Expression of Sucrose Transporter Complementary DNAs Specifically in Companion Cells Enhances Phloem Loading and Long-Distance Transport of Sucrose But Leads to an Inhibition of Growth and the Perception of a Phosphate Limitation

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Sucrose (Suc) is the predominant form of carbon transported through the phloem from source to sink organs and is also a prominent sugar for short-distance transport. In all streptophytes analyzed, Suc transporter genes (SUTs or SUCs) form small families, with different subgroups evolving distinct functions. To gain insight into their capacity for moving Suc in planta, representative members of each clade were first expressed specifically in companion cells of Arabidopsis (Arabidopsis thaliana) and tested for their ability to rescue the phloem-loading defect caused by the Suc transporter mutation, Atsuc2-4. Sequence similarity was a poor indicator of ability: Several genes with high homology to AtSUC2, some of which have phloem-loading functions in other eudicot species, did not rescue the Atsuc2-4 mutation, whereas a more distantly related gene, ZmSUT1 from the monocot Zea mays, did restore phloem loading. Transporter complementary DNAs were also expressed in the companion cells of wild-type Arabidopsis, with the aim of increasing productivity by enhancing Suc transport to growing sink organs and reducing Suc-mediated feedback inhibition on photosynthesis. Although enhanced Suc loading and long-distance transport was achieved, growth was diminished. This growth inhibition was accompanied by increased expression of phosphate (P) starvation-induced genes and was reversed by providing a higher supply of external P. These experiments suggest that efforts to increase productivity by enhancing sugar transport may disrupt the carbon-to-P homeostasis. A model for how the plant perceives and responds to changes in the carbon-to-P balance is presented.

Suc, whether from photosynthesis or storage reserves, is the principal form of assimilated carbon transported throughout plants. Suc distribution follows a diversity of routes and includes movement through the symplast and transport across plasma membranes for intercellular transport via the apoplast and across endomembranes for intracellular compartmentation (Ayre, 2011). As a relatively large and polar molecule, Suc movement across membranes requires facilitators, which may be passive or energized. SWEETs are a recently described family of transporters participating in passive movement (Chen et al., 2012), while Suc/H+ symporters, alternatively called sucrose transporters (SUTs) or sucrose carriers (SUCs), couple the movement of Suc to the proton motive force to allow energized Suc accumulation. SUTs are best characterized for their role in apoplastic phloem loading in which Suc, after release into the apoplast from photosynthetic cells, is actively accumulated to high concentrations in phloem companion cells in preparation for long-distance transport to heterotrophic tissues. Reducing the activities of SUTs involved in phloem loading by mutation or transcript reduction strategies results in dramatically stunted plants and carbohydrate accumulation in source leaves (Kühn et al., 1996; Gottwald et al., 2000; Slewinski et al., 2009). However, SUTs are also found throughout the plant, and their regulation and role in carbohydrate distribution remain active research areas. The physiological and biochemical functions of SUTs have been regularly reviewed (Sauer, 2007; Braun and Slewinski, 2009; Kühn and Grof, 2010; Ayre, 2011).

In all species studied, SUTs form small families whose members have distinct gene expression patterns, and the encoded proteins have distinct kinetic properties and
subcellular localizations. Different research groups divide the family into three to five clades and use different nomenclature for those subdivisions, but the basic structures of the trees are similar (Aoki et al., 2003; Sauer, 2007; Braun and Slewinski, 2009; Kühn and Grof, 2010). Reinders et al. (2012) recently analyzed the SUT clades in the context of land-plant evolution, following the type designation of Aoki et al. (2003; types I, IIA, IIB, and III; see Supplemental Table S1 for cross-referencing designations used by different laboratories). They propose that the earliest land plants had type II transporters localizing to the plasma membrane and type III transporters localizing to the tonoplast. Among angiosperms, eudicots adapted type III SUTs for phloem loading and other high-affinity uptake processes across plasma membranes, probably by losing the tonoplast localization signals, to form the type I clade, while monocots recruited type II SUTs for similar high-affinity uptake processes to form the type IIB branch (Reinders et al., 2012). The type IIA SUTs are more enigmatic; they are found in all land plants, have lower affinity for Suc, and have been postulated to be Suc sensors rather than functional SUTs (Barker et al., 2000; Barth et al., 2003).

Despite the fact that SUTs predate vascular plants, their current roles in Suc distribution and plant productivity made them mainstays of plant physiology well before their molecular characterization (Giaquinta, 1983; Bush, 1999). The physiological function of specific SUTs has been inferred mostly from gene repression and mutations, expression patterns, and protein localization; biochemical activity has been characterized predominantly by electrophysiology in *Xenopus laevis* oocytes and by Suc uptake assays into yeast (*Saccharomyces cerevisiae*). There are relatively few reports of overexpression (OE) in plants to characterize their contribution to growth and development or for possible gains in productivity. Both *AtSUC1* from Arabidopsis (*Arabidopsis thaliana*; type I; Wippel and Sauer, 2012) and *HvSUT1* from barley (*Hordeum vulgare*; type IIB; Reinders et al., 2012) restored phloem loading in an Arabidopsis *Atsuc2–4* mutant when expressed from the companion cell-specific *AtSUC2* promoter (*AtSUC2p*), showing that their biochemical activity in planta can replace that of *AtSUC2*. *AtSUC2* (type I) encodes the exclusive, or at least the predominant, SUT catalyzing phloem loading in Arabidopsis (Gottwald et al., 2000; Srivastava et al., 2009a). With respect to ectopic expression of SUT genes to study the impact on carbon partitioning, constitutive expression of *SoSUT1* (type I) of spinach (*Spinacia oleracea*) from the *Cauliflower mosaic virus* 35S promoter in potato (*Solanum tuberosum*) resulted in shifts in metabolite levels with little effect on tuber morphology (Leggewie et al., 2003), while OE of *OsSUTSZ* (type IIB) and *OsSUT2M* (type III) of rice (*Oryza sativa*) from a tuber-specific promoter was reported to increase tuber yield (Sun et al., 2011). In other species, ectopic expression of potato *SsSUT1* in the storage parenchyma of pea (*Pisum sativum*) cotyledons during seed development enhanced Suc influx and enhanced cotyledon growth rates (Rosche et al., 2002), and ectopic SUT expression in wheat (*Triticum aestivum*) grains increased levels of storage protein, indicating that enhanced Suc transport has benefits beyond carbohydrate accumulation (Weichert et al., 2010).

Altering phloem transport of Suc by manipulating the SUTs involved in phloem loading has been put forward as a means to improve plant productivity. As one example, Suc loading is modified in response to the physiological and environmental needs of the plant to maintain phloem hydrostatic pressure and coordinate source output with sink demand (Ayre, 2011), and it was proposed that heterologous promoters that are uncoupled from this natural regulation may be useful to keep loading rates constantly high (Srivastava et al., 2009b). Also, increased Suc transport from source leaves to sink organs was proposed to be an effective method to enhance crop productivity (Ainsworth and Bush, 2011), especially in conditions where carbohydrate is in excess (Stitt, 2013). This stems from two predictions: more carbohydrate would be sent to sink organs for growth and/or storage, and there would be more primary productivity because enhanced Suc transport would help remove Suc-mediated product inhibition on photosynthesis (Paul and Foyer, 2001; Stitt et al., 2010; Ainsworth and Bush, 2011).

The objectives of the research reported in this manuscript were (1) to identify a promoter that allows consistently high-level and specific expression in companion cells, (2) to systematically test the capacity of representative members from each SUT family subdivision to transport Suc into the phloem and rescue an *Atsuc2–4* mutation when the genes are expressed specifically in companion cells, and (3) to test the ability of those *SUT* genes that rescue the *Atsuc2–4* phenotype to increase phloem loading and enhance productivity when expressed in companion cells, in addition to the endogenous SUT expression of wild-type plants. For the first objective, we show that the companion cell-specific promoter from *Commelina yellow mottle virus* (*CoYMVp*) has regulation distinct from the endogenous *AtSUC2p*, because it is not subject to feedback inhibition by Suc. For the second objective, each representative *SUT* was expressed from *CoYMVP* in the homozygous *Atsuc2–4* background, which is extremely stunted due to an inability to phloem load Suc and transport it long distance (Srivastava et al., 2008). The ability of each SUT to restore phloem loading and rescue the *Atsuc2–4* phenotype was used to gauge the capacity of that SUT for moving Suc across the plasma membrane from the apoplast to symplast of the companion cells in planta. For the third objective, siblings of the plants used in the second objective that were segregating wild type (*AtSUC2*+/+) instead of *Atsuc2–4* were analyzed for the impact of added SUT expression in companion cells on Suc transport and plant growth.

The study shows that despite sequence conservation, comparable biochemical characteristics, and similar physiological functions in dicots (Supplemental Table S1; Reinders et al., 2012), only two of the tested type I SUT genes, *AtSUC2* and *AtSUC1*, rescued the *Atsuc2–4* phenotype, whereas a divergent, monocot-specific type IIB SUT, *ZmSUT1* from *Zea mays*, effectively restored
growth. No or very poor rescue was achieved with representative type IIA \( (AtSUC3\) and tomato \( (Solanum lycopersicum) \) \( LeSUT2\) and type III \( (LeSUT4) \) \( SUT\) genes. \( SUT\) expression from \( CoYMVp\) in wild-type siblings enhanced \( Suc\) loading and transport, but against expectations, the plants nevertheless accumulated \( Suc\) and other carbohydrate in rosette leaves and were stunted. This inhibition of growth was accompanied by increased expression of phosphate starvation-induced (\( PSI\)) genes, and the growth inhibition was relieved by including more \( P\) to the growth medium, implying that plants with enhanced \( Suc\) loading were perceiving a \( P\) deficiency. The implications of \( SUT\) OE to enhance phloem transport are discussed, and a model for how enhanced \( Suc\) transport to sink organs may induce perception of a \( P\) limitation is presented.

RESULTS

The \( CoYMV\) Promoter Is Activated by \( Suc\) while the \( AtSUC2\) Promoter Is Repressed

\( CoYMVp\) is known to confer strong companion cell-specific expression (Medberry et al., 1992; Matsuda et al., 2002). A construct in which \( CoYMVp\) was fused to \( AtSUC2\) complementary DNA (cDNA) was shown to rescue the \( Atsuc2-4^{-/-}\) knockout mutation (Srivastava et al., 2009b). Both \( BvSUT1\) from sugar beet \( (Beta vulgaris) \) (Vaughn et al., 2002) and \( AtSUC2\) (Osuna et al., 2007) are suppressed by high \( Suc\) levels. We previously suggested that the modified fine-tuning of exotic promoters, such as \( CoYMVp\), compared to \( AtSUC2p\), could benefit plant growth by maintaining high \( AtSUC2\) expression under conditions where it is otherwise repressed (Srivastava et al., 2009b).

To establish if \( CoYMVp\) and \( AtSUC2p\) sequences are differentially regulated, quantitative GUS assays were performed with plants grown on increasing levels of \( Suc\) and harboring \( AtSUC2\) cDNA (c\( SUC\)) fused to the \( uidA\) reporter gene (encoding the GUS enzyme) downstream of either \( AtSUC2p\) or \( CoYMVp\). As expected, GUS activity in the \( AtSUC2p::cSUC2::uidA\) line decreased with increasing levels of \( Suc\) but was enhanced in the \( CoYMVp::cSUC2::uidA\) line (Fig. 1). This demonstrates that \( CoYMVp\) does show different fine-tuning in response to carbohydrate (\( Suc\), specifically) and may maintain high expression and transport levels when the natural gene is repressed. Quantitative GUS assays were also performed with plants grown on increasing mannitol levels for osmotic stress and sodium chloride to induce salt stress. In both lines, GUS activity increased with similar patterns (Supplemental Fig. S1, A and B). Therefore, under osmotic or salt stress, \( CoYMVp\) probably will not enhance phloem transport beyond the capacity of the natural \( AtSUC2\) promoter.

Sequence Similarity Is Not a Strong Indicator of \( SUT\) Capacity to Rescue \( Atsuc2^{-/-}\) Mutants

Having established that \( CoYMVp\) can drive \( cSUC2\) expression in a manner sufficient to rescue \( Atsuc2-4^{-/-}\) (Srivastava et al., 2009b) and that, unlike the endogenous \( AtSUC2p\), activity of \( CoYMVp\) is not repressed by high \( Suc\) levels, it was used to test the capacity of other \( SUTs\) to restore or improve phloem loading and long-distance transport in \( Atsuc2-4^{-/-}\). Representative \( SUT\) genes from each of the four major branches of the gene family were subcloned as cDNA downstream of \( CoYMVp\) by Gateway recombination (generically referred to as \( CoYMVp::cSUTX\); Fig. 2). The kinetic properties and physiological role of each have been previously reported (see Supplemental Table S1 for references). The \( CoYMVp::cSUTX\) cassettes were transformed into heterozygous \( Atsuc2-4^{-/-}\) plants, and transgenic progeny (T1) were genotyped as \( Atsuc2-4^{-/-}\) or as \( Atsuc2-4^{-/-}\) or \( Atsuc2-4^{-/-}\) by PCR. For each construct, 25 to 30 independent heterozygous \( Atsuc2-4^{-/-}\) T1 plants were identified that contained transfer DNA (T-DNA) at a single locus, based on a 3:1 segregation for glufosinate ammonium resistance in the T2 generation. From these, T3 or T4 plants that were homozygous \( Atsuc2-4\) knockouts and also homozygous for the rescuing \( CoYMVp::cSUTX\) cassette were identified, and growth parameters were compared in plants that had been grown for 21 d. Independent lines showed a range of phenotype rescue, as expected, and the most robust lines were selected for further analysis. The level of rescue expressed in terms of average rosette area of the two most robust lines for each cassette is shown in Figure 3.

Based on the visual phenotype, the \( SUT\) cDNA cassettes could be readily placed into one of two groups: those that promoted rescue (type I \( AtSUC2\) and \( AtSUC1\) and type IIIB \( ZmSUT1\)) and those that did not (type I \( AtSUC3\), \( LeSUT1\), and tobacco \( [Nicotiana tabacum] \) \( NtSUT3\); type IIA \( AtSUC3\) [also called \( AtSUT2\) and \( LeSUT2\); and type III \( LeSUT4\)]. Transgene expression in the most robust lines of each cassette was determined by reverse transcription-quantitative
PCR (RT-qPCR). In all cases, transcript abundance was 50% to 80% that of AtSUC2 in wild-type plants (Fig. 3C). We previously documented that a full gradient of rescue, from none to near-full rescue, could be observed among independent Atsuc2-4 lines expressing AtSUC2 cDNA (Srivastava et al., 2009b). Based on this, the lack of growth among those that did not rescue is striking and argues that the degree of rescue (i.e. restoration of vegetative growth) is not the direct result of transcript abundance (compare Fig. 3A and Fig. 3C). Possible reasons for the lack of rescue are addressed further in “Discussion” and further analysis focused on the AtSUC2, AtSUC1, and ZmSUT1 lines that were rescued by their respective transgenes.

Each of the rescued lines was smaller than the wild type at 21 d postgermination (Fig. 3), suggesting that although growth was largely restored, there still may be some impairment to Suc transport. When Suc transport is impaired, transient carbohydrates accumulate in photosynthetic tissues. Suc, Glc, Fru, and starch in rosettes were therefore quantified to gauge rescue at the level of Suc export from leaves (Fig. 4). Carbohydrate accumulation showed the expected inverse relationship with rosette growth for impaired export: smaller rosettes (less rescued; e.g. AtSUC1 lines) had more accumulated carbohydrate than larger rosettes (more rescued; e.g. AtSUC2 lines). Collectively, these results suggest partial rescue based on relative expression and imply that the capacities of AtSUC2, AtSUC1, and ZmSUT1 for moving Suc across membranes in plants are roughly equal, consistent with kinetic parameters measured in heterologous systems (Chandran et al., 2003; Carpaneto et al., 2005; Sivitz et al., 2008).

Additional Companion Cell-Specific SUT Expression Enhances Phloem Loading But Not Productivity

The analysis above shows that AtSUC2, AtSUC1, and ZmSUT1 cDNA fused to CoYMVp can restore phloem loading to appreciable levels in the Atsuc2-4-/- background. We hypothesized that an additive capacity to phloem load would be achieved if these cassettes were introduced into the wild type (AtSUC2+/+). Because the CoYMVp::SUTX cassettes were transformed into heterozygous AtSUC2+/- plants, AtSUC2+/- siblings of rescued lines (i.e. the same transformation event) were used to test if additional SUT expression can hyperload the phloem. These AtSUC2+/- lines with CoYMVp::SUTX cassettes thus have SUT expression from both the endogenous AtSUC2 promoter and the differentially regulated CoYMVp, and CoYMVp::AtSUC1 and CoYMVp::ZmSUT1 plants, additionally, have two different symporters (AtSUC2 plus the transgene-encoded SUT), which may have different posttranslational regulation, participating in phloem loading. These lines are referred to as SUT OE lines because they have additional companion cell-specific SUT expression over the natural expression from the endogenous AtSUC2 gene.

Additive SUT expression from the endogenous gene and the cDNAs was confirmed by RT-qPCR. Expression of the cDNA from the companion cell-specific CoYMVp roughly equaled or slightly exceeded that of the endogenous gene from the natural AtSUC2 promoter (Fig. 5A; Supplemental Fig. S2). With additive SUT expression, phloem loading, Suc transport, and growth were expected to exceed or at least equal that of the wild type. Unexpectedly, the rosette areas of wild-type lines expressing SUT cDNA were not only smaller than wild-type plants but were also smaller than those of the rescued Atsuc2-4-/- siblings (Fig. 5, B and C).

To assess if the observed phenotype is linked to carbon partitioning, the major transient carbohydrates were quantified at the end-of-day (ED) and end-of-night (EN) time periods. At 21 d postgermination, SUT OE lines had more Glc (Fig. 6A), Fru (Fig. 6B), and Suc (Fig. 6C) at ED compared with the wild type. The SUT OE lines also contained more soluble sugar than the wild type at EN, but the differences were subtle and, in many cases, not statistically significant. Starch did not show any significant differences between the wild type and the SUT OE lines at ED or EN (Fig. 6D). Apart from the elevated levels of soluble sugar in the SUT OE lines, the diurnal patterns of carbohydrate patterns are consistent with published results: Diurnal starch turnover tends to keep the levels of soluble sugars somewhat constant through the 24-h period (Geiger and Servaites, 1994), with slightly higher levels during the day (Bläsing et al., 2005; Gibon et al., 2009; Sulpice et al., 2014). Photosynthesis was estimated by pulsed-amplitude-modulated fluorescence and revealed reductions consistent with the accumulation of sugar in the rosettes (Supplemental Fig. S3).

To test if carbohydrate accumulation in rosettes was affecting sugars in heterotrophic organs, the wild type

Figure 2. T-DNA cassettes used in this study. A, T-DNA cassette of the Gateway recombination compatible parent vector pGPTV-CoYMVp::ccdB::CmR. B, T-DNA cassettes of the expression vectors used for floral dip transformation and harboring representative SUT cDNA from each of the four branches of the SUT gene family. White indicates type I, light gray indicates type IIa, dark gray indicates type IIb, and black indicates type III. LB, T-DNA left border; RB, T-DNA right border; NOSp-Bar-NOSpA, nopaline synthase promoter-Bialaphos (glutosate ammonium) resistance cDNA-nopaline synthase polyadenylation signal.

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To test if carbohydrate accumulation in rosettes was affecting sugars in heterotrophic organs, the wild type
and two SUT OE lines (At2-1-6 and Zm1-6-8) were grown on one-half-strength Murashige and Skoog (MS) medium without Suc, and shoots and root tips were analyzed separately. One centimeter of root tip was used for analysis because the tip is the strongest sink. Suc levels 4 h into the light period were higher in both the shoots and root tips of SUT OE lines compared with the wild type (Fig. 6, E and F). More Suc in the root provides evidence that transport to sink organs was improved rather than compromised and suggests that sink organ growth patterns may be altered (Kehr et al., 1998). To test for this, a high-resolution time lapse camera was used, and changes in the pattern of diurnal root growth were observed. In standard growth cabinet conditions, wild-type roots have a distinctive elongation pattern: there is a peak when the lights first come on, followed by a rapid drop and leveling off in the remainder of the light period; shortly after the transition to darkness, the root elongation rate drops, followed by recovery as the night period progresses (Yazdanbakhsh et al., 2011). This pattern is observed in the wild type (Fig. 6E) but is altered in the SUT OE lines: instead of showing a transient peak and subsequent decline, growth increases after illumination, but less markedly, and remains fairly constant for the remainder of the light period. All genotypes showed a similar response during the night, with a decrease in the rate of root elongation at the start of the night followed by a partial recovery in the middle of the night and a decrease in the last hours of the night (Fig. 6E). These alterations are consistent with the regulation of the CoYMVp::cSUTX transgenes relative to the endogenous AtSUC2 gene: Suc accumulation during the day may increase SUT cDNA expression from CoYMVp and enhance transport to roots, resulting in more consistent elongation throughout the day compared with the wild type.

To further investigate if SUT OE increases phloem transport from source to sink organs, rosettes were photosynthetically labeled with [14C]CO₂ for 20 min in the middle of the 14-h light period, and roots were harvested to measure transport of label by scintillation counting. As a percentage of total label in the plant, all three SUT OE lines transported more label to the root, indicating that more photoassimilate produced during the 20-min labeling period was loaded into the phloem and allocated to roots (Fig. 7A). When these values were standardized against the wild type, the SUT OE lines transported 25% to 30% more label to the root. There was no significant difference among the SUT OE lines and the indicated rescued Atsuc2-4⁻⁻ line. For scale, the round pots are 5 cm. C, SUTX transcript levels determined by RT-qPCR, relative to the elongation factor 1α (EF1α) transcript (n = 3 individual plants as biological replicates, each with two technical replicates). Variation is expressed as ±s. Different letters represent significant differences based on univariate ANOVA with Scheffe post hoc analysis at the 0.05 level. Note that all transgenic plants are in subgroups B or C with respect to SUTX expression.
wild type for each carbohydrate are based on Student's t test. In these experiments, the rate of 14C exudation over the 20 min of exudation, which would contain the contents of cut cells, was discarded, and 14C exudation over the next two 1-h intervals was measured and expressed as counts per minute exuded per hour per milligram of fresh weight. The WT OE plants had phloem exudation rates 4- to 5-fold higher than the wild type (Fig. 7B), indicating more efficient loading and transport of carbon in the phloem.

Figure 4. Principal soluble sugars and starch in 21-d-old rosettes of the wild type (WT) and the indicated rescued line. A, Glc, Fru, and Suc levels expressed as nanomoles per milligram of fresh weight (FW). B, Starch levels in the same samples represented in A, expressed as nanomoles Glc equivalents per milligram of fresh weight. Variation is se (n = 6 rosettes of individual plants). Significant differences from the wild type for each carbohydrate are based on Student’s t test (*P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001).

lines, indicating that each of the rescuing constructs enhanced phloem transport equally well.

In another method to address the question of whether the SUT OE lines have enhanced long-distance transport, the rate of 14C exuded from cut stems into EDTA solution was measured. In these experiments, the first 20 min of exudation, which would contain the contents of cut cells, was discarded, and 14C exudation over the next two 1-h intervals was measured and expressed as counts per minute exuded per hour per milligram of fresh weight. The SUT OE plants had phloem exudation rates 4- to 5-fold higher than the wild type (Fig. 7B), indicating more efficient loading and transport of carbon in the phloem.

14C allocation to roots and phloem exudation rates both measure long-distance transport, which is dependent on loading efficiency, but neither measure directly addresses phloem loading (Turgeon and Wolf, 2009). To compare loading among the wild type and SUT OE lines, uptake of 14C into leaf veins was measured directly: leaf disks were infiltrated with a [14C]Suc solution for 20 min, washed thoroughly, and freeze dried, and phloem loading was assessed by autoradiography and scintillation counting. The results show that there is more loading in the SUT OE lines compared with the wild type (Fig. 7C). Taken together, the experiments above show enhanced loading and long-distance transport, as well as a higher availability of carbon in both source and root sink tissues.

Enhanced Suc Loading and Transport Triggers Perception of a Phosphorous Limitation

The results above argue that phloem loading and transport are enhanced (Figs. 6 and 7), yet the SUT OE lines are smaller than the wild type and the rescued Atsuc2-4–/– lines (Fig. 5). This is counterintuitive because enhanced transport is predicted to increase carbon supply to growing sink organs while also reducing feedback inhibition on photosynthesis in source leaves (Ainsworth and Bush, 2011). In addition to being important for metabolic activities, sugars provide the majority of biomass for new growth and storage, are important signaling molecules for plant growth and development (Rolland et al., 2006), and interact with other essential nutrients. For example, more carbon from source organs is required for a response to a phosphorous limitation because P deficiencies induce many changes that have high demand for photoassimilate, including enhanced growth of lateral roots and root hairs, changes in gene expression, remodeling of metabolism, secretion of organic acids, and energization of membranes (Misson et al., 2005; Morcuende et al., 2007; Hammond and White, 2008, 2011; Plaxton and Tran, 2011; Veneklaas et al., 2012). Conversely, utilization of sugars and storage carbohydrates requires P for metabolism, which occurs mostly by ATP-dependent formation of phosphoesters. Recently, the mutation hypersensitive to phosphate starvation1 (hps1) was isolated in an activation-tagging screen as having enhanced sensitivity to P deficiency (Lei et al., 2011). Interestingly, hps1 results in ectopic and ubiquitous activation of AtSUC2, and growing hps1 mutants in the presence of excess P reversed the pleiotropic effects. The authors argued that Suc is a global regulator of P starvation (Lei et al., 2011).

hps1 resulted in ectopic SUT OE in all tissues, which is likely to lead to major changes in the distribution of Suc and many pleiotropic responses. Our observation that phloem-specific SUT OE resulted in diminished growth prompted us to test if this also resulted in an increased requirement for P. Wild-type and SUT OE lines were grown on vertical plates with one-half-strength MS, which includes 0.6 mM P, and 0% Suc, with and without additional P. In the absence of additional P, rosettes and roots of SUT OE plants were stunted relative to the wild type. SUT OE roots were approximately two-thirds the length of wild-type roots and had fewer lateral roots (Fig. 8, A and C). On medium supplemented with additional PO43− (ranging from 1.2 to 3 mM total PO4), root length and rosette size of the SUT OE lines recovered and equaled the wild type (Fig. 8, B and C).

It was previously observed that expression levels of PSI genes in Arabidopsis seedlings positively correlate with Suc concentration in the culture medium (Karthikeyan...
et al., 2007). To test if companion cell-specific OE of SUT genes enhances PSI gene expression in the absence of exogenous Suc, representative PSI genes were analyzed by RT-qPCR. \( A:\text{PHT2 (PHT1;4, At2G38940) and PHT2;1 (At3G26570) are inorganic P transporters induced by P starvation (Karthikeyan et al., 2007; Lei et al., 2011). Both had higher expression levels in the SUT OE lines than in the wild type (Fig. 8, D and E).} \)

**Figure 5.** Phloem-specific SUT transcript abundance in the wild-type (WT; \( \text{AtSUC2}^{+/+} \)) background and growth characterization. A, Phloem-specific SUT transcript levels relative to \( \text{EF1a} \) determined by RT-qPCR in the wild-type (\( \text{AtSUC2}^{+/+} \)) background. cDNA expressed from \( \text{CoYMVP} \) (white) is superimposed on the endogenous \( \text{AtSUC2} \) expressed from the natural \( \text{AtSUC2} \) promoter (black). Variation is expressed as se (\( n = 3 \) individual plants as biological replicates, each with two technical replicates). See also Supplemental Figure S2. B, Quantitative comparison of rosette area in 21-d-old \( \text{Atsuc2-4}^{-/-} \) knockout (KO), SUT transgene-rescued \( \text{Atsuc2-4}^{-/-} \) knockout lines (\( \text{At2-1-3, Zm1-6-3, and At1-4-1} \)), the wild type, and wild-type lines with additional companion cell-specific SUT transgene expression (\( \text{At2-1-6, Zm1-6-8, and At1-4-4} \)). Note that rescued knockout lines and the wild type with SUT expression are segregants of the same transformation event and are thus genetically identical with respect to the SUT transgene. Variation is se (\( n = 8 \) plants). Different letters represent significant differences based on univariate ANOVA with Scheffé post hoc analysis at the 0.05 level. C, Representative 21-d-old knockout (\( \text{Atsuc2-4}^{-/-} \)), SUT transgene-rescued \( \text{Atsuc2-4}^{-/-} \) knockout plants (\( \text{At2-1-3, Zm1-6-3, and At1-4-1} \)), the wild type, and wild-type plants over-expressing the SUT transgenes (\( \text{At2-1-6, Zm1-6-8, and At1-4-4} \)), as labeled. The genetic background of each line is indicated in parentheses behind the line designations: \( ^{-/-} \) for the rescued knockout background and \( ^{+/+} \) for the wild-type background with additional companion cell-specific SUT expression. For scale, the round pots have a 5-cm diameter.
secrete acids into the rhizosphere to help solubilize available P (Hammond and White, 2008). The wild type, At2-1-6, and Zm1-6-8 were grown on one-half-strength MS with 0% Suc and supplemented with bromocresol blue indicator dye. Only in the vicinity of the SUT OE lines did the media change from blue to yellow, indicating a drop from the starting pH of 6.8 to less than pH 5.0 and suggesting that these roots were secreting acid in response to perception of a P limitation (Supplemental Fig. S4).

One representative companion cell-specific SUT OE line, At2-1-6, was selected for further study. Plants were grown on a range of P concentrations and photosynthetically labeled with \([^{14}C]CO_2\) for 20 min. Relative to the wild type, At2-1-6 consistently accumulated a larger percentage of total assimilated carbon in the roots, implying enhanced transport to root sinks (Fig. 9A). This increase in \(^{14}C\) export to the roots was seen with all PO\(_4\) supplementations, although it was less pronounced with 3 mM PO\(_4\). Because P deficiency can activate phosphoenolpyruvate carboxylase to incorporate \(^{14}C\) directly into roots independent of photoassimilate transport (Plaxton and Tran, 2011), roots that were freshly severed from their rosettes were also labeled with \([^{14}C]CO_2\). In all experiments, \(^{14}C\) incorporation into severed roots was essentially indistinguishable from background, while roots attached to shoots showed substantial accumulation of \(^{14}C\) (Fig. 9B). This indicates that the higher percentage of label in At2-1-6 roots relative to the wild type was the result of enhanced Suc import.

**SUT OE from the Natural AtSUC2 Promoter Causes the Same Phenotype as Expression from the CoYMV Promoter**

It was previously shown that constitutive OE of AtSUC2 from the Cauliflower mosaic virus 35S promoter could trigger a P limitation response (Lei et al., 2011). However, this might be due to inappropriate expression of Suc transport activity in cells that normally do not accumulate Suc from the apoplast. It is striking from our results that phloem-specific expression has a similar effect. While CoYMVP\(_p\) has the same spatial expression as AtSUC2p, it responds differently to various stimuli, particularly increased Suc levels (Fig. 1). It is possible that differences in the regulation of SUT expression, and not enhanced Suc transport per se, were responsible for oriented plates for access to roots. E, Sugar levels in rosettes expressed as nanomoles per milligram of fresh weight per hour. F, Sugar levels in 1 cm of the primary root tip of the plants in E to emphasize the phloem-unloading zone and strongest heterotrophic sinks, expressed as nanomoles per centimeter of root tip. For A to F, black bars represent the wild type, white represents At2-1-6, light gray represents Zm1-6-8, and dark gray represents At1-4-4 (A-D only); variation is \(\sigma (n = 6\) individual plants). Significant differences from the wild type for each carbohydrate are based on Student’s t test (*\(P \leq 0.05\), **\(P \leq 0.01\), and ***\(P \leq 0.001\)). G, Root elongation expressed as micrometers per hour of the wild-type and SUT overexpressing lines, as indicated, grown on synthetic medium in vertically oriented plates (\(n = 6\) individual plants).}
roots were analyzed separately by scintillation counting, and 14C transported
based on Student’s t test (*t* = 6). B, Phloem exudation from severed rosettes of
soil-grown plants into EDTA-containing solution after photosynthetic label-
ing with [14C]CO2 shows enhanced phloem transport of [14C] relative to the
wild type (WT), expressed as a rate of counts per minute per hour per
milligram of rosette fresh weight (FW). Variation is expressed as $\pm$ (n = 12
exudations for each line; three of each line in four labeling chambers). C,
Uptake of [14C]Suc into leaves of wild-type and SUT OE lines expressed as
counts per minute per square centimeter of leaf area. The results are the
same when expressed as counts per minute per gram of leaf tissue (not
shown). CPM, Counts per minute; Variation is $\pm$ (n = 16 mature leaves from
four plants). For A, B, and C, significant differences from the wild type are
based on Student’s t test (*P* $\leq$ 0.05, **P* $\leq$ 0.01, and ***P* $\leq$ 0.001).

Figure 7. Additional companion cell-specific SUT expression enhances Suc
loading and long-distance transport as determined by [14C] transport to roots,
exudation from cut rosettes, and direct [14C]Suc uptake. A, Plants grown on
vertical plates were photosynthetically labeled with [14C]CO2, shoots and
roots were analyzed separately by scintillation counting, and [14C] transported
to the roots was expressed as a percentage of total [14C] incorporated. Vari-
ation is expressed as $\pm$ (n = 6). B, Phloem exudation from severed rosettes of
soil-grown plants into EDTA-containing solution after photosynthetic labeling
with [14C]CO2 shows enhanced phloem transport of [14C] relative to the
wild type (WT), expressed as a rate of counts per minute per hour per
milligram of rosette fresh weight (FW). Variation is expressed as $\pm$ (n = 12
exudations for each line; three of each line in four labeling chambers). C,
Uptake of [14C]Suc into leaves of wild-type and SUT OE lines expressed as
counts per minute per square centimeter of leaf area. The results are the
same when expressed as counts per minute per gram of leaf tissue (not
shown). CPM, Counts per minute; Variation is $\pm$ (n = 16 mature leaves from
four plants). For A, B, and C, significant differences from the wild type are
based on Student’s t test (*P* $\leq$ 0.05, **P* $\leq$ 0.01, and ***P* $\leq$ 0.001).

DISCUSSION

Solute accumulation in the phloem is a dynamic process that helps coordinate the needs of sink tissues with the output capacity of source tissues and, through osmotic adjustment, maintains phloem hydrostatic pressure during changes in plant water balance. Suc uptake and the activity of the predominant phloem Suc/H+ symporters is regulated by numerous physiological and environmental stimuli, including leaf development, light, diurnal cycles, sugar signaling, and turgor (Vaughn et al., 2002; Ainsworth and Bush, 2011; Ayre, 2011). Dissecting these specific regulations will contribute to our understanding of how plants control transport processes and response to various stimuli and, from an applied perspective, may provide knowledge that can be used to maintain high rates of transport to target biomass partitioning to desired tissue and organs. Enhanced phloem transport has been proposed as a potential means to increase plant productivity by simultaneously enhancing transport to sink organs and reducing sugar-mediated feedback inhibition on photosynthesis (Ainsworth and Bush, 2011).

Plants contain multiple Suc transporters with differing expression patterns, properties, and subcellular locations. It was shown previously that AtSUC2 cDNA expressed from various phloem-specific promoters can rescue Atsuc2-4/−/− knockout plants (Srivastava et al., 2008, 2009b). It was predicted that although these promoters had similar spatial expression as the natural AtSUC2p, differences in responses to stimuli could be used to maintain high levels of SUT expression and, consequently, high levels of phloem transport in conditions where transport may normally be down-regulated (Srivastava et al., 2009b).

CoYMVp is from Commelina yellow mottle virus, but despite its viral origin, it is specific to phloem companion cells (Medberry et al., 1992; Matsuda et al., 2002). Research presented here demonstrates that while AtSUC2p is repressed, CoYMVp is up-regulated in the presence of elevated Suc levels and thus can be used to bypass possible endogenous regulation of AtSUC2 expression at the transcriptional level. In addition to transcriptional regulation, there is evidence for posttranslational regulation of SUT activity through protein-protein interactions (Schulze et al., 2003; Krügel et al., 2008, 2009; Fan et al., 2009). To potentially bypass these levels of regulation, SUT genes from each of the four subfamily types (Reinders et al., 2012) were tested for the capacity to

the increased requirement for external P. To test this, AtSUC2 cDNA was overexpressed from 2 kb of the natural AtSUC2p. Line KD1039 was previously described as an Atsuc2-4/−/− mutant rescued with AtSUC2 cDNA expressed from 2 kb of AtSUC2p (Srivastava et al., 2009b). Line KD476 is a sibling of KD1039 segregating AtSUC2+/−. As shown in Figure 8, KD476 plants overexpressing AtSUC2 cDNA from AtSUC2p have a similar growth phenotype to that of plants expressing AtSUC2 from CoYMVp, implying that they are also perceiving a P limitation. This excludes the possibility that the inhibition of growth in lines in which SUT cDNA is expressed under the control of CoYMVp is due to a subtle difference in the expression pattern between this heterologous promoter and the endogenous AtSUC2p.
**Figure 8.** SUT OE lines perceive a P deficiency as indicated by rescue of wild-type (WT) levels of growth with P supplements and increased expression of PSI genes. A, Wild-type and SUT OE plants, as indicated, grown on one-half-strength MS medium with Gamborg’s vitamins (0.6 mM total P). SUT OE lines have stunted rosettes and shorter roots. B, As in A, with 1.2 mM additional P (1.8 mM total). Rosette and root growth is rescued to wild-type levels. Bars in A and B = 0.5 cm. C, Quantitative assessment of root elongation (cm) with the indicated P concentrations (mM). Variation is se (n = 8 plants per line). Significant differences from the wild type at each P concentration are based on Student’s t test (**P ≤ 0.01 and ***P ≤ 0.001). D to G, Increased expression of PSI genes AtPT2 (D), AtPHT2;1 (E), AtPAP14 (F), and AtPAP24 (G) among SUT OE lines relative to the wild type indicate that SUT OE lines are perceiving a P deficiency. All plants were grown on potting mix for 21 d as described in “Materials and Methods” (n = 2 biological replicates with 2 technical replicates). Significant differences in expression from the wild type are based on Student’s t test (*P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001).
rescue the Atsuc2-4<sup>-/-</sup> mutation and restore efficient phloem transport.

A restoration-of-growth bioassay in Atsuc2-4<sup>-/-</sup> knockout plants was used as a proxy metric for the capacity of each SUT to load Suc into the phloem. The SUTs tested fell into two groups: those that did restore phloem transport and those that did not. As expected, expression of AtSUC2 using CoYMVp complemented the Atsuc2-4<sup>-/-</sup> mutant. However, among the other eudicot-specific type I SUTs tested, only AtSUC1 restored phloem transport. It was previously shown that AtSUC1 cDNA fused to AtSUC2p restores phloem loading in an Atsuc2 mutant (Wippel and Sauer, 2012), and here, we restore phloem transport with AtSUC1 expression driven from a viral promoter. Type IIB SUTs are used for phloem loading and other high-affinity uptake processes in monocots. Type IIB ZmsSUT1 cDNA efficiently rescued homozygous Atsuc2-4<sup>-/-</sup> mutants, even though the monocot type IIB and eudicot type I SUT genes separated in one of the earliest branches of the streptophyte SUT gene family (Reinders et al., 2012). It is also documented that HeSUT1 from barley can restore phloem transport to Atsuc2-4<sup>-/-</sup> knockout mutants (Reinders et al., 2012). In the study reported here, rescued plants were slightly stunted and accumulated carbohydrate in rosette leaves, implying that Suc transport was not fully restored to wild-type levels but was sufficient for near-normal growth. Because RT-qPCR revealed that SUT expression was less than that in the wild type, compromised transport is not surprising.

Type IIA SUTs are characterized as low-affinity/high-capacity plasma membrane SUTs that predate the evolution of vascular plants (Reinders et al., 2012). The kinetic parameters of this group are sufficiently different from those that catalyze phloem loading that it is not surprising that the representative members tested did not rescue the Atsuc2-4<sup>-/-</sup> mutant. Type III SUTs from Arabidopsis, barley, and rice localize to the tonoplast and are proposed to be an ancient tonoplast-localized family, but tomato LeSUT4 and potato StSUT4 are reported to localize to the plasma membrane (Weise et al., 2000; Chincinska et al., 2008). Because of this discordance, we tested the full-length LeSUT4 sequence in Arabidopsis with the rationale that restoration of phloem transport would support plasma membrane localization. However, transport was not restored, and this negative result does not illuminate the question of LeSUT4 localization.

Perhaps the more surprising outcome here is that many other type I SUTs did not restore phloem transport. AtSUC9 has the highest affinity for Suc among the plant SUTs, and the encoding gene is expressed broadly in sink organs, whereas NsSUT3 and LeSUT1 are the major transporters for phloem loading in the Solanaceae species tobacco and tomato (Lemoine et al., 1999; Hackel et al., 2006; Weise et al., 2008). One possible explanation for the lack of complementation is that these constructs are not expressed at a high enough level. However, we previously demonstrated a broad gradient of rescued phenotypes among Atsuc2-4 lines that were independently transformed with constructs for companion cell-specific expression of AtSUC2 cDNA (Srivastava et al., 2009b). This argues that the lack of rescue was not the result of a failure to achieve a required threshold of expression, although this possibility is not formally excluded. Posttranscriptional or posttranslational regulation that limited production, accumulation, or localization of the proteins are also possible explanations for the lack of rescue. For example, SUT1 and BvSUT1 proteins in potato and sugar beet, respectively, were shown to have half lives in the range of several hours (Kühn et al., 1997; Vaughn et al., 2002), and differential rates of degradation among the proteins encoded by the transgenes tested here may have contributed to the lack of rescue. Notwithstanding, these negative results are worthy of mention because each of these SUTs localizes to the plasma membrane and catalyzes membrane transport of Suc in heterologous organisms (yeast and/or X. laevis oocytes), showing that they do not have an obligate need of plant-specific cofactors or protein-protein interactions for activity. An intriguing possibility for why these type I SUTs did not rescue is that a plant-specific cofactor or protein, which is present in the phloem but missing in yeast and X. laevis oocytes, may act as an inhibitor that is removed to activate...
these SUTs in specific organs or with specific stimuli.

We next tested if the three Suc transporters that could rescue $Atsuc2-4^{-/-}$ knockout plants (i.e. $AtSUC1$, $AtSUC2$, and $ZmSUT1$) increased phloem loading and plant growth when overexpressed in companion cells of wild-type $AtSUC2-4^{+/+}$ segregants. Unexpectedly, segregating $AtSUC2^{+/+}$ sibling lines with companion cell-specific expression of SUT cDNA, in addition to the wild-type expression from the endogenous $AtSUC2$ gene, were more stunted and contained higher sugar levels in rosettes than their rescued $Atsuc2-4^{-/-}$ counterparts. Starch accumulation did not differ significantly between wild-type and SUT OE lines. Altered patterns of root elongation were also observed: wild-type plants showed an expected burst of elongation shortly after the onset of day, followed by a gradual decline toward night, but this pattern was distinctly muted in SUT OE lines. A possible explanation for these results is that the use of C for growth, as well as the storage and remobilization of transient C reserves, in both source and sink organs is subject to regulation by the circadian clock (Dodd et al., 2005; Graf et al., 2010; Stitt and Zeeman, 2012; Scialdone et al., 2013), but added SUT expression from the transgenes disrupts the coordinated distribution of C between source and sinks.

Stunting and carbohydrate accumulation in the SUT OE lines was unexpected because our premise was that up-regulating SUT expression should enhance phloem transport and growth. Transcript analysis indicated that introduction of $AtSUC1$, $AtSUC2$, and $ZmSUT1$ cDNA did not lead to silencing of the endogenous $AtSUC2$ gene (Fig. 5). Prior reports argue that SUT proteins can form homodimers and heterodimers when expressed in the same cells, and the ratio of monomers to dimers is influenced by protein abundance (Reinders et al., 2002; Krügel et al., 2008). These putative interactions could impede posttranslational regulation of SUT activity and lead to an inhibition of phloem loading. For this reason, multiple lines of experimentation were carried out to establish that OE of $AtSUC1$, $AtSUC2$, and $ZmSUT1$ cDNA in companion cells leads to enhanced loading and long-distance transport compared with the wild type. First, after labeling intact plants with $^{14}$CO$_2$, a greater percentage of $^{14}$C was transferred to roots in the SUT OE lines than the wild type. Second, in EDTA exudation experiments, in which transport is not limited by sink capacity to receive the contents of the translocation stream, exudation rates of $^{14}$C were 2- to 4-fold higher in the SUT OE lines than the wild type. Third, $^{14}$C-Suc uptake into the veins of leaf disks, which is a direct measure of phloem loading, was enhanced in the SUT OE lines compared with wild-type plants (Fig. 7).

But if phloem loading and transport are more efficient in SUT OE lines, why were the plants debilitated? There is growing evidence that long-distance Suc transport plays an important role in phosphorous responses (Liu et al., 2005; Karthikeyan et al., 2007; Hammond and White, 2008, 2011). The $pho3$ mutation, which was identified in a screen for reduced acid phosphatase activity during P limitations, turned out to be a mutant allele of $AtSUC2$ (Lloyd and Zakhleniuk, 2004). Both a reduction in photosynthesis and stem girdling, which blocks phloem transport, attenuate P deficiency responses in roots, including a decrease in the expression of many PSI genes (Liu et al., 2005; Karthikeyan et al., 2007). In addition, it was recently shown that ubiquitous SUT
OE can induce a P deficiency response (Lei et al., 2011), potentially by disrupting normal Suc distribution patterns. It is understandable that normal and efficient Suc transport is required for an effective response to P limitation, because P limitation induces processes that have a high demand for photoassimilate, such as growth of lateral roots and root hairs, changes in gene expression, remodeling of metabolism, secretion of organic acids, and energetics of membranes (Misson et al., 2005; Morcuende et al., 2007; Plaxton and Tran, 2011; Veneklaas et al., 2012). However, what is significant and different about the result presented here compared to previous studies is that enhanced Suc transport along the normal distribution path triggers a P-limited phenotype. Our results imply not only that Suc is required to mount a response to a P deficiency, but also that too much Suc in sink organs is apparently interpreted as a P limitation. The same phenotype was obtained whether AtSUC2, AtSUC1, or 2mSUT1 cDNA was overexpressed and, for AtSUC2, whether this was done using CoYMVP or AtSUC2p. Achieving the same phenotype with an AtSUC2p::AtSUC2 cDNA construct is also noteworthy because it argues that AtSUC2 expression, phloem loading, and Suc transport are under prominent dosage level control (i.e. the higher the gene copy number, the more severe the phenotype), in addition to transcription and post-transcriptional control.

We envision several possibilities, which are not mutually exclusive, to explain this link between the supply of Suc and the requirement for P (Fig. 11). One possibility is that the association between long-distance Suc transport and P deficiency is predominantly due to metabolic constraints. Although Suc is the major transport carbohydrate, it is not a prominent storage molecule in most species under physiological conditions. Rather, it is metabolized through a plethora of pathways, and many of the intermediates during its metabolism contain P. Thus, enhanced Suc transport to sink organs and its subsequent metabolism may sequester too much P into P-containing molecules. Any resulting restriction on Suc utilization would lead to an accumulation of Suc in sink organs, which might in turn decrease Suc import via the phloem.

Another possibility is that enhanced Suc transport to sinks may increase synthesis of other P-requiring cellular components related to general growth. In a classic study of barley growing on limiting P supply, about 26% was in free P, 17% was in phospholipids, 26% was in P-containing metabolites, including sugar Ps and nucleotide Ps, and 30% was in nucleic acids (Chapin and Bieleski, 1982). The vast majority of nucleic acids are in ribosomes, which are especially abundant in growing tissues (Detchon and Possingham, 1972; Dean and Leech, 1982; Baerenfaller et al., 2012), where they may represent an even greater proportion of the total P. Suc is known to increase gene expression for nucleotide synthesis and coordinately induce genes for ribosomal proteins and ribosome assembly, especially those for cytosolic ribosomes (Conte et al., 2004; Price et al., 2004; Bläsing et al., 2005; Zrenner et al., 2006; Kojima et al., 2007; Osuna et al., 2007; Usadel et al., 2008; Pal et al., 2013). It is thus plausible that an increased supply of Suc may induce pathways for nucleotide synthesis and ribosome biogenesis, which, in turn, creates an increased demand for P in growing sink tissues. Phospholipids are also important metabolites in growing tissues. Interestingly, among Proteaceae species that are adapted to grow on the extremely P-deficient soils in southwestern Australia, phospholipids are almost completely replaced by galactolipids in mature leaves but still represent the majority of the membrane lipids in young leaves. This implies that phospholipids are required in growing plant organs and cannot be substituted during P deficiencies (Lambers et al., 2012).

Although additional P in the media restored growth among the overexpressing lines to wild-type levels, it is noteworthy that growth did not exceed the wild type. It is also noteworthy that growth did not change as P increased from 1.2 to 3.0 mM (Fig. 8C), suggesting that when P is provided in excess, another limitation prevents growth enhancements, despite additional phloem transport of Suc. While our study focused on Suc transport and the observed association with P, Kellermeier et al. (2014) recently reported on how plants integrate combinatorial nutritional stimuli of N, P, K, S, and light on root architecture. Their results confirm that P limitations are associated with reductions in main root growth. They also report that higher levels of light, which was used as a proxy for more photoassimilate, exacerbated the effects of N starvation.

A more speculative possibility is that the apparent P deficiency is not linked directly to metabolism but is...
the result of a signaling pathway that monitors the C-to-P balance (Fig. 11). In this scenario, the higher Suc levels in the transport stream may be perceived as a disruption in C-to-P homeostasis, provoking the plant to prepare for a limitation. The addition of more available P to the medium would alleviate this by putting the C-to-P ratio back into balance. In this regard, Suc may act as a global regulator of P starvation as proposed by Lei et al. (2011). Further experiments will be needed to test an interesting implication, which is that the rate of Suc export from source leaves and the allocation of the Suc to recipient tissues may be subject to feedback control to maintain an appropriate balance between the Suc import and P availability in growing sink tissues.

CONCLUSION

Increasing photosynthesis to enhance plant productivity is a prominent goal of plant biology (Ainsworth and Bush, 2011; Evans and von Caemmerer, 2011), as increasing P use efficiency to minimize the need for this nonrenewable resource in agricultural production and to reduce the deleterious impacts of P used in fertilizers (Veneklaas et al., 2012). The implications of the study presented here are that efforts to enhance photosynthesis and growth by increasing Suc export may be compromised by the plant's perception that it requires more P, unless the links between carbohydrate and P homeostasis are better understood and uncoupled.

MATERIALS AND METHODS

Plasmid Construction

Gateway-compatible (Life Technologies) destination binary vectors with phloem-specific promoters were created by standard techniques (Sambrook and Russell, 2001). The AttR1-CmR-ccdB-AttR2 cassette of pMDC100 (Curtis and Grossniklaus, 2003) was isolated as a KpnI-Sacl fragment and, using the same sites, cloned downstream of the Cucumis melo GALACTINOL SYNTHASE1 promoter (CmGAS1p) and AtSUC2p of pGEM-CmGAS1p::SUC2 and pGEM-SUC2::SUC2 (Srivastava et al., 2008) to create pGEM-CmGAS1p::CmR-ccdB and pGEM-SUC2::CmR-ccdB. CoYMVp was isolated from pGEM-CoYMVp::cSUC2 (Srivastava et al., 2009a) with 5′fl and KpnI and cloned into the same sites of pGEM-SUC2::CmR-ccdB to create pGEM-CoYMVp::CmR-ccdB. The CmGAS1p::CmR-ccdB and SUC2p::CmR-ccdB cassettes were isolated with 5′fl and SacI and cloned into the same sites of pGPTV-bar (Becker et al., 1992) to create the destination vectors pGPTV-CmGAS1p::CmR-ccdB and pGPTV-SUC2p::CmR-ccdB to create destination vector pGPTV-CoYMVp::CmR-ccdB.

pCR8 donor vectors carrying cDNA for AtSUC1 (The National Center for Biotechnology Information nucleotide accession no. NM_105846), AtSUC2 (NM_012896), AtSUC9 (NM_100132), and AtSUT2 (AB169165), and AtSUC29 (NM_120869) were gifts from Dr. John Ward (University of Minnesota). NtSUT3 (AF149981; p195x=AtNtSUT3), LeSUT2 (AF166498; pDR195=LeSUT2), LeSUT2 (AF176950; pDR195-1=LeSUT2), and LeSUT1 (XM_00429302; p121A1=LeSUT1) cDNA were obtained from Dr. John Ward in conventional vectors. ZmSUT1 (AB008646) was obtained in a pENTR donor vector from Dr. David Braun (University of Missouri). AtSUC2 cDNA was previously described (Srivastava et al., 2008). cDNA in pCR8 donor vectors were recombined into pGPTV-CoYMVp::CmR-ccdB by LR recombination using Gateway LR Clonase II enzyme mix (Life Technologies). For those in conventional cloning vectors, the cDNA was PCR amplified with the oligonucleotides indicated in Supplemental Table S2, directionally cloned into pENTR/D TOPO Cloning Kit (Life Technologies), and sequenced to confirm accuracy.

For compatible antibiotic selection, the cDNA in pENTR donor vectors was first recombined into pMDC7 (Curtis and Grossniklaus, 2003) with Gateway LR Clonase II enzyme mix, then into pDonor-Zeo (Life Technologies) with BP Clonase II enzyme mix, and then into pGPTV-CoYMVp::CmR-ccdB with LR Clonase II enzyme mix. Electroporation into Agrobacterium tumefaciens GV3101 mp90, floral dip transformation of Arabidopsis (Arabidopsis thaliana) Atsuc2-4/+ heterozygous plants, and selection and genotyping of transgenic progeny were as previously described (Srivastava et al., 2008).

Plant Material and Growth Conditions

All plant material was Arabidopsis ecotype Columbia derived from the Arabidopsis Biological Research Center CS60000 or its progeny, CS70000. Unless otherwise noted, seeds were stratified for 72 h at 4°C, and plants were grown on Fafard 3B potting mix in Percival AR95L growth chambers with 14 h of light at 150 μmol photons m−2 s−1 and 24°C and 10 h of dark at 20°C. Twenty-five to five more glucuronide ammonium-resistant lines segregating heterozygous Atsuc2-4/+ at the genomic locus were obtained from the T1 generation for each CoYMVp::cDNA construct. These were grown to seed, and among this T2 generation, glucuronide ammonium-resistant siblings were PCR genotyped to identify individual homozygous wild-type Atsuc2-4/+ and knockout (Atsuc2–/–) at the endogeneous locus. These were then taken through subsequent generations to identify lines that were homozygous for the companion cell-specific cDNA based on segregation of glucuronide ammonium resistance. All experiments were conducted with T3 or T4 generation seeds. From the 25 or more independent lines identified for each CoYMVp::cDNA construct, two representative lines were selected for further study based on rescue of the Atsuc2-4/+ phenotype that best approximated the growth habit of the wild-type control. Plants were digitally photographed, and rosette surface area was measured with ImageJ version 1.38x (Rasband, 2007).

Photosynthesis was measured by imaging pulsed amplitude modulated fluorescence on dark-adapted plants (variable fluorescence/maximal fluorescence Fv/Fm) based on the equipment manufacturer's instructions (model IMAG-C, Walz).

For plants grown on sterile synthetic media, surface-sterilized seeds were distributed on one-half-strength MS media with Gamborg's vitamins (Phytotechnology Laboratories) and 1% (w/v) Suc (pH 5.8) and solidified with 2.8 g L−1 gellan gum (Sigma-Aldrich). Seeds were stratified for 72 h and germinated in the horizontal orientation for 7 d. The plants were then transferred to fresh one-half-strength media with appropriate supplements but without added Suc: for all experiments involving growth on synthetic media, plants were grown in the absence of Suc for at least 7 d before analysis. Media used during GUS analysis are described below. For growth on vertically oriented plates, 5.0 g L−1 gellan gum was used for stiffer media and to prevent the roots from growing into the media. Seedlings were transferred such that the rosettes were in a line at the top of the plate and the roots trailed behind on the media surface. The plates were sealed with Parafilm and oriented in the growth chamber 15° from vertical for an additional 7 d. Tissues were harvested between 4 and 6 h into the light period; rosettes and roots were collected and analyzed separately. KH2PO4 was used for P-supplemented media to achieve final PO4 concentrations of 0.6 μmol (no supplement), 1.2 μmol, 1.8 μmol, and 3.0 μmol. Potassium levels were kept constant by balancing KH2PO4 supplements with K2SO4. To test for rhizosphere acidification by transgenic lines relative to wild-type lines, pH indicator dye bromocresol blue was added to one-half-strength MS medium (without Suc or P supplements) as described (Yang et al., 2007). High-resolution analyses of root elongation on vertically oriented plates was as previously described (Yadlanbakhsh et al., 2011)

Quantitative GUS Analysis

The transgenic lines harboring CoYMVp::SUC2=uidA and AtSUC2::CmR-ccdB were used to analyze promoter strength under different environmental conditions were previously described (Srivastava et al., 2008, 2006b). Sterile seeds were stratified and germinated as above, except that full-strength media was used for 10 d. Seedlings were transferred to fresh media without Suc (unless otherwise noted) with different concentrations of Suc (0%, 1%, and 5% [w/v], NaCl (0.5, 100, and 200 μmol), and mannitol (0, 100, 200, and 300 μmol). After 3 d, seedlings were pooled and used in quantitative GUS assays with 4-methylumbelliferylβ-D-glucuronide hydrate (Gold Biotechnology) and a VersaFluor fluorometer (Bio-Rad Life Science Research) as described (Weigel and Glazebrook, 2002).

Transcript Analysis

Total RNA was isolated from rosettes of 21-d-old plants using Trizol (Life Technologies) according to the manufacturer's instructions and treated with

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RNase-Free DNase I (Ambion TURBO DNase). Six hundred nanograms of RNA from each plant was reverse transcribed with 50 μg oligo(dT) and SuperScript III reverse transcriptase (Life Technologies) according to the manufacturer’s instructions. Gene-specific primers used for real-time quantitative PCR are listed in Supplemental Table S3. RT-qPCR was performed with SybrGreen PCR Master Mix (Life Technologies) on an Applied Biosystems ViA 7 instrument (Life Technologies) using the following amplification protocol: 10-min polymerase activation and denaturation at 98°C followed by 40 cycles of 95°C for 10 s, 58°C for 30 s, and 72°C for 30 s. This was followed by a product melt to confirm a single PCR product. The level of SUT gene expression was normalized to that of EF1a by subtracting the cycle threshold value of EF1a from the cycle threshold value of the SUT genes. Three biological and two technical replicates were used for SUT measurements. For the PSI genes, transcript abundance of AIP2 and AIPHT2;1 were determined relative to EF1a, and AIPAP14 and AIPAP24 were determined relative to Ubiquitin10 (UBQ10), using two biological replicates each with two technical replicates, and the transcript abundance in the transgenic lines was expressed relative to the wild type.

Transient Carbohydrate Analysis

Transgenic lines and controls were grown for 21 d on potting mix under the conditions described above or on synthetic medium as described above. All tissues were collected between 4 and 6 h into the light period, unless sampling was done at the end of the day or the end of the night, with plants removed from the growth chamber immediately before sampling. Rosettes (and roots for plants grown on synthetic media) were excised at the hypocotyl, fresh weight was determined, and rosettes and roots were immersed in a minimum of five volumes of ice-cold methanol:chloroform:water extraction solution (12:5:3) containing 10 μl lactose as a standard. Samples were kept on ice until all were collected. Further processing and analysis of the major neutral sugars by high-performance anion exchange chromatography with pulsed amperometric detection was as previously described (Srivastava et al., 2008), except that the Dionex CarboPac PA20 column was held at 30°C. The insoluble fraction of the harvested tissue was analyzed for starch as previously described (Srivastava et al., 2008). Similar experiments were conducted with tissues that were harvested into liquid nitrogen, ground with a pestle, and then extracted and processed as described. Among the carbohydrate analyzed, there was no difference when fresh or frozen tissues were used (not shown).

Radiolabeling and EDTA Exudate Analysis

To investigate transport efficiency among the transgenic lines, an EDTA exudation method (Srivastava et al., 2008) was used to collect phloem sap from severed rosettes of the wild type and representative SUT OE lines after photosynthetic labeling with [14C]CO2. Transparent polyethylene terephthalate containers with hinged lids (16.5 × 12.7 × 5.1 cm), commonly used for catering (Douglas Stevens Plastics), were adapted for plant growth by creating drainage holes and filling one-half full with potting mix. The tight seal of the lid maintained these ideal chambers for phloem extraction labeling with [14C]CO2. Nine transgenic plants, three of each wild type, AtSUT1-6, and ZmSUT1, in a randomized design, were grown in each chamber with the hinged lids open. Four chambers were prepared. For labeling, the lid seal was supplemented with a film of vacuum grease, and the closed chambers were placed 60 cm below a 400-W metal halide lamp: chambers were processed individually so that each was directly exposed to the light source during labeling, and all labeling was conducted between 6 and 8 h into the standard 14-h light period. [14C]CO2 was generated by mixing 5 μl of [14C]NaHCO3 (1 μCi μl−1; 50 mCi mmol−1; MP Biomedicals) with 15 μl of 80% lactic acid in a syringe barrel with a 20-gauge needle extending into the labeling chamber. The needle was removed, and the hole was sealed with modeling clay. Photosynthesis in the presence of [14C]CO2 was used for 20 min, and then the chamber was pumped out through a column containing soda lime for 5 min to capture residual [14C]CO2. The chamber was then opened, and photosynthesis continued for 15 min in room air. The rosettes were cut at the hypocotyl, and fresh weight was determined. The hypocotyls were then cut again under EDTA, and collection of exudates from individual plants was as described (Cao et al., 2013). Twenty-five microliters of exudate solution was mixed with 5 ml of ScintiSafe Plus 50% scintillation cocktail (Fisher Scientific), and counts per minute were determined.

To analyze [14C]Suc uptake studies, individual leaves of 21-d-old plants were harvested by cutting the petioles near the base of the lamina, fresh weight was established, and plants were submersed in MES buffer (20 mM, pH 5.5, with KOH) plus 2 mM CaCl2 supplemented with [14C]Suc (1 μM, 30 kBq mL−1; MP Biomedicals), and weighted down with 4-mm glass beads in wells of 24-well microtiter plates. Each replicate contained four pooled leaves from the same plant. The leaves were vacuum infiltrated for 5 min and incubated at room temperature for 20 min, followed by three washes in fresh buffer without sugar. The leaves were gently blotted dry after washing, placed between sheets of filter paper, and frozen in powdered dry ice. Frozen rosettes were lyophilized in a −60°C chamber for 48 h, pressed flat between steel plates in a vice, and exposed to x-ray film (Kodak BioMax MR Film) for 24 h. The leaves were then cleared with 1 mL of 95% ethanol for 30 min and bleached with 1 mL of commercial bleach for 15 min. Five milliliters of scintillation fluid was added and [14C]Suc uptake calculated as counts per minute per milligram of fresh weight (not shown) or counts per minute per leaf surface area (Fig. 7C). The leaf surface area was measured with ImageJ version 1.43u (Rashband, 2010).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Comparison of CoYMVp and AtSUC2 companion cell-specific promoter activities in the presence of different stimuli.

Supplemental Figure S2. RT-qPCR analysis of AtSUC1, AtSUC2, and ZmSUT1 transcript levels in wild-type plants and wild-type lines with additional companion cell-specific SUT expression.

Supplemental Figure S3. Photosynthesis is reduced in SUT overexpressing lines.

Supplemental Figure S4. Increased rhizosphere acidification by plants with additional SUT expression in companion cells.

Supplemental Table S1. Oligonucleotides used to create the indicated SUT cDNA by PCR for TOPO directional cloning.

Supplemental Table S2. Oligonucleotides used for RT-qPCR analysis of expression for the indicated genes.

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