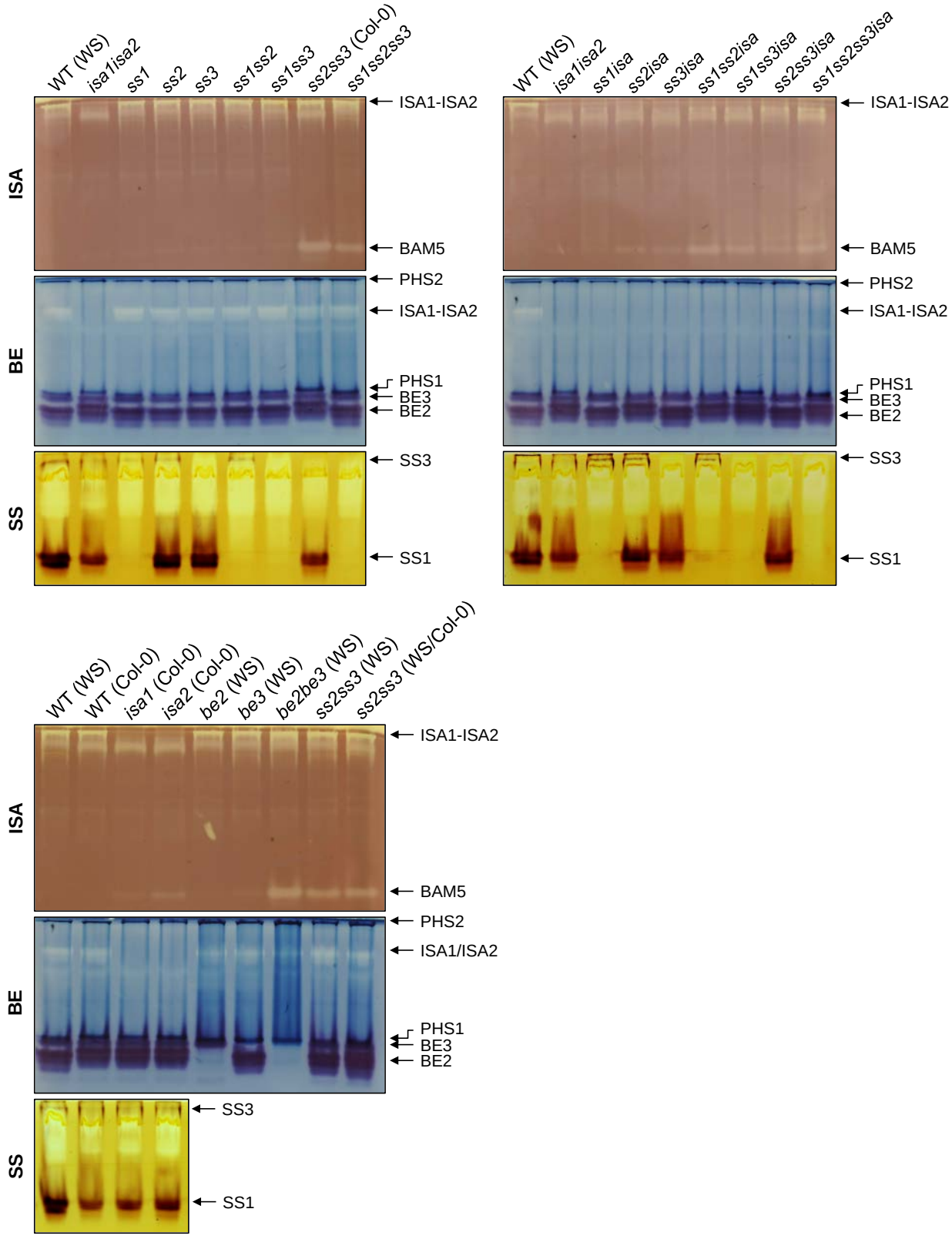
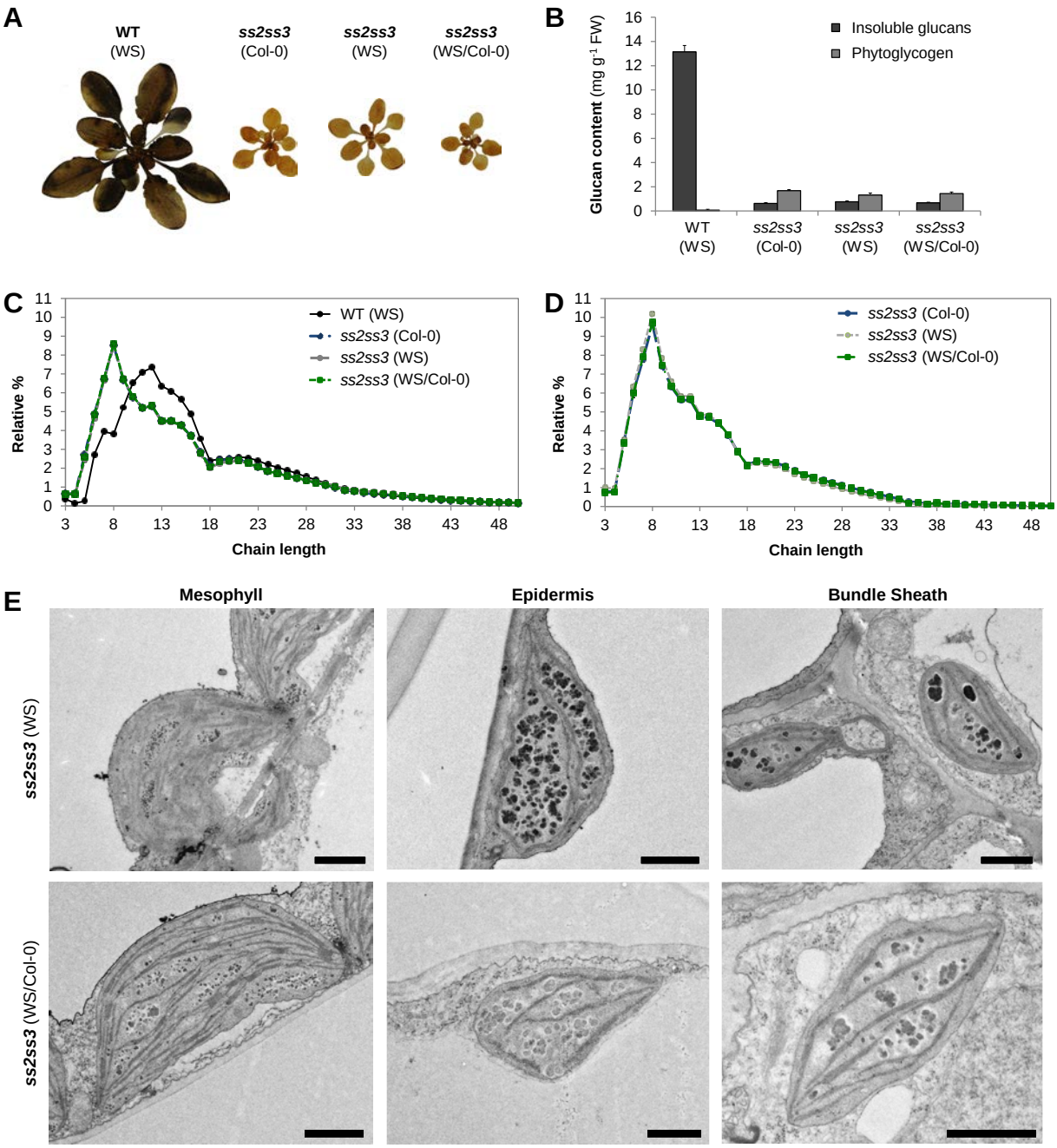

WT (WS)
isa1isa2
ss1
ss2
ss3
ss1ss2
ss1ss3
ss2ss3 (Col-0)
ss1ss2ss3
ISA
BE
SS
ISA1-ISA2
BAM5
PHS2
ISA1-ISA2
PHS1
BE3
BE2
SS3
SS1
WT (WS)
isa1isa2
ss1isa
ss2isa
ss3isa
ss1ss2isa
ss1ss3isa
ss2ss3isa
ss1ss2ss3isa
ISA1-ISA2
BAM5
PHS2
ISA1-ISA2
PHS1
BE3
BE2
SS3
SS1
WT (WS)
WT (Col-0)
isa1 (Col-0)
isa2 (Col-0)
be2 (WS)
be3 (WS)
be2be3 (WS)
ss2ss3 (WS)
ss2ss3 (WS/Col-0)
ISA
BE
SS
ISA1-ISA2
BAM5
PHS2
ISA1/ISA2
PHS1
BE3
BE2
SS3
SS1

Supplemental Figure S1. Native PAGE of *ssisa* Mutant Extracts.

Proteins extracted from the indicated lines were separated by native PAGE with different substrates for hydrolytic activity (upper panels), branching activity (middle panels) or synthase activity (lower panels)(see Materials and Methods). Note that BE2 and BE3 migrate slightly differently depending on the ecotype background, with BE2 and BE3 from WS being slightly faster. In addition, SS1 activity appears stronger in WS than in Col-0. PHS1 and PHS2 are the plastidial and cytosolic isoforms of phosphorylase, respectively (Zeeman et al., 2004).


A
WT
(WS)
ss2ss3
(Col-0)
ss2ss3
(WS)
ss2ss3
(WS/Col-0)
B
Glucan content (mg g-1 FW)
Insoluble glucans
Phytoglycogen
WT (WS)
ss2ss3 (Col-0)
ss2ss3 (WS)
ss2ss3 (WS/Col-0)
C
Relative %
Chain length
WT (WS)
ss2ss3 (Col-0)
ss2ss3 (WS)
ss2ss3 (WS/Col-0)
D
Relative %
Chain length
ss2ss3 (Col-0)
ss2ss3 (WS)
ss2ss3 (WS/Col-0)
E
Mesophyll
Epidermis
Bundle Sheath
ss2ss3 (WS)
ss2ss3 (WS/Col-0)

Supplemental Figure S2. Three *ss2ss3* Lines Have Identical Phenotypes.

The three *ss2ss3* lines have different ecotype backgrounds (see Supplemental Table S2).

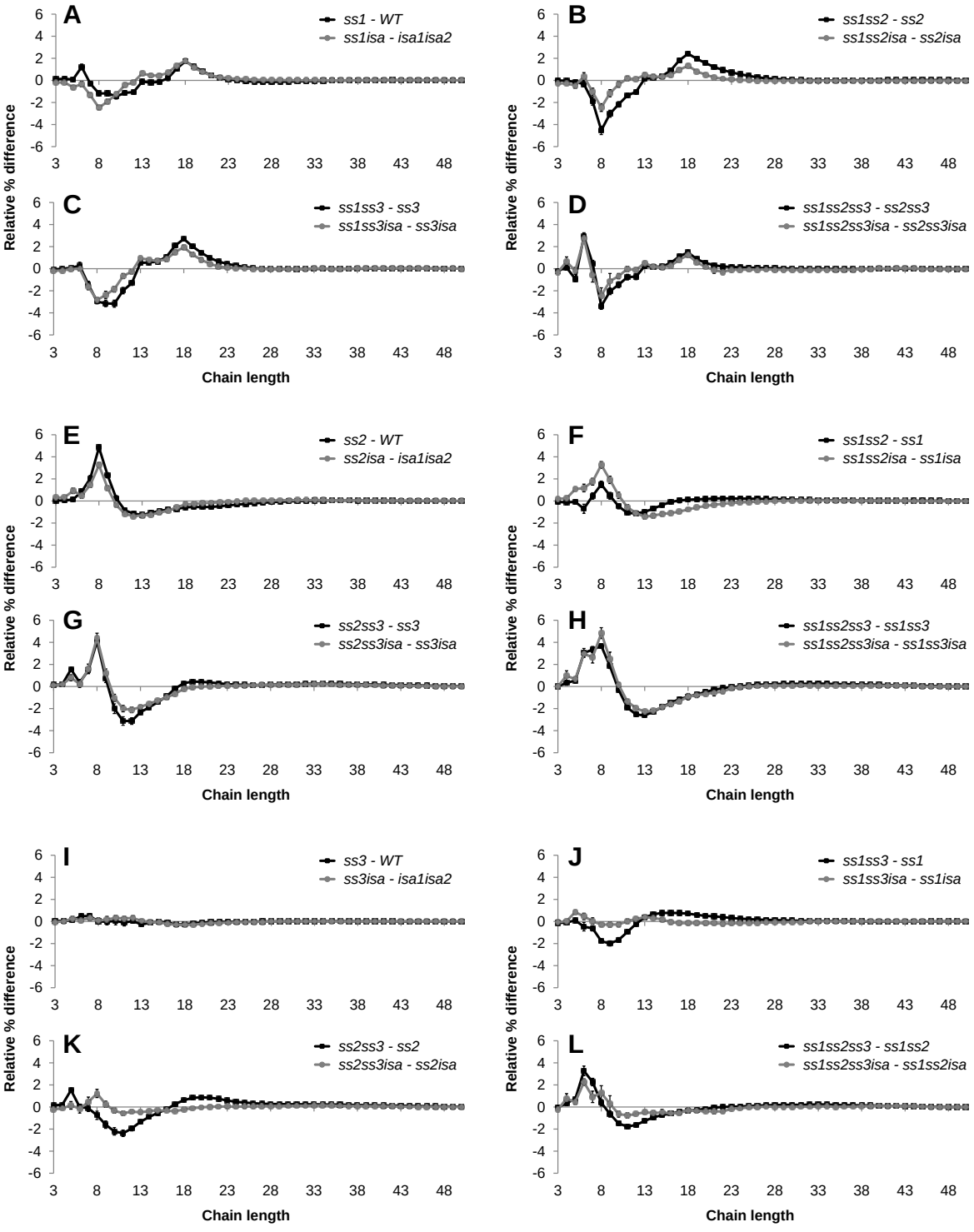
(A) Iodine staining of whole rosettes harvested at the end of the day.

(B) Insoluble and soluble glucan content at the end of the day (measured as described in Figure 2). Values are means ± SE (n=4 for W and n=5 for the *ss2ss3* lines).

(C) Chain length distributions from insoluble glucans (obtained as described in Figure 3). Shown are means ± SE (n=4 for WT and *ss2ss3* WS, n=5 for the *ss2ss3* Col-0 and *ss2ss3* WS/Col-0).

(D) Chain length distributions of soluble glucans (obtained as described in Figure 4). Values are means ± SE (n=4 for WT and n=5 for the *ss2ss3* lines).

(E) Transmission electron micrographs (obtained as described in Figure 7) of the plastids in the indicated lines and leaf cell types. Bars = 1 μm.


A
ss1 - WT
ss1isa - isa1isa2
B
ss1ss2 - ss2
ss1ss2isa - ss2isa
C
ss1ss3 - ss3
ss1ss3isa - ss3isa
D
ss1ss2ss3 - ss2ss3
ss1ss2ss3isa - ss2ss3isa
E
ss2 - WT
ss2isa - isa1isa2
F
ss1ss2 - ss1
ss1ss2isa - ss1isa
G
ss2ss3 - ss3
ss2ss3isa - ss3isa
H
ss1ss2ss3 - ss1ss3
ss1ss2ss3isa - ss1ss3isa
I
ss3 - WT
ss3isa - isa1isa2
J
ss1ss3 - ss1
ss1ss3isa - ss1isa
K
ss2ss3 - ss2
ss2ss3isa - ss2isa
L
ss1ss2ss3 - ss1ss2
ss1ss2ss3isa - ss1ss2isa
Relative % difference
Chain length
6
4
2
0
-2
-4
-6
3
8
13
18
23
28
33
38
43
48

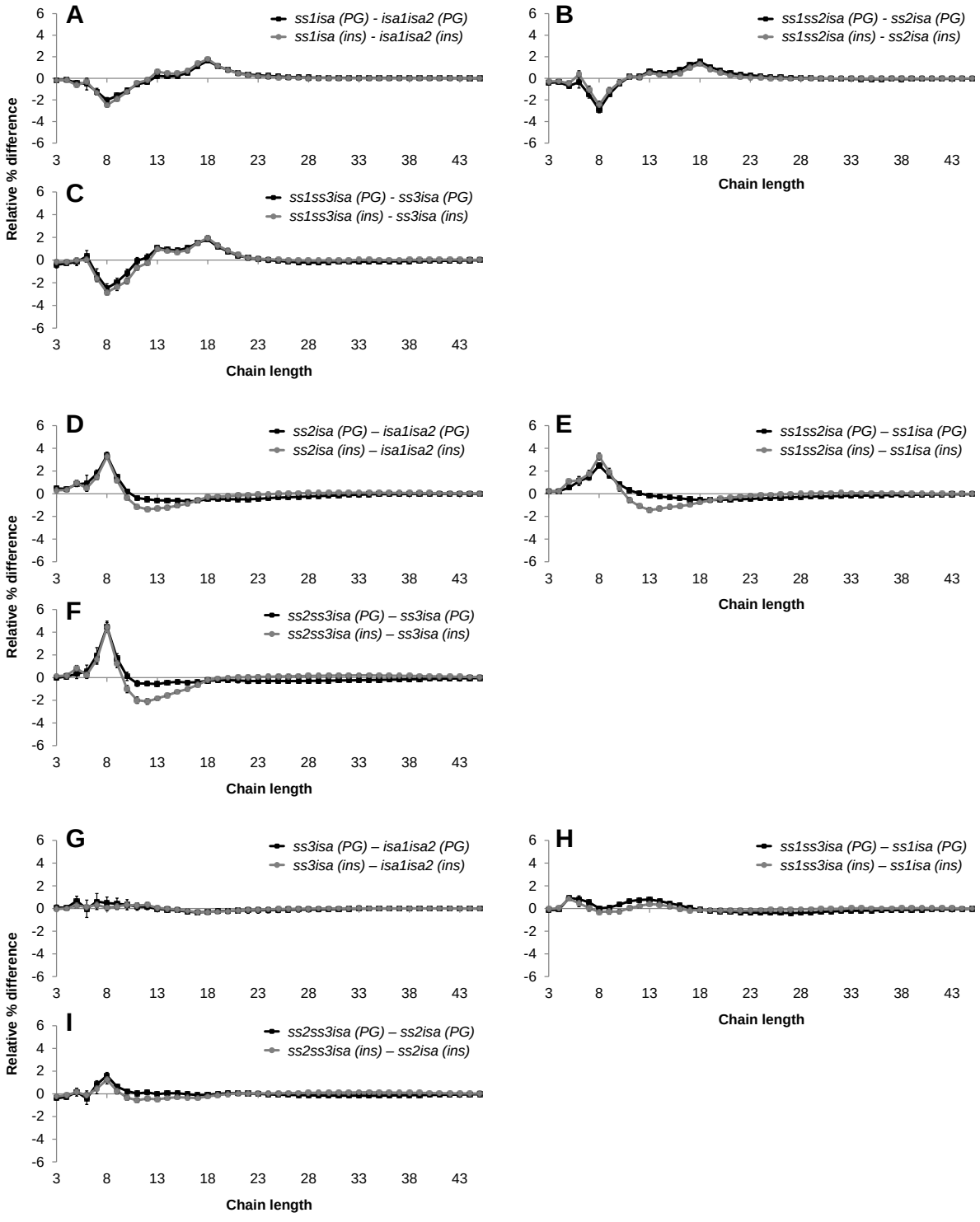
Supplemental Figure S3. The Loss of Individual Starch Synthases Results in Distinct Chain Length Distribution Patterns.

Average CLDs of insoluble glucans (obtained as in Figure 3) from one genotype was subtracted from the average CLD of another genotype to visualize the differences between them; i.e. in "*ss1* – WT" the CLD of WT was subtracted from the CLD of the *ss1* mutant, revealing the effect of loss of SS1 activity. Error bars show the summed standard errors of the lines compared. WT, WS wild type;

(A-D) Loss of SS1 results in a similar pattern in all available backgrounds: in all cases, chains from DP 6/7 to 12/13 are decreased (with the minimum mostly at DP 8) and chains from DP 13 to approximately DP 23 are increased (with the maximum at DP 18). This suggests that SS1 is involved in producing short chains from DP 6 to 12.

(E-H) Loss of SS2 results in an increase of chains around DP 8 and in a decrease of chains from DP 10/11 to approximately DP 18. This suggests that SS2 preferentially elongates chains of approximately 8 glucose units to chains of around 11 to 18 units.

(I-L) For SS3, the situation is more complex. Lack of SS3 in the presence of all other starch synthases does not result in a change of CLD (I). If SS1 or SS2 are absent as well, an effect of loss of SS3 is visible, but the nature of the changes depends on the background. For example, *ss1ss3* has even fewer short chains (DP 7-12) than *ss1*, indicating a partial overlap between SS1 and SS3 in production of these short chains. This effect, however, is not obvious when ISA activity is also missing (i.e. in *ss1ss3isa – ss1isa*). In the *ss2* or *ss2isa* background, additional lack of SS3 enhances the phenotype of *ss2*, suggesting redundant functions here as well (see *ss2ss3 – ss2* and *ss2ss3isa – ss2isa*; also compare Figure 3E and 3F). Thus, when considering these chain lengths, the role of SS3 seems to overlap with the functions of both SS1 and SS2.


A
ss1isa (PG) - isa1isa2 (PG)
ss1isa (ins) - isa1isa2 (ins)
B
ss1ss2isa (PG) - ss2isa (PG)
ss1ss2isa (ins) - ss2isa (ins)
C
ss1ss3isa (PG) - ss3isa (PG)
ss1ss3isa (ins) - ss3isa (ins)
D
ss2isa (PG) – isa1isa2 (PG)
ss2isa (ins) – isa1isa2 (ins)
E
ss1ss2isa (PG) – ss1isa (PG)
ss1ss2isa (ins) – ss1isa (ins)
F
ss2ss3isa (PG) – ss3isa (PG)
ss2ss3isa (ins) – ss3isa (ins)
G
ss3isa (PG) – isa1isa2 (PG)
ss3isa (ins) – isa1isa2 (ins)
H
ss1ss3isa (PG) – ss1isa (PG)
ss1ss3isa (ins) – ss1isa (ins)
I
ss2ss3isa (PG) – ss2isa (PG)
ss2ss3isa (ins) – ss2isa (ins)
Relative % difference
Chain length
6
4
2
0
-2
-4
-6
3
8
13
18
23
28
33
38
43

Supplemental Figure S4. Effects of Losing SS1, SS2 or SS3 on Chain Length Distributions are Similar in Phytoglycogen and Insoluble Glucans.

Average CLDs of phytoglycogen (*PG*, shown as black plots and obtained as described in Figure 4) from one genotype was subtracted from the average CLD of another genotype to visualize the difference between them. The corresponding data from insoluble glucan (*ins*, shown as grey plots and obtained as described in Supplemental Figure 3 online) are shown for direct comparison. Upon loss of SS1 (A-C), SS2 (D-F) or SS3 (G-I), respectively, the patterns from phytoglycogen and insoluble glucans are similar, indicating that these starch synthases act in a similar manner on both glucans.

Supplemental Methods

Analysis of Enzymatic Activity by Native PAGE

Rosette material from five 24-day old plants per genotype was harvested at the middle of a 12-h day, pooled and frozen in liquid N_2. Proteins were extracted using an all-glass homogenizer in 100 mM Tris-HCl, pH 7.0, 1 mM EDTA, 5 mM DTT, 10% (v/v) glycerol containing protease inhibitors (Complete EDTA-free; Roche). For isoamylase activity, 25 µg proteins were loaded on a 7.5% native polyacrylamide gel containing 0.1% potato amylopectin (Sigma-Aldrich) and run at 10 V cm^{-1} for 3 h at 4°C. The gel was washed briefly in 100 mM Tris HCl, pH 7.0, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM DTT and then incubated in the same buffer for 2 h at 37°C.
For branching enzyme activity, 25 µg of proteins were separated on a 7.5% native polyacrylamide gel containing 0.02% oyster glycogen (Sigma-Aldrich) and run at 10 V cm^{-1} for 3 h at 4°C. The gels were washed for 30 min at 4°C in 50 mM Hepes-NaOH pH 7.0 and 10% (v/v) glycerol, then incubated overnight at 20°C with gentle shaking in 50 mM Hepes-NaOH, 10% (v/v) glycerol, 2.5 mM AMP (Sigma-Aldrich), 50 mM glucose 1-phosphate and 28 U (per gel) phosphorylase *a* (from rabbit muscle, Sigma-Aldrich).
For starch synthase activity, 70 µg of proteins were loaded on a 7.5% native polyacrylamide gel containing 0.3% oyster glycogen (Sigma-Aldrich) and separated at 10 V cm^{-1} for 2 h at 4°C. The gels were incubated overnight at 20°C with gentle shaking in 100 mM HEPES-NaOH pH 7.5, 2 mM DTT, 10% (v/v) glycerol, 0.5 mM EDTA, 0.5 M trisodium citrate and 1.5 mM ADP-glucose. In all cases, protein activity was visualized by staining the gels with Lugol's solution.

Supplemental Literature Cited

Zeeman SC, Thorneycroft D, Schupp N, Chapple A, Weck M, Dunstan H, Haldimann P, Bechtold N, Smith AM, Smith SM (2004) Plastidial α-glucan phosphorylase is not required for starch degradation in Arabidopsis leaves but has a role in the tolerance of abiotic stress. Plant Physiol **135**: 849–858

Supplemental Table S1. Starch Synthase and Debranching Enzyme Genes, Respective Mutant Alleles, and Primers Used for their Identification.WS, Wassilewskija; Col-0, Columbia-0. To amplify the wild-type allele, the forward (fwd) and reverse (rev) primers were used. To amplify the mutant allele, the primers in bold were used.

Gene AGI code	**Mutation type, position**	**Mutant allele, Line identifier (other name)**	**Primers used to select mutant alleles (shown 5' to 3')**	**Ecotype**	**Reference**
SS1 At5g24300	T-DNA Insertion in intron 1	*ss1*-1, Genoplante_203C08	**Fwd**: TTTCCGTCCGATCGCCAGTCTC Rev: TACGCCAAAGTCAGCCATTACAA **T-DNA**: CTACAAATTGCCTTTTCTTATCGAC	WS	Delvallé et al., 2005
SS2 At3g01180	T-DNA Insertion in exon 8	*ss2*-3, Genoplante_549A11	Fwd: CCTAGTGGTGGAAAATTAGGGG **Rev**: AACCGAGAATCCAACCCATC **T-DNA**: CTACAAATTGCCTTTTCTTATCGAC	WS	Zhang et al., 2008
	T-DNA Insertion in exon 2	*ss2*-1, Salk_065639	Fwd: GTTTCTTACCATGATTTGCCTTCTG **Rev**: GCTGCTACCAATATCACATTCATGAC **T-DNA**: TGGTTCACGTAGTGGGCCATCG	Col-0	Zhang et al., 2008
SS3 At1g11720	T-DNA Insertion in exon 12	*ss3*-3, Genoplante_117H05	**Fwd**: TTACGCGCTTAACACACCAGAAG Rev: ATTCATCTTAGAGCTTCCATTTTA **T-DNA**: CTACAAATTGCCTTTTCTTATCGAC	WS	Szydlowski et al., 2009
	T-DNA Insertion in exon 13	*ss3*-1, SALK_065732	Fwd: TTACGCGCTTAACACACCAGAAG **Rev**: TCTTGCTCCATCACCGTCTT **T-DNA**: TGGTTCACGTAGTGGGCCATCG	Col-0	Zhang et al., 2005
ISA1 At2g39930	T-DNA Insertion in exon 13	*isa1*-1, SALK_042704	**Fwd**: GGGACAGCCTATGTGATCTGCC Rev: TGGGAAACCATGAGGGAAACA **T-DNA**: GCGTGGACCGCTTGCTGCAACT	Col-0	Delatte et al., 2005
ISA2 At1g03310	X-ray Single base pair deletion in exon 1	*isa2*-1 (*dbe1*-1)	Fwd: GGTGACGTATTTACCGATGGA Rev: TGACACTTTGAGCAGCAACC The *isa2*-1 amplicon is cut by *Nla*IV	Col-0	Delatte et al., 2005
BE2 At5g03650	T-DNA Insertion in intron 19	*be2-1* Genoplante_347B03	Fwd: TGAAGACTGGCAAATGGGCGACA **Rev:** CCATTTCCAAGACCGGCCAGC **T-DNA:** CTGATACCAGACGTTGCCCGCATAA	WS	Dumez et al., 2006
BE3 At2g36390	T-DNA Insertion in exon 15	*be3-2* Genoplante_456C03	Fwd: TCGCACCTAGCAGCCGCTTT **Rev:** CGGTCTACACGCGGAGTGGC **T-DNA:** CTACAAATTGCCTTTTCTTATCGAC	WS	Dumez et al., 2006

Supplemental Table S2. Genotypes and Ecotypes of the Mutant Lines Used in this Study. WS, Wassilewskija; Col-0, Columbia-0. Lines with WS/Col-0 background were obtained from the cross between *ss1ss2ss3* (WS) with *isa1isa2* (Col-0).

Mutant name	Genotype	Underlying mutant alleles	Ecotype
isa1	*isa1*	*isa1*-1	Col-0
isa2	*isa2*	*isa2*-1	Col-0
isa1isa2	*isa1isa2*	*isa1*-1*; isa2*-1	Col-0
ss1	*ss1*	*ss1*-1	WS
ss2	*ss2*	*ss2*-3	WS
ss3	*ss3*	*ss3*-3	WS
ss1ss2	*ss1ss2*	*ss1*-1*; ss2*-3	WS
ss1ss3	*ss1ss3*	*ss1*-1*; ss3*-3	WS
ss2ss3 or *ss2ss3* (Col-0)	*ss2ss3*	*ss2*-1; *ss3*-1	Col-0
ss2ss3 (WS)	*ss2ss3*	*ss2*-3*; ss3*-3	WS
ss2ss3 (WS/Col-0)	*ss2ss3*	*ss2*-3*; ss3*-3	WS/Col-0
ss1ss2ss3	*ss1ss2ss3*	*ss1*-1*; ss2*-3; *ss3*-3	WS
ss1isa	*ss1isa1isa2*	*ss1*-1*; isa1*-1; *isa2*-1	WS/Col-0
ss2isa	*ss2isa1isa2*	*ss2*-3; *isa1*-1; *isa2*-1	WS/Col-0
ss3isa	*ss3isa1*	*ss3-3*; *isa1*-1	WS/Col-0
ss1ss2isa	*ss1ss2isa1isa2*	*ss1*-1*; ss2*-3; *isa1*-1; *isa2*-1	WS/Col-0
ss1ss3isa	*ss1ss3isa1*	*ss1*-1*; ss3-3*; *isa1*-1	WS/Col-0
ss2ss3isa	*ss2ss3isa1*	*ss2*-3; *ss3-3*; *isa1*-1	WS/Col-0
ss1ss2ss3isa	*ss1ss2ss3isa1*	*ss1*-1*; ss2*-3; *ss3*-3; *isa1*-1	WS/Col-0
be2	*be2*	*be2*-1	WS
be3	*be3*	*be3*-2	WS
be2be3	*be2be3*	*be2*-1;*be3*-1	WS

Supplemental Table S3. Starch and Phytoglycogen Content of *ssisa* Mutants at the End of the Night. Glucans were measured as described in Figure 2. Values are means ± S.E. (n=4). WS, Wassilewskija; Col-0, Columbia-0; FW, fresh weight.

Genotype	**Starch content (mg g^{-1} FW)**	**Phytoglycogen content (mg g^{-1} FW)**
Wild type (WS)	0.79 ± 0.09	0.00 ± 0.00
Wild type (Col-0)	1.09 ± 0.23	0.00 ± 0.00
isa1	0.13 ± 0.05	0.00 ± 0.00
isa2	0.16 ± 0.03	0.00 ± 0.00
isa1isa2	0.60 ± 0.42	0.00 ± 0.00
ss1	1.37 ± 0.02	0.00 ± 0.00
ss2	1.38 ± 0.19	0.01 ± 0.01
ss3	0.72 ± 0.12	0.00 ± 0.00
ss1ss2	1.97 ± 0.16	0.00 ± 0.00
ss1ss3	0.29 ± 0.06	0.00 ± 0.00
ss2ss3	0.03 ± 0.02	0.00 ± 0.00
ss1ss2ss3	0.01 ± 0.00	0.00 ± 0.00
ss1isa	0.39 ± 0.08	0.00 ± 0.00
ss2isa	0.01 ± 0.01	0.01 ± 0.01
ss3isa	0.15 ± 0.04	0.00 ± 0.00
ss1ss2isa	0.00 ± 0.01	0.00 ± 0.00
ss1ss3isa	0.03 ± 0.03	0.00 ± 0.00
ss2ss3isa	0.00 ± 0.00	0.00 ± 0.00
ss1ss2ss3isa	0.00 ± 0.00	0.00 ± 0.00