Starch Synthase 4 and Plastidal Phosphorylase Differentially Affect Starch Granule Number and Morphology[1]

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The process of starch granule formation in leaves of Arabidopsis (*Arabidopsis thaliana*) is obscure. Besides STARCH SYNTHASE4 (SS4), the PLASTIDIAL PHOSPHORYLASE (PHS1) also seems to be involved, since *dpe2-1/phs1a* double mutants lacking both PHS1 and the cytosolic DISPROPORTIONATING ENZYME2 (DPE2) displayed only one starch granule per chloroplast under normal growth conditions. For further studies, a *dpe2-1/phs1a/ss4* triple mutant and various combinations of double mutants were generated and metabolically analyzed with a focus on starch metabolism. The *dpe2-1/phs1a/ss4* mutant revealed a massive starch excess phenotype. Furthermore, these plants grown under 12 h of light/12 h of dark harbored a single large and spherical starch granule per plastid. The number of starch granules was constant when the light/dark regime was altered, but this was not observed in the parental lines. With regard to growth, photosynthetic parameters, and metabolic analyses, the triple mutant additionally displayed alterations in comparison with *ss4* and *dpe2-1/phs1a*. The results clearly illustrate that PHS1 and SS4 are differently involved in starch granule formation and do not act in series. However, SS4 appears to exert a stronger influence. In connection with the characterized double mutants, we discuss the generation of starch granules and the observed formation of spherical starch granules.

Starch plays an important role in the plant life cycle. The process of starch granule formation, however, remains obscure. Neither the physicochemical mechanisms of starch granule formation nor all necessary proteins/enzymes have been identified. However, for the synthesis of amylopectin as well as amylose, a carbohydrate primer seems necessary. The strongest alteration in starch granule formation in Arabidopsis (*Arabidopsis thaliana*) observed so far occurs when plants lack an isoform of soluble starch synthase, STARCH SYNTHASE4 (SS4; Roldán et al., 2007). This mutant contains, in most cases, only one (or two) starch granule(s) per chloroplast. Null mutants of the other soluble starch synthases (*ss1*, *ss2*, and *ss3*) revealed no effect on starch granule number (Roldán et al., 2007). Thus, SS4 may have a specific function in starch granule formation. Interestingly, immature leaves of *ss4* have no starch granules but accumulate large amounts of ADPglucose (Crumpton-Taylor et al., 2013; Ragel et al., 2013). The accumulation of ADPglucose was proposed to sequester a large part of the chloroplastidial adenine nucleotide pool, resulting in limited photophosphorylation and reduced growth (Crumpton-Taylor et al., 2013; Ragel et al., 2013).

Starch degradation is initiated by a phosphorylation/dephosphorylation cycle at the starch granule surface (Hejazi et al., 2012; Mahlow et al., 2016). Subsequently, starch is degraded predominantly by β-amylolytic degradation, resulting in the formation of maltose that is then transported into the cytosol by the MALTOSE EXCESS1 (MEX1) transporter (Fulton et al., 2008; Nittylä et al., 2004). In addition to this main pathway, the transport of minor amounts of Glc and Glc phosphates into the cytosol during starch degradation has been reported (Weber et al., 2000; Kunz et al., 2010; Fettke et al., 2011a). In the cytosol, the exported maltose is metabolized by DISPROPORTIONATING ENZYME2 (DPE2; Chia et al., 2004; Lu and Sharkey, 2004; Fettke et al., 2006), transferring the nonreducing glucosyl residue of maltose to a cytosolic heteroglycan (Fettke et al., 2007; Fettke and Fernie, 2015) and releasing the other moiety as free Glc.

Surprisingly, analyses of a double mutant lacking DPE2 and PHS1 point to the importance of another glucan-synthesizing enzyme, the PLASTIDIAL

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PHOSPHORYLASE (AtPHS1) (Zeeman et al., 2004), during starch granule formation. The double mutant has an uneven distribution of starch, with starch being restricted to young leaves (Malinova et al., 2014). A more detailed analysis of this mutant revealed that each chloroplast of young leaves contains only one starch granule per chloroplast, in contrast to wild-type plants, which typically contain five to seven granules per chloroplast. No starch granules were detectable in mature leaves, and the chloroplasts were disintegrated (Malinova et al., 2014). It was concluded that the formation of starch granules in *dpe2-1/phs1a* is controlled by an as yet unidentified signal related to the dark phase and located at least partly in the cytosol (Malinova et al., 2014).

Taken together, both PHS1 and SS4 appear, at least under certain conditions, to be involved in starch granule initiation. However, the precise role of each enzyme in this process is unclear. In order to analyze their roles in more detail, a *dpe2-1/phs1a/ss4* triple mutant as well as the corresponding *dpe2-1/ss4* and *phs1a/ss4* double mutants were generated and characterized.

RESULTS

Additional Elimination of SS4 in the *dpe2-1/phs1a* Background Causes the Recovery of a Starch Excess Phenotype

Constitutive triple knockout Arabidopsis mutants, lacking DPE2, PHS1, and SS4 (designated as *dpe2-1/phs1a/ss4*), were generated by crossing the *dpe2-1/phs1a* double knockout mutant (Malinova et al., 2014) and the single knockout mutant lacking SS4 (Roldán et al., 2007). The absence of DPE2 and PHS1 activity was confirmed by zymograms, whereas the lack of SS4 was confirmed by SDS-PAGE and western blotting using antibody directed against SS4 (Fig. 1A).

When plants were cultivated in soil under a 12-h-light/12-h-dark regime, the macroscopic features of the various mutants differed. *dpe2-1* and *ss4* had smaller shoot size compared with the wild type, whereas *phs1a* was indiscernible from the wild type, as reported previously (Chia et al., 2004; Roldán et al., 2007; Malinova et al., 2014). All double mutants showed a reduced shoot size compared with the wild type (Fig. 1B). *dpe2-1/phs1a* revealed the strongest dwarf phenotype and pale leaves, as reported previously (Malinova et al., 2014). *dpe2-1/ss4* exhibited a growth phenotype similar to the *dpe2-1* single knockout mutant. The *phs1a/ss4* double knockout mutant showed some variations even under identical growth conditions; thus, it was sometimes similar in growth to the *ss4* mutant (data not shown) but in most cases had a shoot size even bigger than that of *ss4* (Fig. 1B). The growth phenotype of *dpe2-1/phs1a/ss4* differed from that of *dpe2-1/phs1a* in that it exhibited a larger shoot size.

When comparing *dpe2-1/phs1a* and *dpe2-1/phs1a/ss4* in more detail, the triple mutant revealed a higher average fresh weight per plant (Fig. 1C) and a higher starch content at the ends of both the light and dark periods (Fig. 1D). Interestingly, *dpe2-1/phs1a/ss4* had an even higher starch content than *dpe2-1* but had less starch than the double knockout mutant *dpe2-1/ss4* (Fig. 1D). The *dpe2-1/ss4* double mutant displayed exceptionally high starch content, which was more than twice that of *dpe2-1*. Furthermore, all lines lacking DPE2 retained more starch at the end of the dark phase than the wild type, likely reflecting an interference in starch degradation. The *ss4* mutant had less starch than the wild type at the end of the light phase, as reported previously (Szydlowski et al., 2009). In contrast, at the end of the dark phase, *ss4* retained more starch than the wild type. A minor yet significant reduction in starch content at the end of the light period was observed for *phs1a/ss4* in comparison with the wild type. Nevertheless, at the end of the dark phase, an increased starch level also was detected in this double mutant compared with the wild type and *phs1a*.

Starch turnover during the light/dark cycle was observed for all genotypes analyzed. When grown in a 12-h photoperiod, wild-type plants typically degrade around 90% of their transitory starch during the night, and starch turnover in *phs1a* was similar to that of the wild type (Fig. 1D). The *ss4* and *phs1a/ss4* mutants degraded less of their starch than the wild type, both in absolute terms and as a percentage of the end-of-day starch content. However, the greatest reduction in starch degradation was observed in the lines lacking DPE2, in which the starch turnover was around 15% to 30%.

For *dpe2-1/phs1a*, it was shown that starch was distributed unevenly through the plant rosette; thus, young leaves contained starch, whereas in older leaves, no starch was detected (Malinova et al., 2014). For comparison, rosettes of *dpe2-1/phs1a* and *dpe2-1/phs1a/ss4* were harvested at 9.5 h during the light period, decolorized, and stained with iodine. Interestingly, in contrast to the double mutant, *dpe2-1/phs1a/ss4* revealed a more or less homogenous distribution of starch (Fig. 1E).

PHS1 and SS4 Cannot Substitute for Each Other with Regard to Starch Granule Formation

Previously, it was shown that SS4 coordinated starch granule formation during leaf expansion, as only mature leaves of *ss4* contained starch granules (Crumpton-Taylor et al., 2013). The opposite effect was observed for *dpe2-1/phs1a*, as only young leaves contained starch granules (Fig. 1E; Malinova et al., 2014). Mature leaves of *dpe2-1/phs1a* were essentially starch free, and this feature was accompanied by the disintegration of chloroplasts (Malinova et al., 2014). Thus, the question appears whether two independent ways of generating starch granules exist or if both routes are linked. Therefore, it is conceivable that no starch granules would be detected in the *dpe2-1/phs1a/ss4* triple mutant if the pathways were independent and both were disrupted. However, the results above clearly demonstrated that all leaves in the rosettes of the triple mutant contained starch (Fig. 1E). All lines were subjected to

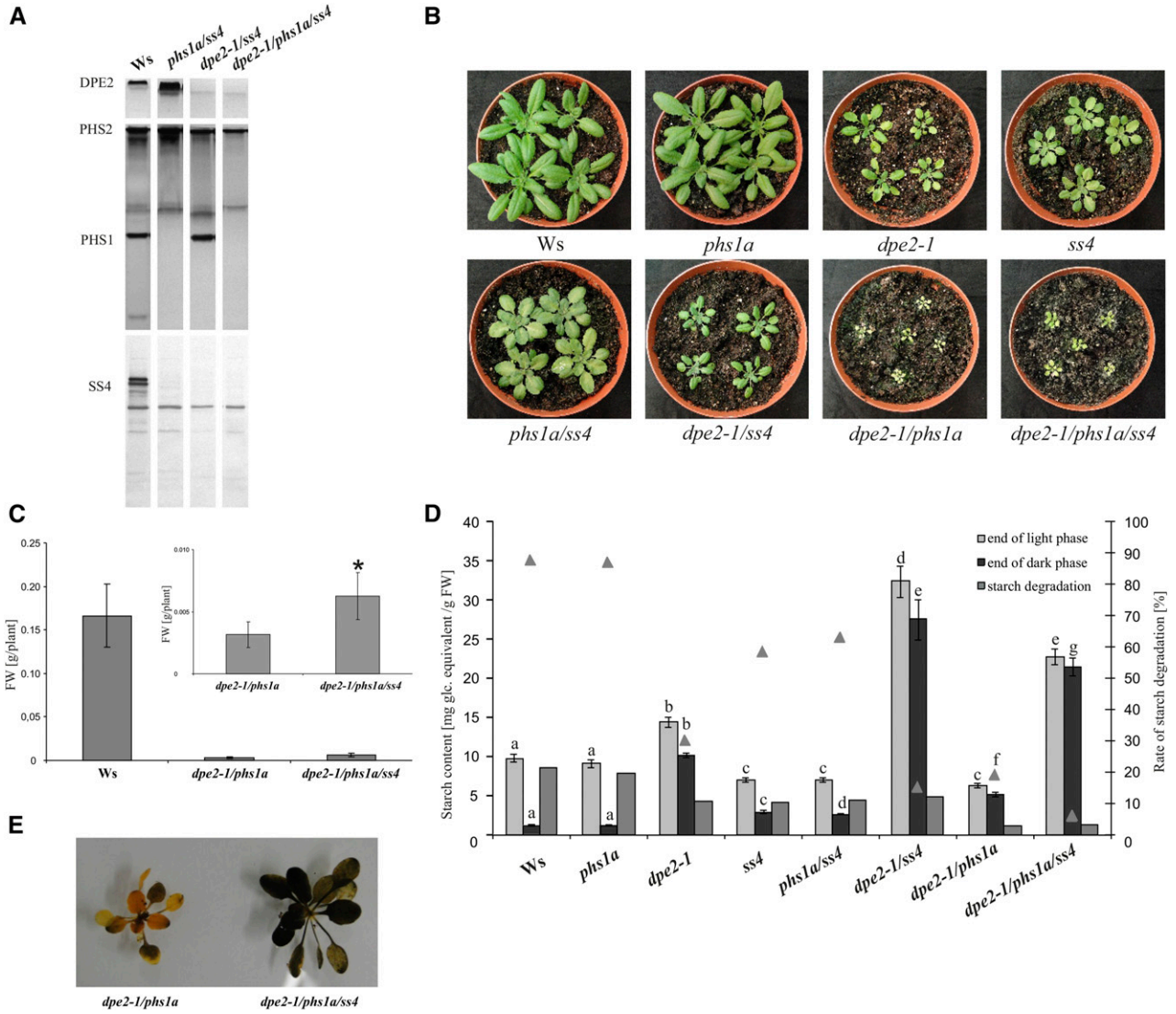

Figure 1. Analysis of double, triple, and parental single mutants and wild-type Wassilewskija (Ws) plants grown under 12-h-light/12-h-dark conditions. A, Screening of the generated double and triple mutants. For each mutant, one leaf (approximately the same size) was harvested, and proteins were extracted in a volume of 100 μL. Twenty microliters of each was loaded per lane. For DPE2 and PHS1, discontinuous native PAGE containing 0.2% (w/v) glycogen was used followed by activity staining. For DPE2, the separation gel was incubated in a mixture of 100 mM citrate-NaOH (pH 6.5) and 20 mM maltose at room temperature overnight. For PHS, gels were incubated in a mixture of 100 mM citrate-NaOH (pH 6.5) and 20 mM Glc-1-P at room temperature overnight. Subsequently, gels were stained with iodine solution. For SS4 detection, protein extracts were denatured and loaded on the SDS-PAGE gel followed by western blot. For immunodetection, a polyclonal anti-AtSS4 antibody was used. B, Growth phenotypes of the plants. Photographs of 5-week-old plants were taken. C, Fresh weight (FW) of *dpe2-1/phs1a*, *dpe2-1/phs1a/ss4*, and wild-type plant rosettes. Values are means ± SD ($n = 24$–37). Plants were harvested 4 weeks after planting. The asterisk indicates a significant difference from *dpe2-1/phs1a* (Student's *t* test, $P < 0.01$). D, Starch content in the analyzed plant lines. All values are means ± SD ($n = 3$–4 replicates from a mixture of at least four to 20 plants each). Letters indicate samples that were not significantly different ($P \leq 0.05$) according to one-way ANOVA with Holm-Sidak posthoc testing. The third bar in each group represents the amount of starch degraded (calculated by starch content at the end of the light minus starch content at the end of the night). Triangles indicate percentages of the total starch content degraded by the end of the dark phase. E, Semiquantitative starch determination in plants. Plants were harvested at the end of the light phase (after 9.5 h of illumination), decolorized, and stained with iodine solution for starch.

transmission electron microscopy (TEM) to analyze the starch granule number per chloroplast as well as the chloroplast ultrastructure. Leaf material from the various genotypes was harvested in the middle of the light phase (after 6 h of illumination). The chloroplasts of the triple mutant mostly contained only a single starch granule, except in some of the older leaves, where up to two starch granules per chloroplast were present (Fig. 2).

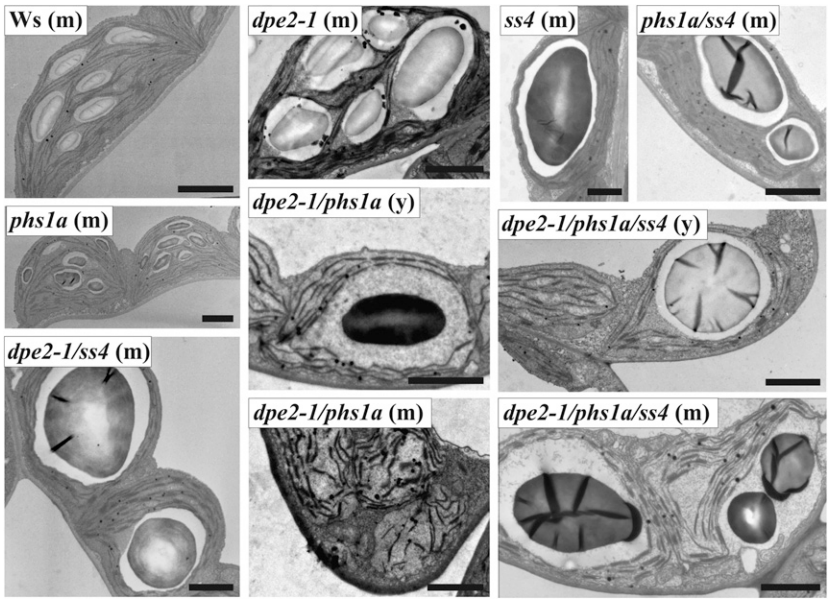

Figure 2. TEM analyses of mesophyll leaf sections. Plant were grown in a 12-h-light/12-h-dark regime for 5 weeks and harvested in the middle of the light phase. m, Mature leaves; Ws, Wassilewskija; y, young leaves. Bars = 2 μm.

In young leaves of *dpe2-1/phs1a*, there also was a single starch granule per chloroplast, whereas in older leaves, no starch granules at all were detected (Fig. 2). Whereas wild type, *phs1a*, and *dpe2-1* chloroplasts contained the expected four to seven starch granules (Crumpton-Taylor et al., 2012), all other mutants showed a strong reduction in the number of starch granules per chloroplast (Fig. 2). In agreement with previous reports, older leaves of *ss4* contained up to two large starch granules per chloroplast (Roldán et al., 2007); however, in our investigations, we also observed some starch granules in young leaves (Supplemental Fig. S1). This difference from the data observed by Crumpton-Taylor et al. (2013) is likely linked to the variability of the definition of young and old leaves as well as young and old leaf parts. However, *phs1a/ss4* and *dpe2-1/ss4* contained one or two starch granules per chloroplast (Fig. 2). No difference in the number of granules per chloroplast between young and mature leaves was observed in either of the double mutants (Supplemental Fig. S1). Although young leaves of both double mutants did contain starch granules, some starch-free chloroplasts also were observed (Supplemental Fig. S1). Thus, all double and triple mutants lacking SS4 revealed a strongly reduced granule number per chloroplast.

The observed disintegration of the chloroplasts in *dpe2-1/phs1a* was not seen in the triple mutant (Fig. 2). Thus, the lack of SS4 in the triple mutant counteracted the disintegration of the chloroplasts expected from the joint loss of DPE2 and PHS1.

Reduction of the Starch Granule Number per Chloroplast Combined with Lack of DPE2 Results in Abnormally Large and Spherical Starch Granules

In addition to the TEM analyses, starch granules were isolated from leaves and analyzed using scanning electron microscopy (SEM). The wild type, *phs1a*, and *dpe2-1* possessed flattened, discoid granules that are typical for Arabidopsis leaf starch (Fig. 3; Zeeman et al., 2002; Mahlow et al., 2014). However, in agreement with previous reports (Crumpton-Taylor et al., 2013), *ss4* had more rounded and thicker starch granules than the wild type. Starch granules from *phs1a/ss4* were similar in shape to those from *ss4* (Fig. 3). Major differences in starch granule morphology were observed for *dpe2-1/ss4*, *dpe2-1/phs1a*, and the triple mutant. Granules from *dpe2-1/ss4* and *dpe2-1/phs1a/ss4* were almost perfectly spherical (Fig. 3). Additionally, granules from *dpe2-1/ss4* (6.8 $\pm$ 1.6 μm; n = 25) were much larger than wild-type granules (1.8 $\pm$ 0.3 μm; n = 25), while those from the triple mutants were intermediate in size (4.8 $\pm$ 0.8 μm; n = 25). Thus, the loss of both DPE2 and SS4 resulted not only in a lower starch granule number per chloroplast but also in a strongly altered granule morphology.

To investigate if the observed changes of the starch granule morphology were accompanied by an alteration of the inner granule structure, chain length distribution (CLD) profiles were determined. Isolated starch granules were heat solubilized and treated with isoamylase, and the released glucan chains were labeled with aminofluorophore 8-amino-1,3,6-pyrenetrisulfonic acid before analysis by capillary electrophoresis with laser-induced fluorescence detection. Only minor differences in CLD profiles were observed (Supplemental Fig. S2). The *phs1a* mutant displayed higher amounts of short glucan chain (i.e. with a degree of polymerization up to 14). In contrast, *ss4* starches had a smaller proportion of short glucan chains than the wild type, in agreement with the report of Roldán et al. (2007). The *dpe2-1* mutant had fewer short glucan chains than the wild type but also slightly more longer glucan chains (Supplemental Fig. S2). Interestingly, the CLD profile of *phs1a/ss4* was

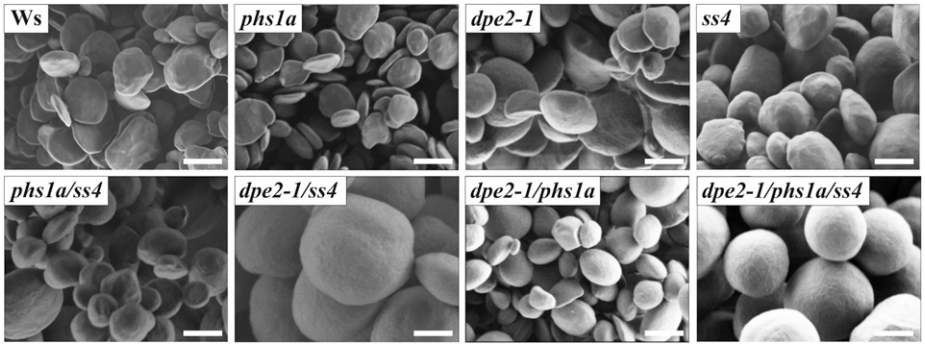

Figure 3. Morphology of isolated starch granules analyzed by SEM. Starch granules were isolated from leaves of 5-week-old plants harvested at the end of the light phase. Ws, Wassilewskija. Bars = 2 μm.

similar to that of *phs1a*. In contrast, *dpe2-1/phs1a* and the triple mutant had CLD profiles that were more similar to those of the *dpe2-1* and *ss4* single mutants. The *dpe2-1/ss4* mutant, like *phs1a*, had an increased number of shorter glucans.

Starch-related protein amounts and enzyme activities were next determined (Supplemental Fig. S3). *dpe2-1/phs1a* and the triple mutant revealed slight reductions in SS1 activity (Supplemental Fig. S3A). Furthermore, for *dpe2-1/phs1a*, reductions in the protein amounts of both starch-related dikinases (GWD and PWD) were detected (Supplemental Fig. S3B). The soluble SS2 protein amount was variable in the various mutants. Thus, the triple mutant revealed a protein amount like the wild type, whereas *dpe2-1*, *ss4*, *dpe2-1/ss4*, and *phs1a/ss4* revealed higher SS2 protein amounts and *phs1a* and *dpe2-1/phs1a* revealed much lower SS2 protein amounts (Supplemental Fig. S3B). Thus, in the latter case, the protein was hardly detected. However, when we analyzed the SS2 protein attached to the starch granules also for these mutants, SS2 was clearly detected (Supplemental Fig. S3C).

Native gel electrophoresis revealed no detectable branching enzyme activity in either *dpe2-1/phs1a* or *dpe2-1/phs1a/ss4*, despite both mutants containing SBE2 and SBE3 proteins, as detected by immunoblotting (Supplemental Fig. S3, D and E). Interestingly, *phs1a/ss4* revealed only one band of branching enzyme activity. However, SBE2 also was detected strongly bound to the starch granule surface (Supplemental Fig. S3C).

dpe2-1/phs1a and *dpe2-1/phs1a/ss4* Reveal Distinct Metabolic Differences

As reported previously, *dpe2-1/phs1a* has exceptionally high levels of maltose and longer maltodextrins (Malinova et al., 2014). The accumulation of longer maltodextrins is associated with chloroplast degradation in *dpe2-1/phs1a* and *mex1/dpe1* and is postulated to be a causal factor (Stettler et al., 2009; Malinova et al., 2014). In sharp contrast, little or no evidence of chloroplast disintegration was observed in the triple *dpe2-1/phs1a/ss4* mutant (Figs. 1E and 2). Therefore, we next isolated and analyzed the soluble glucans. Analysis revealed that *ss4* and *phs1a/ss4* have slightly increased levels of maltodextrins compared with the wild type. However, considerably higher levels of maltodextrins were found in the *dpe2-1* and *dpe2-1/ss4* lines (Fig. 4A). The *dpe2-1/phs1a* and *dpe2-1/phs1a/ss4* mutants exhibited the highest levels of maltodextrins. Indeed, despite the fact that both mutants displayed considerable variability in total maltodextrin content, no significant alterations were detected (Fig. 4A). The maltodextrin profiles (analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection [HPAEC-PAD]) of the *dpe2-1/phs1a* and triple mutants were broadly similar, but the triple mutant had slightly lower amounts of maltose and maltodextrins (degree of polymerization 4–6) than *dpe2-1/phs1a* (Fig. 4B).

The *phs1a* mutant had similar levels of soluble sugars compared with wild-type plants at the ends of both the light and dark periods (Table I), as reported previously (Malinova et al., 2014). Likewise, in agreement with previous reports (Lu and Sharkey, 2004; Malinova et al., 2014), *dpe2-1* had more Glc and Fru than the wild type but less Suc (a typical pattern for mutants with defects in starch degradation). The soluble sugar content of the *ss4* and *phs1a/ss4* mutants was similar and differed only slightly from the wild type. By contrast, the sugar profile of *dpe2-1/ss4* was more similar to that of *dpe2-1*, although the double mutant had somewhat lower hexoses but more Suc than *dpe2-1* (Table I). The *dpe2-1/phs1a* and triple mutants also resembled *dpe2-1* but had even higher Glc levels but slightly less Suc than *dpe2-1*, especially at the end of the night. A broader profiling of metabolites was performed on rosettes of wild-type and mutant plants harvested in the last hour of the light phase. However, no specific metabolic alteration was detected between the genotypes that explain the observed difference. All of the mutants that lacked DPE2 had elevated levels of soluble sugars (maltose, Glc, Fru, and trehalose), the main exception being Suc, which was lower or unchanged (*dpe2-1/ss4*; Supplemental Fig. S4). The metabolite profile of *phs1a/ss4* was comparable to those of the two parental lines, *ss4* and *phs1a*. The profile of *dpe2-1/ss4* was likewise similar to that of its *dpe2-1* parent and very different from that of *dpe2-1/phs1a*, which showed the most divergent metabolite profile, with large differences from the wild type for

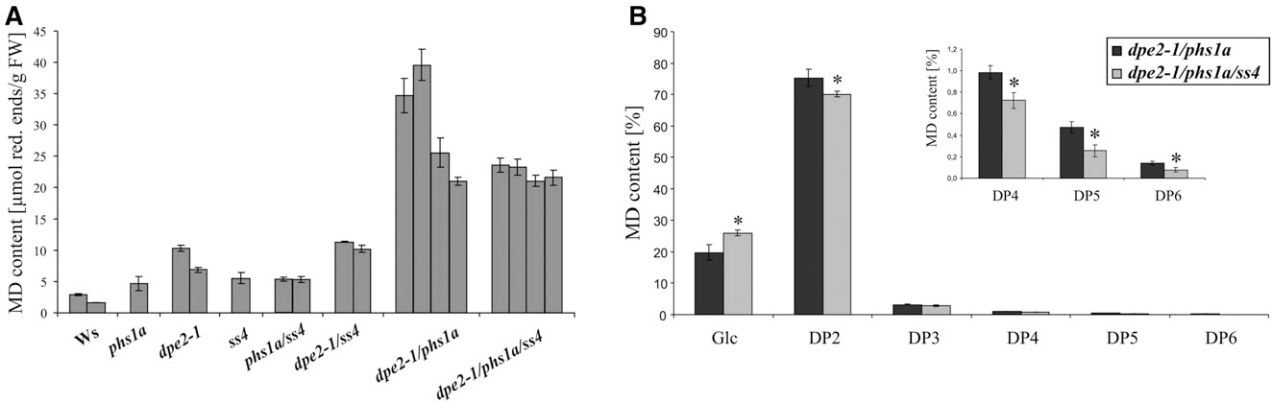

Figure 4. Analyses of the soluble oligosaccharides. Plants were grown under a 12-h-light/12-h-dark regime and harvested at the end of the light phase, and oligosaccharides were isolated. A, Total content of the reducing oligosaccharides per fresh weight (FW). Bars represent different plant batches. All values are means ± SD ($n = 3$ technical replicates from a mixture of at least 15–20 plants). Ws, Wassilewskija. B, Maltodextrin (MD) pattern. Maltodextrins were analyzed using HPAEC-PAD. Carbohydrates (20 nmol) were applied to the column. Asterisks indicate significant differences from *dpe2-1/phs1a* (Student's *t* test, $P < 0.05$). DP, Degree of polymerization.

almost all of the detected metabolites (Supplemental Fig. S4). The profile of the triple mutant resembled that of *dpe2-1/phs1a*, although many of the differences from the wild type were less pronounced in the triple mutant, except for raffinose and some amino acids.

It was reported previously that a lack of SS4 results in a massive accumulation of ADPglucose (Crumpton-Taylor et al., 2013; Ragel et al., 2013); thus, we tested whether all of the mutants (lacking SS4) were similar in this regard. ADPglucose and other phosphorylated intermediates were measured in all of the genotypes in rosettes harvested at the end of the light phase. The *ss4* mutant, as expected, had an exceptionally high amount of ADPglucose (Table II). Interestingly, *phs1a/ss4* contained even higher levels of ADPglucose than the *ss4* single mutant (Table II). Loss of SS4 in the *dpe2-1/phs1a* background also led to the accumulation of ADPglucose, but to a much lesser extent than in the *ss4* and *phs1a/ss4* mutants (Table II). The *dpe2-1/ss4* mutant had elevated ADPglucose levels compared with the wild type but was not significantly different from its *dpe2-1* parent or the *dpe2-1/phs1a* mutant.

The lower level of ADPglucose in the triple mutant compared with the *ss4* and *phs1a/ss4* parental lines was unexpected, suggesting that the accumulation of ADPglucose associated with defective starch synthesis in the *ss4* mutant was, to a large extent, reversed by additional loss of DPE2. To investigate if this could be linked to a change in starch synthesis, we measured the activity and allosteric effectors of ADPglucose pyrophosphorylase (AGPase), the enzyme responsible for ADPglucose synthesis. There was no significant difference in the maximal in vitro activity of AGPase in the triple mutant compared with *ss4* or *dpe2-1/phs1a*, and the activities in all three mutants were indistinguishable from those of the wild type (Supplemental Fig. S5A). However, the triple mutant had less than half the level of 3-phosphoglycerate (3PGA; an allosteric activator of AGPase) compared with the *ss4* and *phs1a/ss4* lines

Table I. *Soluble sugars*

Plants were grown under 12-h-light/12-h-dark conditions and harvested after 5 weeks. All values are means ± SD ($n = 3$–4 replicates from a mixture of at least four to 20 plants each). Letters indicate samples that were not significantly different ($P \leq 0.05$) according to one-way ANOVA with Holm-Sidak posthoc testing.

Genotype	End of the Light			End of the Dark		
	Glc	Fru	Suc	Glc	Fru	Suc
	µmol g^{-1} fresh wt					
Wassilewskija	0.34 ± 0.03 a	0.07 ± 0.03 a	2.43 ± 0.06 a	0.13 ± 0.01 a	0.10 ± 0.01 a	1.75 ± 0.02 a
phs1a	0.33 ± 0.06 a	0.09 ± 0.06 a	2.17 ± 0.06 b	0.12 ± 0.05 a	0.13 ± 0.09 a	1.77 ± 0.10 a,c
dpe2-1	4.97 ± 0.68 b	0.94 ± 0.41 b	1.36 ± 0.18 c	1.33 ± 0.11 b	0.35 ± 0.04 b	1.29 ± 0.11 b
ss4	0.71 ± 0.08 a	0.09 ± 0.04 a	2.21 ± 0.13 a,b	0.10 ± 0.04 a	0.05 ± 0.03 a	1.78 ± 0.09 a,c
phs1a/ss4	0.71 ± 0.07 a	0.08 ± 0.01 a	2.53 ± 0.01 a	0.14 ± 0.03 a	0.05 ± 0.01 a	1.66 ± 0.13 a
dpe2-1/ss4	2.83 ± 0.09 c	0.29 ± 0.05 a	2.00 ± 0.10 b	1.05 ± 0.09 c	0,27 ± 0.05 b	1.99 ± 0.06 c
dpe2-1/phs1a	6.36 ± 0.29 d	0.63 ± 0.05 b	1.15 ± 0.18 c	2.54 ± 0.29 d	0.34 ± 0.09 b	0.90 ± 0.13 d
dpe2-1/phs1a/ss4	6.20 ± 0.28 d	0.80 ± 0.10 b	1.21 ± 0.09 c	3.05 ± 0.23 d	0.55 ± 0.05 c	0.71 ± 0.14 d

Table II. *Phosphorylated metabolites*

Plants were grown under 12-h-light/12-h-dark conditions and harvested in the last hour of the light phase. Plants were 5 weeks old. All values are means ± SD ($n = 4$ replicates from a mixture of at least four to 20 plants each). Letters indicate samples that were not significantly different ($P \leq 0.05$) according to one-way ANOVA with Holm-Sidak posthoc testing.

Genotype	ADPglucose	3PGA	Glc-6-P	Glc-1-P	Trehalose 6-Phosphate
	nmol g^{-1} fresh wt				
Wassilewskija	1.9 ± 1.0 a,e	153.1 ± 15.0 a	98.7 ± 8.8 a	15.1 ± 1.2 a	0.4 ± 0.1 a
phs1a	1.3 ± 0.5 a	130.7 ± 31.2 a	93.8 ± 4.6 a	12.8 ± 1.1 a,b	0.2 ± 0.0 b
dpe2-1	4.4 ± 1.6 b	136.0 ± 36.6 a	84.0 ± 12.3 a,c	13.5 ± 2.2 a,c	0.2 ± 0.0 b
ss4	163.9 ± 17.0 c	141.7 ± 22.1 a	153.3 ± 22.1 b	24.6 ± 1.5 d	0.4 ± 0.0 a
phs1a/ss4	264.4 ± 51.8 d	151.2 ± 21.1 a	90.7 ± 11.7 a,c	16.1 ± 1.1 a	0.6 ± 0.0 c
dpe2-1/ss4	6.2 ± 3.8 b,e	135.8 ± 52.1 a	72.7 ± 9.3 a,c	11.6 ± 0.9 b,c	0.3 ± 0.1 a
dpe2-1/phs1a	5.3 ± 1.0 b	50.3 ± 6.2 b	64.1 ± 7.0 c	8.5 ± 0.6 e	0.1 ± 0.0 d
dpe2-1/phs1a/ss4	9.5 ± 2.9 f	62.8 ± 5.1 b	79.9 ± 11.0 a,c	10.1 ± 1.7 b,e	0.1 ± 0.0 d

(Table II), while orthophosphate (Pi; an allosteric inhibitor of AGPase) was higher in the triple mutant (Supplemental Fig. S4). The 3PGA-Pi ratio also was decreased in the *dpe2-1/phs1a* mutant (Table II; Supplemental Fig. S4). The decreased 3PGA-Pi ratios in the *dpe2-1/phs1a* and triple mutants suggest that the in vivo activity of AGPase would be lower in these two mutants than in the other genotypes, including *ss4* and *phs1a/ss4*, thus limiting the accumulation of ADPglucose compared with these two parental lines. However, the Pi values represent the amounts of entire cells and not the Pi amounts in the plastids. No major alterations in the activity status of AGPase were detected for the various mutants (Supplemental Fig. S5B).

Glc-6-P and Glc-1-P, which are precursors of ADPglucose in the pathway of starch synthesis, also were elevated in the *ss4* mutant compared with the wild type but not in the *phs1a/ss4* mutant (Table II). In contrast, *dpe2/ss4* and *dpe2-1/phs1/ss4* had lower Glc-6-P and Glc-1-P levels than *ss4* and the wild type. Trehalose 6-phosphate is a Suc-signaling metabolite that has been implicated in the regulation of transitory starch degradation (Martins et al., 2013; Yadav et al., 2014). Trehalose 6-phosphate content was significantly lower in the *phs1*, *dpe2-1*, and *dpe2-1/phs1a/ss4* mutants than in the wild type, reflecting the lower levels of Suc in those lines, whereas trehalose 6-phosphate and Suc were both higher in the *phs1a/ss4* mutant than in the wild type (Tables I and II).

Young Leaves of *dpe2-1/phs1a/ss4* Exhibited a Saturation of the Photosynthetic Linear Electron Flow at Lower Light Intensities Compared with *dpe2-1/phs1a*

The starch granule number and metabolite profiles of the *dpe2-1/phs1a/ss4* mutant were similar to those of the *dpe2-1/phs1a* line, but the triple mutant had more starch and grew faster than *dpe2-1/phs1a* (Fig. 1, C and D). To understand these differences, we analyzed the photosynthetic performance of the mutants (Fig. 5). In mature leaves of the *dpe2-1/phs1a* and *dpe2-1/phs1a/ss4* mutants, photosynthetic linear electron flux was saturated by an irradiance of about 500 μmol photons m^{-2} s^{-1}, whereas photosynthesis became saturated around 800 μmol photons m^{-2} s^{-1} in wild-type plants (Fig. 5A).

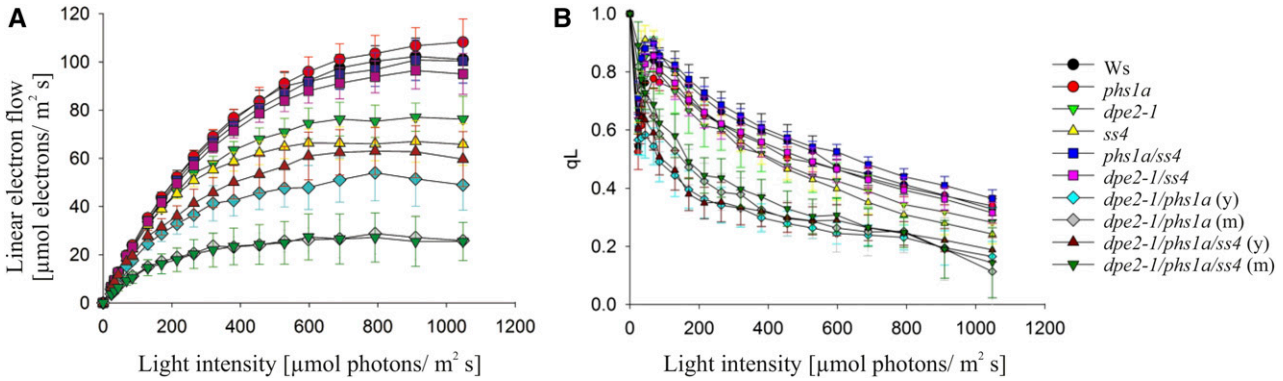

Figure 5. Photosynthetic parameters of leaves of the various Arabidopsis lines. Light-response curves of linear electron flux (A) and the chlorophyll *a* fluorescence parameter qL (B) of 5-week-old plants are shown. At least eight independent replicates were measured for the wild type and the various mutants. Linear electron flux was corrected for differences in leaf absorptance. Error bars represent SD. Ws, Wassilewskija.

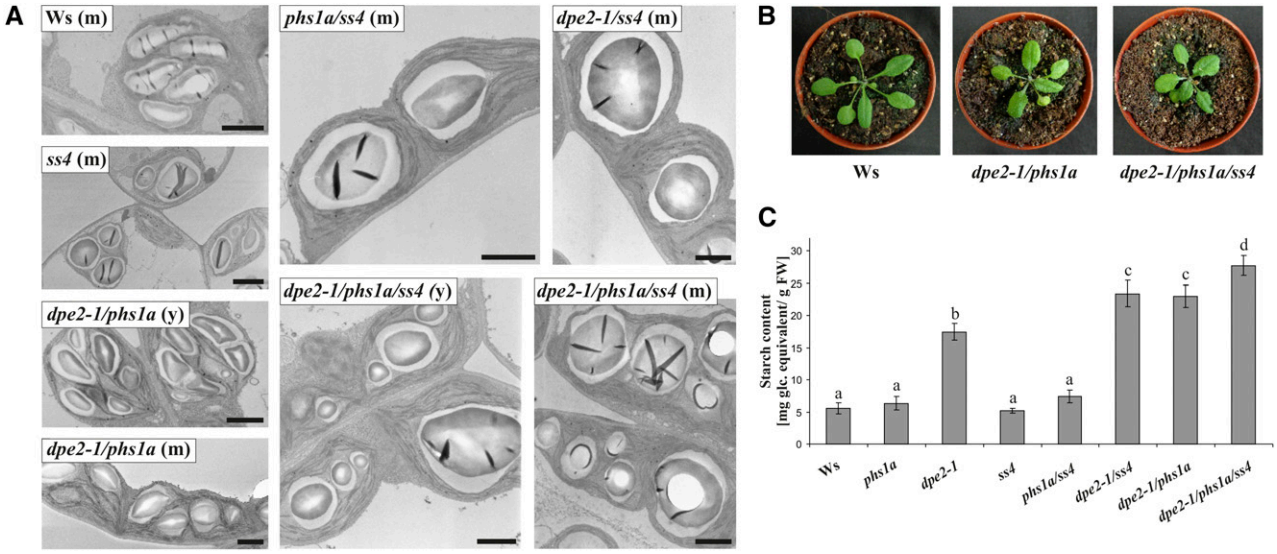

Figure 6. Analysis of the various plant lines grown under continuous light. A, TEM analyses of mesophyll leaf sections. m, Mature leaves; y, young leaves. Bars = 2 μm. B, Growth phenotypes of wild-type Wassilewskija (Ws), *dpe2-1/phs1a*, and *dpe2-1/phs1a/ss4* mutants. Plants were 3 weeks old. C, Starch content of the analyzed lines. All values are means ± SD (n = 3–4 replicates from a mixture of two plants each). Letters indicate samples that were not significantly different ($P \leq 0.05$) according to one-way ANOVA with Holm-Sidak posthoc testing. FW, Fresh weight.

The light-saturated rates of electron flow in mature leaves of the *dpe2-1/phs1a* and *dpe2-1/phs1a/ss4* mutants were similar to each other but about 75% lower than those in wild-type plants (Fig. 5A). By contrast, the light-saturated rates of photosynthesis of young leaves from the *dpe2-1/phs1a* and *dpe2-1/phs1a/ss4* mutants were only about 40% and 25% lower than in the wild type, respectively. Photosynthetic rates were lower than in the wild type in the *dpe2-1* and *ss4* mutants, as reported previously (Stettler, 2009; Ragel et al., 2013), but very similar to the wild-type rate in *phs1a* (Fig. 5A). The redox state of the PSII acceptor side (qL; Fig. 5B) was substantially more reduced than in the wild type in young as well as mature leaves of *dpe2-1/phs1a* and *dpe2-1/phs1a/ss4*. Interestingly, *phs1a/ss4* and *dpe2-1/ss4* revealed photosynthetic activities more similar to those in the wild type than to those in *ss4* and *dpe2-1*, respectively.

The Starch Granule Number per Chloroplast in *dpe2-1/phs1a* Is More Strongly Affected by Alterations of the Light/Dark Regime Than in Mutants Lacking SS4

For *dpe2-1/phs1a*, we showed that a lack of the dark phase resulted in the prevention of chloroplast disintegration and an increase of the starch granule number per chloroplast comparable to the wild type (Malinova et al., 2014). Therefore, we investigated the responses of *dpe2-1/phs1a* and *dpe2-1/phs1a/ss4* to growth in long-day conditions (14 h of light/10 h of dark) or continuous light with all other growth conditions being kept the same as in the experiments described so far. All analyses were performed with at least four independent plants and more than 60 chloroplasts each. Under long-day conditions, an increased starch granule number per chloroplast was observed in young leaves of the *dpe2-1/phs1a* mutant (one to four granules per chloroplast; Supplemental Fig. S6A), whereas chloroplasts from mature leaves contained only a single granule and there was less disintegration of the thylakoids compared with the plants grown in a 12-h photoperiod. When grown in long days, most of the chloroplasts in the triple mutant contained only a single starch granule in young as well as mature leaves (Supplemental Fig. S6A); thus, the granule number remained constant. Additionally, the triple mutant revealed a higher starch content than *dpe2-1/phs1a* (Supplemental Fig. S6B). Under continuous light, the *dpe2-1/phs1a* mutant had an increased number of starch granules per chloroplast (Fig. 6A), with granule number (four to seven granules per chloroplast) being independent of leaf age and similar to that in wild-type plants. The *dpe2-1/phs1a/ss4* triple mutant grown in continuous light had between two and four granules per chloroplast, which was higher than in plants grown in a 12-h photoperiod but still less than in the wild type. In contrast to the growth retardation observed in a 12-h photoperiod, the growth of the *dpe2-1/phs1a* and *dpe2-1/phs1a/ss4* mutants was indistinguishable from that of the wild type under continuous light (Fig. 6B). Nevertheless, the starch-excess phenotype of both mutants was conserved and was similar to that of *dpe2-1/ss4* (Fig. 6C). The levels of sugar phosphates and ADPglucose generally were elevated in all genotypes grown under continuous light compared with a 12-h photoperiod, but the qualitative differences between genotypes were

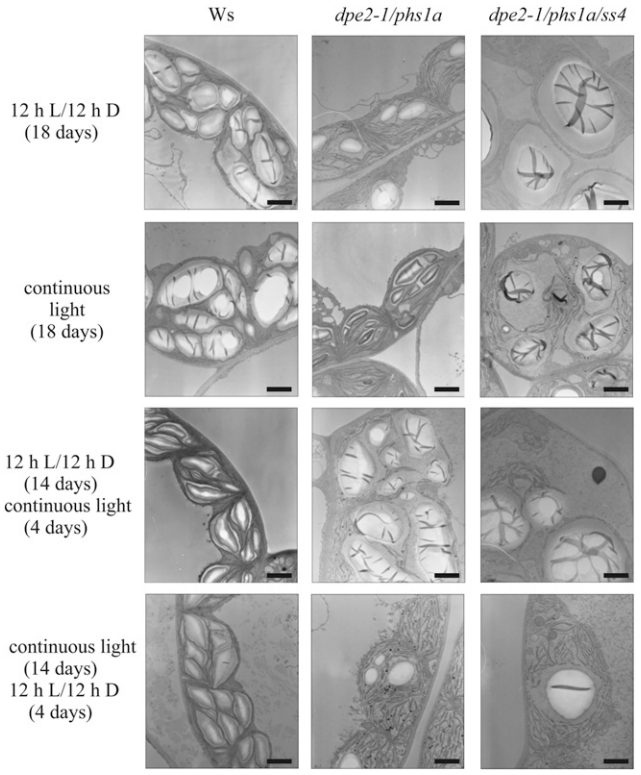

Figure 7. Analysis of starch granule number per chloroplast in plants during the transition from light/dark to continuous light conditions. Plants were grown either in a 12-h-light/12-h-dark regime or under continuous illumination for 14 d and then transferred for 4 d to the opposite light conditions. As a control, plants were grown for 18 d under continuous illumination or 12-h-light/12-h-dark. Plants were harvested in the middle of the light phase corresponding to the 12-h-light/12-h-dark regime. Ws, Wassilewskija. Bars = 2 μm.

similar under both light regimes (compare Table II and Supplemental Table S1). There was a general tendency for soluble sugars to be higher in continuous light-grown plants (Supplemental Table S2) than in plants grown in a 12-h photoperiod and harvested toward the end of the day (Table I), but Glc and Fru were lower in continuous light in all of the genotypes that lacked DPE2. In continuous light, the level of Glc in *dpe2-1/phs1a* was substantially higher than in the triple mutant (Supplemental Table S2).

The *phs1a/ss4* and *dpe2-1/ss4* mutants contain one or occasionally two starch granules per chloroplast in young (data not shown) and mature leaves (Fig. 6A) under continuous illumination. However, the observed increased ADPglucose amount in *phs1a/ss4* in comparison with *ss4* was not detected under continuous light. Indeed, under continuous light, *ss4* displayed the highest ADPglucose level (Supplemental Table S1). Interestingly, in continuous light, *ss4* also displayed an increased number of starch granules, with up to three granules per chloroplast.

The *dpe2-1/phs1a* and *dpe2-1/phs1a/ss4* mutants had increased starch granule numbers per chloroplast when grown in continuous light compared with a 12-h photoperiod, although the increase was less pronounced in the triple mutant.

In these experiments, the plants were grown under continuous illumination or in a light/dark regime throughout their lives. We investigated whether the phenotypic differences between the two light regimes could be reversed by switching between growth conditions. Wild-type plants and the *dpe2-1/phs1a* and *dpe2-1/phs1a/ss4* mutants were grown under either 12 h of light/12 h of dark or continuous illumination for 14 d and then swapped to the other light regime for 4 d. Control plants were grown either under continuous illumination or the 12-h-light/12-h-dark regime for 18 d. When plants were grown under the 12-h-light/12-h-dark regime or continuous illumination, the number of starch granules per chloroplast was the same as before (Fig. 7). Swapping the growth regime had no effect on the starch granule number per chloroplast in either the triple mutant or the wild type. Thus, the triple mutant contained a single granule per chloroplast while the wild-type plants contained four to seven granules per chloroplast under all conditions. In contrast, switching *dpe2-1/phs1a* from the 12-h-light/12-h-dark regime to continuous illumination increased the starch granule number from one or two granules per chloroplast to four to seven granules, as in the wild type (Fig. 7). Likewise, the reverse transition from continuous light to the 12-h-light/12-h-dark regime resulted in a strong reduction of granule number per chloroplast. Thus, the number of granules per chloroplast in *dpe2-1/phs1a* was highly flexible and reflected the current rather than the previous light regime. Furthermore, these experiments clearly demonstrated that the increase in the number of granules was determined by the dark phase.

In summary, these experiments revealed that the granule number per chloroplast is not fixed in *dpe2-1/phs1a* and can respond to a change in the light regime, but this flexibility is lost, or at least decreased, following the additional loss of SS4 in the *dpe2-1/phs1a/ss4* mutant.

DISCUSSION

Lack of SS4 and PHS1 Individually or in Combination Does Not Prevent Starch Granule Formation

This study was initiated to analyze in more detail the observation that SS4 and, in part, also PHS1 (in the background of the *dpe2-1/phs1a* mutant) play a role in the regulation of starch granule number per chloroplast and to understand whether they act independently or together. For this purpose, the expression of SS4 was eliminated in the background of the *dpe2-1/phs1a* mutant, whose chloroplasts contained a single starch granule, when the plants were grown in a 12-h-light/12-h-dark regime. Combining mutations that individually perturb starch granule formation was expected to disrupt starch granule formation even further or even abolish it altogether. However, the triple *dpe2-1/phs1a/ss4* mutant not

only contained starch but also revealed significant differences from both *dpe2-1/phs1a* and *ss4*. First, the triple mutant grew better than the parental mutants, resulting in a higher total fresh weight than *dpe2-1/phs1a* (Fig. 1C). This improved performance is likely to be related to the absence of chloroplast degradation in mature leaves of the triple mutant, in contrast to *dpe2-1/phs1a* (see below). The latter, like all of the double mutants that lack DPE2 activity as well as the *dpe2-1* mutant itself, displayed reduced growth compared with the wild type, probably caused by a depletion of carbohydrates during the night resulting from a block in starch-related maltose metabolism (Chia et al., 2004; Lu and Sharkey 2004). Second, the starch content (on a per gram fresh weight basis) of the triple mutant was higher than in either of the parental lines (Fig. 1D). Furthermore, iodine staining of the triple mutant revealed the presence of starch in all leaves throughout the rosette. This distribution was similar to that of wild-type plants but different from *dpe2-1/phs1a*, which had some leaves with no detectable starch (Fig. 1E). Third, the starch granule number per chloroplast differed between the *ss4*, *dpe2-1/phs1a*, and *dpe2-1/phs1a/ss4* mutants and was dependent on the light regime (Figs. 2, 6A, and 7; Supplemental Fig. S6A). Thus, all of these mutants revealed increases in starch granule number when the photoperiod was extended from 12 to 14 h or when the plants were grown in continuous light. To our knowledge, this response of granule number in *ss4* to length of day has not been reported previously. Extension of the photoperiod improved the growth of the mutants, and in continuous light their growth was similar to that of the wild type (Fig. 6B). Starch granule number in the *dpe2-1/phs1a* mutant was sensitive to a change in photoperiod during the growth period, but this effect was diminished, or even abolished, in the triple mutant (Fig. 7). The *dpe2-1/ss4* and *phs1a/ss4* double mutants contained a single starch granule per chloroplast irrespective of the light regime during growth (Fig. 6A). From these observations, we conclude that SS4 and PHS1 act independently in the regulation of starch granule number, and neither enzyme acts downstream of the other. Furthermore, SS4 seems to exert a stricter regulation of starch granule number then PHS1, as mutants lacking PHS1, but not SS4, showed greater flexibility in adjusting the granule number in response to changes in photoperiod.

Spherical Starch Granules Are Formed When Both the Starch Granule Number per Chloroplast and the Rate of Starch Degradation Are Reduced

Additionally, we observed that the *dpe2-1/phs1a/ss4* mutant formed large (4.8 ± 0.8 μm diameter [$n = 25$]), spherical starch granules. As revealed by an analysis of the various other double mutant combinations, the observed shape also was detected in the *dpe2-1/ss4* and *dpe2-1/phs1a* mutants, although in the latter, the granules were less round than in *dpe2-1/phs1a/ss4* and *dpe2-1/ss4*. These mutants revealed a strongly reduced starch degradation, given that the starch amount at the end of the light phase and at the end of the dark phase differed by 5% to 18% (Fig. 1D). Similarly, *dpe2-1* revealed a starch degradation of only around 30%, but the obvious difference between *dpe2-1* and the above-mentioned double and triple mutants is that the latter have a strongly reduced granule number per chloroplast. Thus, it could be speculated that the storage of higher amounts of starch resulted in a shift from a discoid to a spherical granule shape. These spherical starch granules seen in the *dpe2-1/ss4* and *dpe2-1/phs1a/ss4* mutants appear to be unique and are not typical of other Arabidopsis mutants that also reveal strongly reduced starch degradation, such as *sex1-8*, *sex1-1*, *sex4*, *lsf1*, *sex1/lsf1* (Zeeman et al., 2002; Comparot-Moss et al., 2010; Mahlow et al., 2014), *mex1*, and *bam3* (data not shown), which all were characterized by flat granules. However, it remains necessary to prove that the observed spherical starch granules were not found exclusively in mutants lacking DPE2. Thus, additional combinations of *phs1a* and *ss4* with other starch-degrading mutants that lack enzymes such as GWD, PWD, or BAM3 need to be generated and their starch granule morphology examined. With regard to the inner structure of the starch granules, only small alterations of the glucan chain distribution pattern were detected, in spite of the fact that the morphology of the starch granules was so different. However, with regard to the ongoing discussion about the function of the plastidial phosphorylase in starch metabolism, it should be noted that, for *phs1a*, a higher number of short glucan chains (degree of polymerization up to 14) was observed (Supplemental Fig. S2), similar to published data for potato (*Solanum tuberosum*) plastidial phosphorylase antisense lines (Orawetz et al., 2016).

We also analyzed several enzymes related to starch metabolism. We observed some alterations in the localization of SS2. Thus, in *dpe2-1/phs1a* and the triple mutant as well as in *phs1a*, SS2 was detected mainly bound to starch granules, whereas in *dpe2-1*, *ss4*, *phs1a/ss4*, and *dpe2-1/ss4*, a significant amount of SS2 also was detected in a soluble state. However, no correlation with an altered inner starch structure was observed, although *ss2* was reported to have a severe starch phenotype (Szydlowski et al., 2011).

SBE2 and SBE3 proteins were clearly detectable by immunoblotting in all of the analyzed lines (Supplemental Fig. S3E). However, despite this, we observed no, or only weak, branching activity in *dpe2-1/phs1a* and *dpe2-1/phs1a/ss4* using native gels (Supplemental Fig. S3D). One potential explanation for this apparent discrepancy is that both SBE2 and SBE3 activities were strongly reduced in both mutants, perhaps due to some posttranslational modifications. However, that the enzymes were inactive during the entire growth period is very unlikely, as in both lines starch granules were formed and it was shown that the lack of both branching enzymes resulted in a starch-free Arabidopsis plant (Dumez et al., 2006). Furthermore, the assay used for branching enzyme activity detection, which stimulates the activity of phosphorylase *a*, seems

to be specific for SBE2, as only the lack of SBE2, but not that of SBE3, was reported to result in a lack of branching enzyme activity in the gel (Dumez et al., 2006). Thus, at least SBE2 may be strongly reduced in activity in *dpe2-1/phs1a* and *dpe2-1/phs1a/ss4* at the time of harvesting. A second possibility also is related to the phosphorylase *a*-based assay, which depends on the elongation of intrinsic glucans bound to the branching enzymes (Brust et al., 2014). Thus, if these glucans were altered or missing altogether in the mutants, the branching activity assay might not detect activity even if the SBE2 and SBE3 enzymes were active. A change in the localization of the branching enzymes to a starch granule-bound state, as observed for the *phs1a/ss4* mutant, was excluded (Supplemental Fig. S3C).

Chloroplast Degradation in the *dpe2-1/phs1a* Background Is Counteracted by the Additional Loss of SS4

It was postulated that the reason for the observed chloroplast disintegration in *dpe2-1/phs1a* is related to the accumulation of maltodextrins (Malinova et al., 2014). However, our results argue against this hypothesis, since the maltodextrin content and profile of the triple and *dpe2-1/phs1a* mutants often were similar but only the latter showed severe chloroplast disintegration. Furthermore, in mature leaves of the triple mutant, normal starch granules and chloroplasts were detected. We also tested the possibility that phosphorylated maltodextrins were accumulated, as described for SEX4 (Kötting et al., 2009), by dephosphorylation of the isolated glucans with a phosphatase; however, no differences were detected (data not shown). From $^{14}CO_2$-labeling experiments of intact plants (data not shown), we found no evidence of an increased turnover of maltooligosaccharides in the triple mutant acting to bypass the partial block in starch metabolism. Thus, we exclude this as a potential explanation for the alleviation of the chloroplast disintegration phenotype in the triple mutant.

ADPglucose Accumulation Is Not Linked Directly to a Lack of SS4

In the *ss4* mutant, massive amounts of ADPglucose were detected, as described previously in relation to wild-type plants (Crumpton-Taylor et al., 2013; Ragel et al., 2013). Ragel et al. (2013) postulated that the accumulation of ADPglucose sequesters much of the stromal Pi pool, limiting photophosphorylation and causing photoinhibition, which leads to chlorosis and reduces growth. Interestingly, although the *dpe2-1/ss4* and *dpe2-1/phs1a/ss4* mutants had elevated ADPglucose compared with the wild type, the levels were nowhere near as high as in *ss4* (Table II). However, the *phs1a/ss4* mutant did have very high levels of ADPglucose, but its growth rate was less reduced than in *ss4* and its starch metabolism was more similar to that of the wild type. In addition, *phs1a/ss4* was characterized by a similar photosynthesis to that exhibited by the wild type (Fig. 5). Moreover, under continuous light, the ADPglucose levels were even more elevated, pointing to a lack of a strict connection between high ADPglucose levels and reduced plant growth.

Our data show that the loss of DPE2, which blocks maltose catabolism in the cytosol and also inhibits starch degradation, largely abolishes the accumulation of ADPglucose observed in mutants lacking SS4. Thus, starch degradation and related processes in the dark appear to have influence on the accumulation of ADPglucose in the light. Furthermore, all mutants lacking DPE2 revealed a strong increase in Glc levels both in the light and in the dark (Table I), possibly reflecting an increased flux of the initial products of starch degradation into Glc, which can be exported from the chloroplasts and metabolized in the cytosol, thereby partially bypassing the block in maltose catabolism caused by the loss of DPE2.

dpe2-1/phs1a and *dpe2-1/phs1a/ss4* Exhibit Highly Complex Phenotypes

In summary, the observed data for both mutants point to several independent but connected phenotypical traits, such as starch granule number per chloroplast, starch granule morphology, and chloroplast intactness. Two further inferences can be made: (1) these traits are linked in some way to interactions between the chloroplasts and cytosol; and (2) factors associated with starch degradation at night influence ADPglucose, starch synthesis, and granule formation during the day. In other words, there are both spatial and temporal interactions underlying these complex phenotypes. Arabidopsis accessions revealed variability in starch metabolism (Cross et al., 2006; Fettke et al., 2009; Sulpice et al., 2009). Thus, we additionally generated the *dpe2-5/phs1b* mutant in the background of Columbia-0. The complex phenotype, including the strong reduction of the starch granule number per chloroplast to mostly one, also was observed for this mutant (Supplemental Fig. S7).

The *mex1/phs1b* mutant accumulates maltose, like *dpe2-1/phs1a*, but shows no difference from the wild type in the number of starch granules per chloroplast (Malinova et al., 2014). This suggests that altered granule number in the *dpe2-1/phs1* and *dpe2-1/phs1a/ss4* mutants is linked to the defect in maltose catabolism in the cytosol. We might speculate that this generates a signal in the cytosol during the night that is transmitted to the chloroplasts and affects starch synthesis and granule formation during the day. The nature of this signal remains obscure. Mutants defective in processes downstream of DPE2 may shed light on this assumption, especially if combined with mutants lacking PHS1 activity. However, the direct impact of starch-derived maltose entering the cytosol could be excluded, as, in

initial analyses, the triple mutant *dpe2-5/phs1b/mex1* (where MEX1 is a maltose exporter in the chloroplast inner envelop membrane; Niittylä et al., 2004) revealed a reduced number of starch granules per chloroplast (Supplemental Fig. S7).

MATERIALS AND METHODS

Plant Material and Growth Conditions

Knockout lines of Arabidopsis (*Arabidopsis thaliana*) *dpe2-1*, *phs1a*, *ss4*, and *dpe2-1/phs1a* in the Wassilewskija-0 background were described previously (Lu and Sharkey 2004; Zeeman et al., 2004; Roldán et al., 2007; Malinova et al., 2014). *phs1a/ss4* and *dpe2-1/ss4* were generated by crossing the respective homozygous single mutants and self-pollination of the F1 generation. The *dpe2-1/phs1a/ss4* mutant was generated by crossing *dpe2-1/phs1a* and *ss4* and self-pollination of the F1 generation.

To screen the F2 generation, activities of DPE2 and phosphorylases were detected by native gels and zymograms. Analysis of SS4 protein was performed by SDS-PAGE, western blotting, and immunodetection. Plants were grown in a light/dark regime (12 h of light, 20°C, 110 μmol m^{-2} s^{-1}/12 h of dark, 16°C; relative humidity throughout the light/dark cycle was kept at 60%) unless stated otherwise.

Starch, Oligoglucans, and Metabolite Profiling

Starch and oligosaccharides were isolated and quantified according to Malinova et al. (2014). Ion chromatography (HPAEC-PAD) was performed as described elsewhere (Fettke et al., 2004).

Native starch granules were isolated from leaves according to Ritte et al. (2000), except that the extraction buffer used was 20 mM HEPES-KOH, pH 7.4, 0.4 mM EDTA, and 0.05% [v/v] Triton X-100.

Gas chromatography-mass spectrometry analyses were performed as described previously (Fettke et al., 2011b). ADPglucose and phosphorylated intermediates were measured as described by Lunn et al. (2006) with modifications as described by Figueroa et al. (2016).

Native PAGE and Zymograms

Native PAGE was performed as described (Fettke et al., 2005). For DPE2 and phosphorylase activities, see Malinova et al. (2014). For starch synthase and branching enzyme activity staining, see Brust et al. (2014) and Dumez et al. (2006), respectively.

SDS-PAGE and Western Blotting

SDS-PAGE and western blotting were performed as described by Mahlow et al. (2014).

SEM and TEM

SEM analysis was performed as described by Mahlow et al. (2014). Starch granule diameters were estimated using SmartTiff Software (Zeiss). TEM samples were analyzed as described by Malinova et al. (2014) using a Philips CM100 transmission electron microscope operated at 100 kV.

Photosynthesis Measurements

Chlorophyll-a fluorescence parameters were measured on five weeks old plants using the MAXY version of the Imaging-PAM (M series, Heinz Walz GmbH, Effeltrich, Germany). The system was equipped with a highly sensitive CCD camera (IMAG-K6) to obtain high-quality chlorophyll *a* fluorescence data even from very small leaf areas of *dpe2-1/phs1a* and the triple mutant. Light-response curves of the chlorophyll *a* fluorescence parameter qL (Kramer et al., 2004) and linear electron flux were measured. Linear electron flux was corrected for leaf absorptance.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. TEM sections of mesophyll chloroplasts.

Supplemental Figure S2. Relative differences of the chain length distribution pattern of leaf starch.

Supplemental Figure S3. Analyses of starch-related enzymes.

Supplemental Figure S4. Heat map of metabolite changes using a false-color scale.

Supplemental Figure S5. ADPglucose pyrophosphorylase activity.

Supplemental Figure S6. Analysis of *dpe2-1/phs1a*, *dpe2-1/phs1a/ss4*, and wild-type plants grown under a 14-h-light/10-h-dark regime.

Supplemental Figure S7. TEM analyses of mesophyll leaf sections.

Supplemental Table S1. Phosphorylated metabolites.

Supplemental Table S2. Soluble sugars.

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