in translational fusions between the ORFs and GFP, or the YFP/CFP moieties, driven by the CaMV 35S promoter. The chloroplast transient peptide (CTP) of SS4 was fused to the SS4 C-terminal construct to lead the fusion protein to the chloroplast. The cDNA fragment coding for the CTP was PCR-amplified and a *BamH*I restriction site added at the 3' end of the fragment. Another *BamH*I site was added at the 5' end of the cDNA fragment coding for the C-terminal part of SS4, and both fragments were fused by digestion with *BamH*I and subsequent ligation. The resulting cDNA fragment was

cloned into pDONR221 as described above. The primers used in these steps are listed in Table S2.

For Y2H screening, the cDNA fragments coding for full-length SS4 and its N-terminal (both without the CTP region) and C-terminal regions were amplified from *Arabidopsis* cDNA using the primers listed in Table S2. These primers introduced *Nco*I and *BamH*I restriction sites at the 5' and 3' ends of these fragments respectively. The fragments amplified were cloned into the yeast vector pGBKT7 (Clontech) by restriction with *Nco*I and *BamH*I and subsequent ligation.

For the pull-down assay, a fragment of SS4 cDNA from 619 to 1578 bp of the coding sequence (amino acids 184 to 503), which contains the coiled-coil regions of SS4, was amplified using primers SS4CC_attB1_F and SS4CC_attB2_R (Table S2) and cloned into pDEST15 vector (Invitrogen). This vector allows fusion of the SS4 fragment to the GST protein. The FBN1b cDNA fragment found in the yeast two-hybrid screening (from 95 bp to the stop codon of the coding sequence) was amplified using primers FBN1b_Y2Hclone_F and FBN1b_Y2Hclone_R (Table S2) and cloned into the *E. coli* expression vector pET45b(+) (Novagen).

Transient expression in *N. benthamiana* The corresponding vectors were electroporated into *Agrobacterium tumefaciens* strain C58 (Wood et al., 2001). The saturated overnight bacterial cultures carrying the GFP or YFP/CFP construct moieties were each adjusted to a final $O.D._{600nm}$ of 0.2 and then co-infiltrated with equal amounts of an *Agrobacterium* suspension carrying a p19 suppressor of post-transcriptional gene silencing, following the method of Silhavy et al. (2002). The *Agrobacterium* suspensions were then infiltrated into the leaves of three- to four-week-old *N. benthamiana* plants as previously described (Marillonnet et al., 2005).

The infiltrated plants were kept in a controlled growth chamber under the above conditions for 2 days until analysis by confocal microscopy.

Yeast Two-Hybrid screening. The screening of a commercial, normalized, *Arabidopsis* cDNA library in the yeast expression vector pGADT7-RecAB (Clontech) was performed using the Matchmaker Gold Yeast Two-Hybrid system (Clontech), following the manufacturer's instructions. The baits used in the screening were the full-length SS4 protein, the N-terminal region of SS4 (amino acid residues 43 to 502) (both baits without the CTP), and the C-terminal part of SS4 (amino acid residues 543 to 1040). The cDNA encoding for the respective baits was cloned into the yeast cloning vector pGBKT7 as described above.

Confocal microscopy. A DM6000 confocal laser scanning microscope (Leica Microsystems) equipped with a 63x water-immersion objective was used to examine protein localization by GFP fusion or protein-protein interaction in BiFC assays involving *N. benthamiana* mesophyll cells. GFP or YFP/CFP expression and chlorophyll autofluorescence imaging was performed by excitation with a 488 nm argon laser; fluorescence was detected at 500-525 nm and 630-690 nm respectively.

Chloroplast isolation. 20 g of *Arabidopsis* rosette leaves were disrupted with a tissue mixer in 100 ml of chloroplast isolation buffer (CIB) (300 mM sorbitol, 20 mM Tricine pH 8.4, 10 mM EDTA, 10 mM $NaHCO_3$, 40 mM ascorbic acid, 0.05% bovine serum albumin [BSA] and 1 mM PMSF). The resulting mixture was filtered through Miracloth (100 µm pore) (Millipore. MA, USA) and centrifuged at 1000 x *g* for 10 min at 4ºC. The supernatant was discarded and the pellet resuspended in 1 ml 1xCIB.

The chloroplast-containing extract was loaded into a discontinuous 40-85% Percoll gradient in 1xCIB and centrifuged at 2500 x *g* for 20 min at 4ºC. Intact chloroplasts were recovered at the 40-85% Percoll interface. The chloroplasts were washed three times with 20 ml 1xCIB (the last wash without BSA and ascorbic acid) by centrifugation at 700 x *g* at 4ºC, and resuspended in 0.5-1 ml of 1xCIB without BSA and ascorbic acid.

Analysis of the chloroplast sub-fractions. Chloroplast sub-fractionation was performed according to Block et al. (1983) with some modifications. Thus, isolated chloroplasts (purified from 20 g of rosette leaves as described above) were resuspended in 3 ml lysis buffer (62.5 mM Tris pH 7.5, $MgCl_2$ 2 mM), supplemented with 15 µl protease inhibitor cocktail (SIGMA, P9599), and disrupted in a French press at 20,000 psi. The chloroplast extract was centrifuged at 10,000 x *g* for 10 min at 4ºC and the pellet (containing starch granules) discarded. The supernatant was loaded onto a discontinuous sucrose gradient (0.4-1.0 M) in lysis buffer. This gradient was then centrifuged in an SW41Ti rotor (Beckman) at 90,000 x *g* for 90 min at 4ºC and three fractions collected. The first fraction, above the 0.4 M sucrose layer, corresponded to stroma components; the second fraction, at the interface between the 0.4 and 1 M sucrose layers, corresponded to the chloroplast envelopes; finally, the third fraction was the pellet, which contained the thylakoid membranes. This pellet was resuspended in 500 µl lysis buffer supplemented with 1 µl protease inhibitor cocktail. The protein concentration of each fraction was determined according to the Lowry method (Lowry et al., 1951) in order to ensure the same amount of protein was used in immunoblot analysis of the fractions. For amyloglucosidase treatment, the supernatant resulting from centrifugation of the

chloroplast extracts at 10,000 x *g* was supplemented with 100 mM (final concentration) sodium acetate pH 4.5 and 30 U amyloglucosidase from *Rhizopus* sp. (Megazyme). The supernatant was incubated at 55ºC for 1 h and then loaded onto a discontinuous sucrose gradient as described above.

Immunoblot analysis. Proteins were transferred from an SDS-polyacrylamide gel to a nitrocellulose membrane by electroblotting using a Trans-Blot SD transfer cell (Bio-Rad) according to the manufacturer's instructions. Blots were probed with rabbit anti-SSIV (Roldán et al., 2007) (1:5000 dilution), anti-PsbA (Agrisera) (1:10000 dilution), anti-PRK (Agrisera) (1:1000 dilution) or anti-FBN1a (Agrisera) (1:5000 dilution) followed by a horseradish peroxidase-conjugated goat anti-rabbit IgG serum (Bio-Rad), and detected using ECL Plus Advanced Western Blotting Reagent (GE Healthcare). Chemiluminiscence was visualized using a Chemidoc XRS (Bio-Rad) apparatus running Quantity One software (Bio-Rad).

SSs localization analysis by immunoblotting. Complete coding sequences of SS1, SS2, SS3, and the coding sequences of the N-, and C-terminal regions of SS4, cloned into pEarlyGate 103 vector, were transiently expressed in *Nicotiana benthamiana* leaves by agro-infiltration as described above. Thus, 500 mg of material was harvested 2 days after infiltration and disrupted in liquid nitrogen with a mortar and pestle. Extracts were resuspended in 500 µl PD buffer (20 mM HEPES-KOH, pH 7.6, 80 mM NaCl, 1 mM MgCl2, and 1 mM DTT) supplemented with 2 µl protease inhibitor cocktail (SIGMA). Samples were centrifuged (12,000 x g for 20 min) and the supernatants collected ("Soluble Fraction"). Pellets were washed with 1 ml PD buffer, centrifuged at 12,000 x g for 20 min and the resulting pellets

resuspended in 500 µl PD buffer with 0.2% Triton X-100. The resulting mixtures were filtered through Miracloth (Milipore. MA, USA). These extracts were placed over 1 ml Percoll 90% diluted in PD buffer and centrifuged at 12,000 x g for 45 min. The upper phase ("Membrane Fraction") was collected and starch, which was pelleted, was also isolated and resuspended in 20 µl preloading buffer (50 mM Tris-HCl, pH 6.8 and 2% SDS) and boiled for 10 minutes. The samples were then centrifuged at 12,000 x g for 10 min to eliminate debris and the supernatants, which constitute the "Starch Fractions", isolated. The protein concentration in each sample was determined according to the Lowry method (Lowry et al., 1951). 30 µg of proteins from the Soluble and Membrane Fractions and 2 µg from the Starch Fraction were analyzed by SDS-PAGE and immunoblot using anti-His_5 antibody (QIAGEN).

Bacterial expression and *in vitro* Pull-Down assay. The FBN1b cDNA fragment cloned into pET45b(+), SS4 cDNA fragment coding for the coiled-coil region (residues 184 to 503) cloned into pDEST15, and plasmid pGEX-4T2, which allows the expression of GST protein, were transformed into *E. coli* BL21 strain (Invitrogen). Five ml overnight cultures were transferred to 50 ml Luria-Bertani (LB) medium containing 100 µg/ml ampicillin and incubated at 37°C to OD_{600} 0.5. The recombinant proteins were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 28 °C for 12 h. Bacteria were harvested by centrifugation (12,000 x g for 10 min) and washed with 2 ml 50 mM HEPES-NaOH, pH 7.5. After centrifugation (12,000 x g for 10 min), pellets were resuspended in 2 ml PD buffer (20 mM HEPES-KOH, pH 7.6, 80 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, 0.1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM DTT). Bacterial cells were lysed using a French press (10,000 psi), the extract was centrifuged at 12,000 x g for 10 min and the pellet discarded.

100 µg of proteins from each extract were used in the Pull-Down assays. Extracts containing the coiled-coil region of SS4 fused to GST or GST alone were incubated with N-terminal His_5-tagged FBN1b fragment and 20 µl 50% Glutathione Sepharose 4B (GE healthcare) in PD buffer at 4°C under gentle rotation. After 2 h, the Sepharose beads were washed three times with 1 ml PD buffer. After the final wash, the beads were resuspended with 20 µl 2x loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol), boiled for 5 min, and pelleted. The supernatant was subjected to immunoblot analysis to detect the bound protein using anti-His_5 antibody (Qiagen).

Starch determination. The starch content of the *Arabidopsis* leaves was determined as described by Szydlowski et al. (2009).

Zymogram of starch synthases. The starch synthases activities in native polyacrylamide gel were detected according to the method described by Delvallé et al. (2005).

Electron microscopy analysis. Fully expanded leaves from plants cultured under a 16 h light/8 h dark photoperiod were collected at the indicated times. Small pieces (2 mm^2) of leaves were cut with a razor blade and immediately fixed in 3% glutaraldehyde in 0.05 M Na-cacodylate buffer, pH 7.4, and embedded in Araldite Durcupan ACM, as described by Lucas et al. (1998). Ultrathin (60 nm) sections were cut with a Leika Ultracut microtome (Vienna, Austria) fitted with a diamond knife. Sections were contrasted with 2% aqueous uranyl acetate and lead citrate (Reynolds, 1963). Observations were performed using a STEM LEO 910 electron

microscope (Oberkochen, Germany) at 80 kV, equipped with a Gatan Bioscan 792 digital camera (Pleasanton, CA, USA). Different sections from at least three different leaf samples were analyzed.

Real-time RT-PCR. The isolation of total RNA from the *Arabidopsis* leaves was performed using TRIsure reagent (Bioline Ltd., London, UK) following the manufacturer's instructions. Single-stranded cDNA was synthesized from total RNA using the Quantitec Reverse Transcription kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Quantitative real-time PCR (QRT-PCR) assays were performed using an iCycler (Bio-Rad). The PCR mixture, contained in a total volume of 25 µl, included 5 µl cDNA, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, a 1:100,000 dilution of SYBR Green I nucleic gel stain (Molecular Probes, Eugene, OR)/fluorescein calibration dye (Bio-Rad), 0.3 units of Taq polymerase, 2.5 µl 10x Taq polymerase buffer, and 0.2 µM of each primer. The specific oligonucleotides used are listed in Table S3.

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

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SUPPORTING INFOMATION

Table S1. Primers used in the genotyping of *fbn1a*, *fbn1b* and *fbn1a/fbn1b* mutants.

Table S2. Primers used in plasmid construction

Table S3. Primers used in QRT-PCR analysis

Figure S1. Identification of single and double *fbn1a*, *fbn1b* mutants

Figure S2. SDS-PAGE analysis of *in vitro* pull-down assays of $SS4_{184\text{-}503}$-GST and FBN1b proteins

Figure S3. Time-course of starch content over a day in *Arabidopsis* wild-type and *fbn1a/fb1nb* plants.

Figure S4. mRNA levels of starch metabolism genes in *fbn1a/fbn1b* mutants.

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FIGURE LEGENDS

Figure 1. Localization of SS4 in the thylakoid fraction. **a**. Chloroplasts isolated from *Arabidopsis* rosette leaves harvested at the end of the day were disrupted and the stroma, chloroplast envelopes, and thylakoid fractions separated by ultracentrifugation in a discontinuous sucrose gradient. The different fractions were analysed by immunoblotting using antibodies specific to the markers of each compartment. S: stromal fraction; E: chloroplast envelope fraction; T: thylakoid fraction; PRK: phosphoribulokinase. **b**. Localization of SS4 in crude plastid extracts

treated with amyloglucosidase. Chloroplasts isolated from rosette leaves harvested at the end of the day were disrupted and the crude extract treated with 30 units of amyloglucosidase for 1 h. The stroma and thylakoid membrane fractions were separated by ultracentrifugation in a discontinuous sucrose gradient. The different fractions were analysed by immunoblotting using antibodies specific to SS4. Tc: thylakoid fraction of a control, untreated, chloroplast crude extract; Tt: thylakoid fraction of extracts treated with amyloglucosidase; St: stromal fraction of extracts treated with amyloglucosidase.

The bands corresponding to SS4 are indicated with asterisks.

Figure 2. Localization of different SS4 constructs fused to GFP in *Nicotiana benthamiana* chloroplasts. cDNA coding for the full-length SS4 protein (a), its N-terminal region (b) and its C-terminal region (c) was fused to GFP protein and transiently expressed in *N. benthamiana* leaves. Images are combinations of chlorophyll autofluorescence and GFP fluorescence images. The different constructs are schemed. The black box indicates the chloroplast transit peptide. Dark gray boxes indicate the predicted coiled-coil regions in the N-terminal part of SS4. Light gray boxes indicate the C-terminal domain of SS4. Numbers indicate the amino acid positions.

Figure 3. Localization of SS1, SS2 and SS3 in *Nicotiana benthamiana* chloroplasts. cDNAs coding for the full-length SS1, SS2 and SS3 proteins were fused to GFP and transiently expressed in *Nicotiana benthamiana* leaves. Localization of the different constructs was monitored by confocal microscopy. **a**, **b** and **c**: GFP signal, chlorophyll autofluorescence and merged images, respectively,

for the SS1-GFP construct. **d**, **e** and **f**: SS2-GFP construct. **g**, **h** and **i**: SS3-GFP construct. **j**, **k** and **l**: Magnification of Panels g, h and i respectively

Figure 4. Distribution of starch synthases in the different chloroplastic compartments. cDNAs coding for the full-length SS1, SS2, SS3, and the cDNA fragments coding for the N- and C-terminal part of SS4 were cloned into the pEarlyGate 103 vector and transiently expressed in *Nicotiana benthamiana*. The different fractions (Soluble (S), Membranes (M) and Starch (St)) were analysed by SDS-PAGE and immunoblot using anti-His_5 antibody.

Figure 5. Interaction between SS4 and FBN1b. cDNAs coding for the full-length SS4 protein (**a**, **b** and **c**), its N-terminal region (**d**, **e**, and **f**), and its C-terminal region (**g**, **h** and **i**), were fused to the N-terminal half of YFP and co-transformed into *N. benthamiana* leaves together with cDNA coding for FBN1b fused to the C-terminal moiety of CFP. **a**, **d** and **g** show YFP/CFP fluorescence, **b**, **e** and **h** chlorophyll autofluorescence. **c**, **f** and **i** are merged images of **a** and **b**, **d** and **e**, and **g** and **h**, respectively

Figure 6. Interaction between SS4 and FBN1a. cDNAs coding for the full-length SS4 protein (a, b and c) and its N-terminal region (d, e, and f) were fused to the N-terminal half of YFP and co-transformed in *N. benthamiana* leaves together with the cDNA coding for FBN1a fused to the C-terminal moiety of CFP. **a** and **d** correspond to YFP/CFP fluorescence, and **b** and **e** to chlorophyll autofluorescence. **c** and **f** are merged images of **a** and **b**, and **d** and **e**, respectively.

Figure 7. Localization of SS4 in the thylakoids of wild-type and *fbn1a/fbn1b* plants. Chloroplasts isolated from rosette leaves of wild-type and *fbn1a/fbn1b* mutant plants harvested at midday were disrupted and the stroma and membrane fractions separated by ultracentrifugation in a discontinuous sucrose gradient. The different fractions were analysed by immunoblotting using antibodies specific to markers for each compartment. S: stromal fraction; T: thylakoid fraction; PRK: phosphoribulokinase. The positions of the different proteins are indicated by asterisks

Figure 8. Transmission electron microscopy analysis of leaf sections from *fbn1a/fbn1b* mutant and wild-type plants. Leaves from plants cultured under a 16 h light/8 h dark photoperiod were collected at midday and subsequently fixed, embedded and sectioned as described in Experimental Procedures. **a** and **c**: WT plants; **b** and **d**: *fbn1a/fbn1b* mutant leaves.