Arabidopsis Sucrose Transporter AtSUC1 Is Important for Pollen Germination and Sucrose-Induced Anthocyanin Accumulation[1[OA]]

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The Arabidopsis (*Arabidopsis thaliana*) sucrose transporter *AtSUC1* (At1g71880) is highly expressed in pollen; however, its function has remained unknown. Here, we show that *suc1* mutant pollen is defective in vivo, as evidenced by segregation distortion, and also has low rates of germination in vitro. AtSUC1-green fluorescent protein was localized to the plasma membrane in pollen tubes. *AtSUC1* is also expressed in roots and external application of sucrose increased *AtSUC1* expression in roots. *AtSUC1* is important for sucrose-dependent signaling leading to anthocyanin accumulation in seedlings. *suc1* mutants accumulated less anthocyanins in response to exogenous sucrose or maltose and microarray analysis revealed reduced expression of many genes important for anthocyanin biosynthesis. The results indicate that *AtSUC1* is important for sugar signaling in vegetative tissue and for normal male gametophyte function.

Arabidopsis (*Arabidopsis thaliana*) Suc transporter AtSUC1 (At1g71880) is a proton-coupled Suc uptake transporter (Sauer and Stolz, 1994; Zhou et al., 1997). *AtSUC1* is expressed in pollen (Stadler et al., 1999), trichomes, and roots (Sivitz et al., 2007). In pollen, AtSUC1 has been proposed to function in Suc uptake during germination. *AtSUC1* mRNA accumulates during pollen maturation (Stadler et al., 1999; Bock et al., 2006), but AtSUC1 protein is not detectable in pollen until germination (Stadler et al., 1999). Such delayed translation of transcripts in pollen is well known. In this article, we tested the hypothesis that AtSUC1 functions in pollen germination using *atsuc1* insertional mutants and show that *atsuc1* mutant pollen is defective in germination both in vivo and in vitro.

AtSUC1 expression was long considered to be pollen specific. However, microarray data revealed expression also in leaves and roots (Schmid et al., 2005). This discrepancy was recently resolved: *AtSUC1* is expressed in developing trichomes and roots (Sivitz et al., 2007). Initial experiments were done using the glabrous ecotype C24 (Stadler et al., 1999) and therefore trichome expression was not observed. Expression of *AtSUC1* in roots was shown to be controlled by intragenic sequences: The AtSUC1 promoter directs expression only in pollen and trichomes, whereas whole-gene constructs show expression additionally in roots (Sivitz et al., 2007). This explains why early work using AtSUC1 promoter-GUS did not detect expression in roots (Stadler et al., 1999).

In this article, we used *AtSUC1* insertional mutants to investigate potential functions of AtSUC1 in Suc-dependent signaling leading to anthocyanin production in the shoot. Suc is an important signaling compound in plants (Koch, 1996; Smeekens, 2000) and results from numerous studies show that Suc plays a role in germination, senescence, flowering, phosphate starvation responses, and anthocyanin production (Ohto et al., 2001; Gibson, 2004; Pourtau et al., 2004; Teng et al., 2005; Karthikeyan et al., 2006). Suc-induced anthocyanin accumulation is considered to be one of the few signaling pathways that are Suc specific: Glc does not cause an increase in anthocyanins. This has been described in detail (Teng et al., 2005; Solfanelli et al., 2006), but no mutants affected in this response to Suc have been isolated to date.

RESULTS

Identification of *suc1* Mutant Lines in Col-0 Ecotype

Two insertional mutant alleles, *suc1-1* (SM_3_19971) and *suc1-2* (SM_3_20664), were used in this study. Homozygous lines were identified using PCR on genomic DNA (see "Materials and Methods"). Figure 1A shows a diagram of the *AtSUC1* gene containing three exons with the *suc1-1* insertion in the first exon and the *suc1-2* insertion in the second exon. Growth of homozygous *suc1* mutant plants was not different from wild

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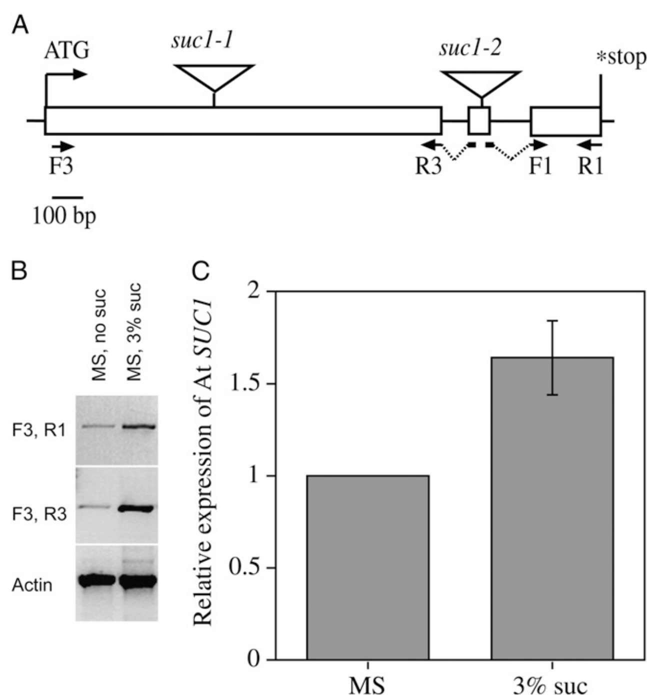

Figure 1. AtSUC1 expression is induced by Suc. A, Diagram showing the position of the *suc1-1* and *suc1-2* insertions and primers used for RT-PCR. Dotted lines indicate that primers span, but do not include, intron sequence. The insertions and the primers are not to scale. B, RT-PCR of RNA extracted from 5-d-old liquid-grown Col-0 seedlings. PCR product sizes were 1,497, 1,235, and 1,033 bp for the F3-R1, F3-R3, and Actin-11 primer pairs, respectively. C, Quantitative RT-PCR on Col-0 using primers F1 and R1 (235-bp product) shows a 1.64 ± 0.20 fold increase in *SUC1* transcript level when Suc is present in the growth medium. Data come from three technical replicates each for two biological replicates and are presented as an average fold increase ± SE.

type. *suc1* seedlings did not differ from wild type in primary root length: *suc1-1* seedlings had a root length of 49.4 ± 1.4 mm (n = 53), *suc1-2* had an average root length of 43.8 ± 1.1 mm (n = 54), and the root length for Columbia (Col-0) was 46.6 ± 1.6 mm (n = 44) after 11 d of growth on 0.5× Murashige and Skoog (MS) medium. No differences in fertility were observed for *suc1* mutants compared to wild type; this is of interest considering that AtSUC1 is expressed in pollen (Stadler et al., 1999). Both the number of seeds per silique and silique length were not statistically different in Col-0 and *suc1-2*: Col-0 had 49.7 ± 8.9 seeds/silique (n = 23) and siliques were 12.2 ± 1.4 mm long (n = 24), whereas *suc1-2* had 44.8 ± 8.6 seeds/silique (n = 20) and siliques were 11.8 ± 1.0 mm long (n = 19). *AtSUC1* is also expressed in young trichomes (Sivitz et al., 2007), suggesting a role in trichome development. However, the *suc1* mutants displayed no obvious defects in trichome development, perhaps due to redundant expression of other Suc transporters in trichomes.

Expression of *AtSUC1*

Reverse transcription (RT)-PCR indicated that wild-type (Col-0) seedlings grown in liquid MS supplemented with 3% Suc produced more *AtSUC1* mRNA than those grown on MS alone (Fig. 1B). Quantitative RT-PCR confirmed this finding; *AtSUC1* expression levels were 1.64 ± 0.20-fold higher in Suc-grown plants than in those grown without Suc (Fig. 1C). Suc-induced expression of *AtSUC1* was also found in transgenic plants expressing pSUC1::SUC1::GUS fusions (this construct includes 2 kb upstream of the ATG, all introns and exons, and a translational fusion to GUS; Sivitz et al., 2007). Figure 2 shows an increase in GUS enzyme activity in plants grown either for 3 d (Fig. 2B) or 24 h (Fig. 2, C, E, and H) in medium containing Suc compared to plants grown in medium lacking Suc (Fig. 2, A, D, and G). Plants transformed with an *AtSUC1* promoter-GUS construct do not have detectable GUS activity in roots (Sivitz et al., 2007) and Suc application did not induce GUS expression in roots in *AtSUC1* promoter-GUS lines (data not shown).

AtSUC1 expression in root tissue was analyzed at the cellular level using vibratome sections of GUS-stained transgenic Arabidopsis lines expressing pSUC1::SUC1::GUS. Sivitz et al. (2007) previously showed that *AtSUC1* is expressed in the root vasculature in the elongation zone of the root and sites of lateral root formation (Fig. 2, F and I). Root cross sections show that *AtSUC1* expression is not limited to cells within the vascular cylinder (Fig. 2D): *AtSUC1* is expressed in the pericycle and endodermis as well. Treatment with Suc, such as in Figure 2C, resulted in extension of *AtSUC1* expression to the cortex and epidermis (Fig. 2E).

AtSUC1 is highly expressed in pollen (Stadler et al., 1999; Fig. 2J). Figure 3 shows confocal images of pSUC1::SUC1::GFP-expressing pollen tubes where AtSUC1 was localized in the plasma membrane (Fig. 3, A–E). Fluorescence was also observed in unidentified internal structures (Fig. 3, A and B) associated with callous plugs (Fig. 3E), and cytoplasmic fluorescence was more pronounced at the tip (Fig. 3D) probably associated with plasma membrane biosynthesis and membrane trafficking.

***suc1* Insertional Mutants Have Defective Pollen**

When *suc1-2* plants were backcrossed to wild type, the F_2 population did not show normal Mendelian segregation. Only 4% of the F_2 plants were homozygous for *suc1-2* instead of the expected 25% (n = 28). To determine whether this was caused by embryo lethality or a gametophyte defect, reciprocal crosses of *suc1-2* heterozygous (het) plants to Col-0 wild type were made. When the pollen for the cross was from the *suc1-2* het, only 6% of the progeny were het for the *suc1-2* allele instead of the expected 50% of the progeny (n = 284; Table I), indicating that transmission of the mutation through the male gametophyte was reduced. Whereas when the female parent was the *suc1-2* het, 50% of the plants in the F_1 generation were het for the mutation (n = 147). The pollen defect could be rescued by expressing *AtSUC1* under its own promoter in the

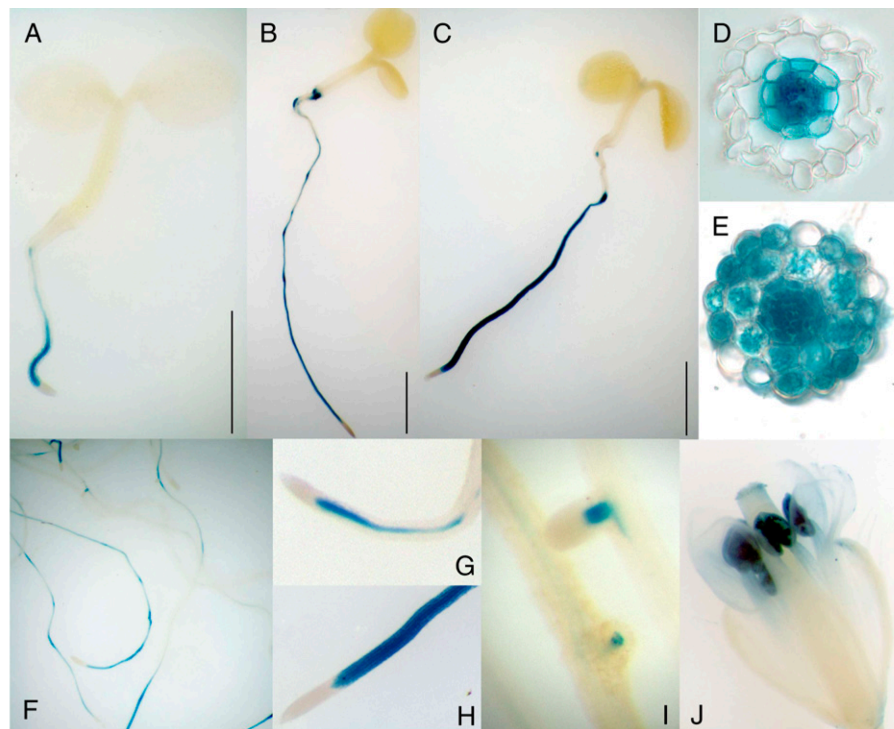

Figure 2. Analysis of AtSUC1 expression. pSUC1::SUC1::GUS expressing plants were incubated for 48 h in GUS-staining solution. A to E, Three-day-old seedlings. Bars indicate 2 mm. Seedlings were grown continuously in 0.5× MS (A), 0.5× MS supplemented with 3% Suc (B), or 0.5× MS for 2 d, then transferred to 0.5× MS supplemented with 3% Suc for 24 h (C). D and E, Vibratome sections (100 μm thick) of GUS-stained roots imbedded in 4% agarose. D, Plants were grown under the same conditions as in A. E, Plants were grown under same conditions as in C. F, Roots from an adult plant showing expression only in some root sections, but not in the mature roots. G, Close-up of root tip of plant grown as in A. H, Close-up of root tip grown as in C. I, Close-up of the lateral root nodes in F, showing *SUC1* expression in the lateral root bud. J, Flower showing *SUC1* expression in anthers.

suc1-2 mutant background. *suc1-2* homozygous mutants carrying one copy of the *AtSUC1* transgene (het for the transgene) do not show normal Mendelian segregation of the transgene. Mutants carrying one copy of *AtSUC1* transgenically should have a higher rate of transgene transmission through the male gametophyte because the pollen carrying the transgene (one-half of all pollen grains) should show greater rates of transmission than the mutant pollen without the complementing transgene. As expected, significantly more than 75% of the progeny of three independent lines of *suc1-2* mutants het for a transgenic *AtSUC1* construct carried the transgene (Table II). One line (C) did not show complementation probably due to an incomplete transgene insertion or positional effects and was not investigated further.

Because *AtSUC1* is highly expressed during pollen maturation (Stadler et al., 1999; Schmid et al., 2005; Bock et al., 2006), the effect of the *suc1* mutation on pollen germination was analyzed. In vitro, only 7.9% of *suc1-2* pollen and 13.1% of *suc1-1* pollen germinated, whereas 39.0% of wild-type pollen germinated (Fig. 4). This was not due to low viability of the mutant pollen; there was no difference in the viability of *suc1-2* pollen compared to wild type based on Alexander's viability stain (data not shown). To test for complementation, *suc1-2* mutant lines homozygous for either pSUC1::SUC1 or pSUC1::SUC1::GFP were generated. Both constructs were able to complement the mutant phenotype as indicated by pollen germination rates that were similar to wild type (Fig. 4A). Replacing Suc in the germination medium with equimolar Glc did not improve the germination rate of *suc1-2* pollen; however, there was a reduction in wild-type pollen germination rates (from 39% to 24%; Fig. 4B).

The lack of rescue of the pollen germination phenotype with Glc indicated that potentially pollen development was affected because Suc transporters do not transport Glc. To attempt to characterize an effect of *AtSUC1* mutation on pollen development, we looked for ultrastructural defects in mutant pollen by transmission electron microscopy. The pollen coat is important for proper pollen grain adhesion and hydration (Fiebig et al., 2000), but appeared identical in both the *suc1* mutant and the wild type (data not shown). No differences between mutant and wild-type pollen were observed. Fatty acid accumulation occurs during pollen development and oil bodies supply energy required for germination and pollen tube growth. To assess the stored energy status of the pollen, the fatty acid content of pollen grains was examined. Fatty acids were extracted from both wild type and the *suc1-2* mutant pollen grains and analyzed by gas chromatography (Table III). There was no difference in the types of fatty acids present (Table III; % total fatty acid). Total fatty acids in mutant pollen were not significantly different compared to wild type (Col-0 had 2.74 ± 0.14 μg fatty acid/μg protein, whereas *suc1-2* had 2.25 ± 0.23 μg fatty acid/μg protein).

suc1-2 Plants Accumulate Fewer Anthocyanins in Response to Suc

Teng et al. (2005) reported that Arabidopsis accumulates anthocyanins in response to Suc. The authors

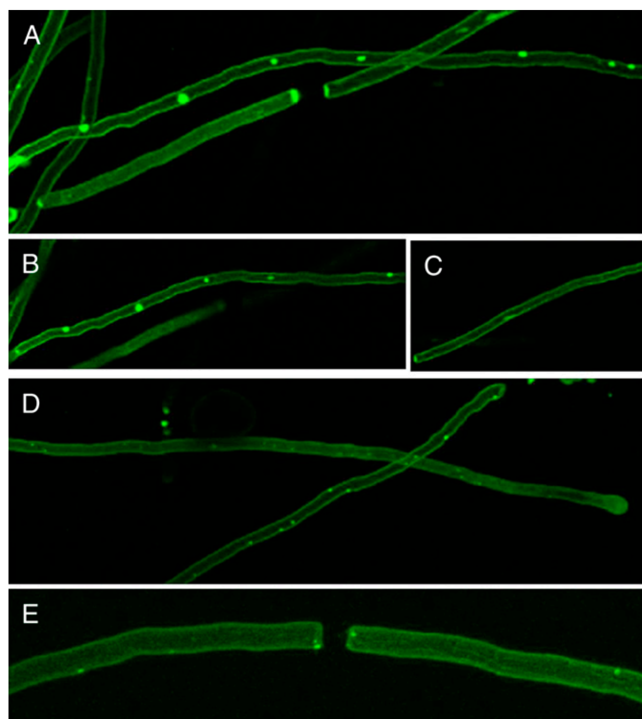

Figure 3. AtSUC1 is localized to the pollen plasma membrane. Pollen from Col-0 expressing the pSUC1::SUC1::GFP construct was germinated overnight in pollen germination medium. Pollen was imaged using a Nikon C1si laser-scanning confocal microscope. A, D, and E are z projections. B and C are individual optical sections from A.

noted that the effect was Suc specific (although, to a lesser extent, it was also observed with maltose) and not observed in response to Glc, Fru, or sorbitol. Figure 5, A to C, shows plants grown with 3% Suc. Wild-type and het plants accumulated visible anthocyanins in cotyledons and at the apex of the hypocotyl, whereas *suc1-2* mutants (Fig. 5B) did not, indicating that the phenotype is recessive. All plants looked similar when grown on medium lacking Suc (Fig. 5, insets). *suc1-2* plants accumulated anthocyanins in response to high light (data not shown), indicating that *suc1-2* plants are not impaired in anthocyanin biosynthesis in response to an alternative stimulus.

Figure 6 shows quantification of accumulated anthocyanins. When *suc1-2* seedlings were grown continuously on 3% Suc or 3% maltose, they did not accumulate as many anthocyanins as wild-type seedlings (Fig. 6A). However, when Suc concentration was raised to 5%, there was no difference in anthocyanin accumulation between wild type and *suc1-2* mutants (Fig. 6A). Nevertheless, mutants and wild type showed differences in anthocyanin accumulation at 5% Suc if the plants were assayed after being grown first on MS medium without Suc, and then incubated with 5% Suc for 24 h (Fig. 6A). *suc1-1* also showed reduced anthocyanin accumulation when grown continuously on 3% Suc (Fig. 6B). And, as expected, two independent lines of *suc1-2* plants expressing an *AtSUC1* transgene showed wild type-like Suc-induced anthocyanin accumulation (Fig. 6B). Assaying radioactive Suc uptake into whole seedlings grown for 3 d in liquid MS showed that *suc1-2* mutants did not accumulate less Suc than wild type. After 15-min incubation in 0.044 mM ^{14}C-labeled Suc, wild type accumulated 6.3 ± 0.4 pmol/seedling (n = 4), whereas *suc1-2* accumulated 7.5 ± 0.9 pmol/seedling (n = 4).

suc1-2 Mutants Have Altered Expression of Anthocyanin Biosynthetic Genes

Expression of many genes involved in anthocyanin biosynthesis is increased by exogenous Suc (Solfanelli et al., 2006). To determine whether AtSUC1 is necessary for this process, we performed microarray analysis on *suc1-2* and wild-type plants that were grown in liquid 0.5× MS medium for 3 d and then 24 h in liquid 0.5× MS medium supplemented with 3% Suc. Labeled RNA was hybridized to Affymetrix GeneChip ATH1 microarrays for each of the two genotypes (each with two biological replicates) to find up- and down-regulated genes. Table IV shows normalized signal from individual hybridizations for the *suc1-2* mutant and Col-0 treated with 3% Suc for 24 h and data from Solfanelli et al. (2006) for Arabidopsis wild-type seedlings treated similarly (without or with 3% Suc applied for 6 h). Genes in Table IV were identified by Solfanelli et al. (2006) as regulated by Suc and involved in anthocyanin biosynthesis. The data are sorted by fold signal difference between *suc1-2* and Col-0. Dihydroflavonol 4-reductase is a downstream enzyme in the anthocyanin biosynthesis pathway that is regulated by the PAP1 complex (for review, see Koes et al., 2005). TT3 (At5g42800) is a Suc-induced dihydroflavonol reductase (Solfanelli et al., 2006) and is expressed at a 3.6-fold higher level in Col-0 versus *suc1-2*. Many other genes show a similar pattern: TT6 (At3g51240), flavonol 3-*O*-glucosyltransferase (At5g54060), cinnamate-4-

Table I. *Reciprocal crosses demonstrate that suc1-2 mutants segregate aberrantly*

Numbers in parentheses indicate the number of plants with an insertion/total progeny counted. *P* value is calculated from a two-tailed *t* test.

Pollen Donor	Female Plant	Progeny Containing T-DNA			*P*
		Expected	Observed	X^2	
Col-0	*suc1-2* het	50%	49% (72/147)	0.06	0.804
suc1-2 het	Col-0	50%	6% (17/284)	220	<.0001

Table II. *Complementation of suc1-2 mutants with the wild-type SUC1 gene or SUC1-GFP fusion*

Numbers in parentheses indicate the number of plants resistant to hygromycin/total progeny counted. *P* value is calculated from a two-tailed *t* test. Four independent T_2 transgenic lines were selfed and the percent of the progeny that were hygromycin resistant were counted (transgene marker).

Line	Construct	Hygromycin-Resistant Progeny			*P*
		Expected	Observed	X^2	
A	pSUC1::SUC1::GFP	75%	84.6% (160/189)	9.18	0.0025
B	pSUC1::SUC1	75%	90.8% (216/238)	32.18	<0.0001
C	pSUC1::SUC1	75%	78.4% (120/153)	0.88	0.3495
D	pSUC1::SUC1	75%	91.1% (194/213)	29.04	<0.0001

hydroxylase (At2g30490), flavonol synthase (At5g08640), chalcone isomerase (At3g55120), and TT4 chalcone synthase (At5g13930) are all strongly induced by Suc (Solfanelli et al., 2006) and show lower expression in *suc1-2* as compared to Col-0. At1g53520, encoding a putative chalcone isomerase, was the only anthocyanin biosynthetic gene found to be expressed at a higher level in the mutant than wild type. On a whole-genome level, a contingency table and Fisher exact test (*P* value < 2.2 e-16) showed that genes up-regulated by Suc have higher expression in Col-0 compared to *suc1-2*. Likewise, genes down-regulated by Suc showed lower expression in Col-0 compared to *suc1-2* (*P* value < 2.2 e-16). In summary, microarray analysis revealed that *suc1* mutants have reduced response to exogenous Suc.

DISCUSSION

Based on *AtSUC1* expression in pollen and the timing of the appearance of AtSUC1 protein during pollen germination, Stadler et al. (1999) proposed a function for AtSUC1 in pollen germination. That hypothesis was tested here using *AtSUC1* insertional mutants. Het *suc1* mutants produced a low number of homozygous progeny, indicating either embryo or seedling lethality or a gametophytic phenotype. Results from reciprocal crosses (Table I) showed that the segregation distortion was due to defective *suc1* mutant pollen. This phenotype was confirmed in vitro using two independent insertional mutants (Fig. 4A): Pollen from homozygous *suc1-1* and *suc1-2* mutants had a lower germination rate compared to wild type. The linkage between this phenotype and mutations in the AtSUC1 gene was further confirmed by complementation of the pollen germination defect with both the wild-type gene and by a GFP fusion to AtSUC1 expressed under the control of the native promoter (Fig. 4A). These results indicate that AtSUC1 is important for pollen germination; the observed segregation distortion showed that *suc1* mutant pollen does not compete well with wild-type pollen, but is capable of fertilization. No differences in fertility were observed between wild type and homozygous *AtSUC1* mutants, although stress conditions were not tested. It should be noted that *hap3*, a mutant with pollen tube growth defects, was reported to carry a T-DNA insertion 500 bp upstream of At1g66570, AtSUC7 (Johnson et al., 2004), further implicating Suc transporters in pollen function.

These data support a function for AtSUC1 in Suc uptake into germinating pollen. No obvious develop-

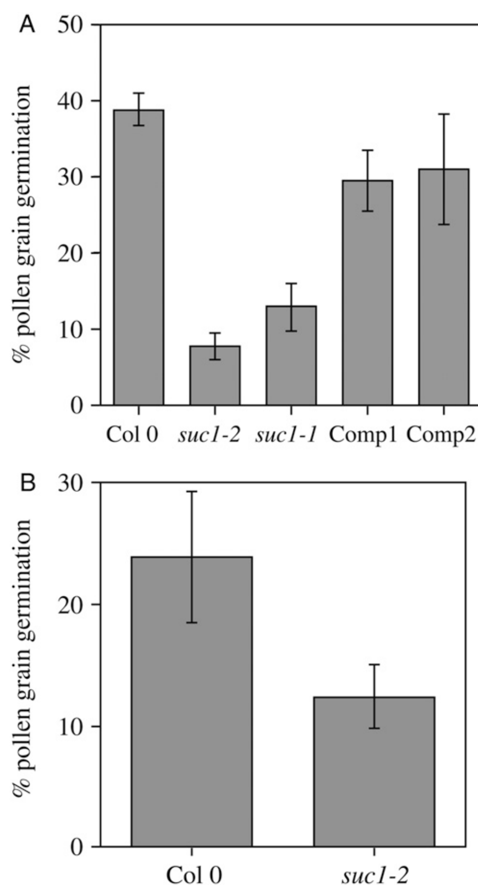

Figure 4. *suc1* mutant pollen does not germinate as well as wild type in vitro. Pollen from stage 14 flowers was incubated for 22 h in liquid germination medium (see "Materials and Methods" for details). The number of germinated and nongerminated pollen grains was counted and the relative germination rate determined. Each bar represents mean ± SE. A, Pollen germination in medium containing Suc. Comp1 is a transgenic *suc1-2* line expressing pSUC1::SUC1::GFP$^{+/+}$; Comp2 is a transgenic *suc1-2* line expressing pSUC1::SUC1$^{+/+}$ ($n = 4$–12; each replicate had at least 200 pollen grains). B, Pollen germination when the Suc in the medium was replaced with Glc. Each bar represents mean ± SE ($n = 4$).

Table III. *Fatty acid content of Col-0 and suc1-2 pollen*

Fatty Acid	Col-0		*suc1-2*		
	% Total Fatty Acid[a]	Fatty Acid (μg)/ Protein (μg)	% Total Fatty Acid	Fatty Acid (μg)/ Protein (μg)	*P* Value[b]
16:1	23.54	0.65	23.51	0.53	0.21
18:0	2.58	0.07	1.99	0.04	0.38
18:1	3.78	0.10	4.01	0.09	0.34
18:2	11.05	0.30	11.35	0.26	0.40
18:3	59.06	1.62	59.14	1.33	0.12
Total		2.74		2.25	0.15

[a]Means of three replicates. [b]Two-tailed Student's *t*-test.

mental defects were observed in *suc1* mutant pollen. Pollen grain ultrastructure as observed by electron microscopy appeared similar to wild type (data not shown) and no differences were found in lipid content of mature pollen compared to wild type. AtSUC1 was localized to the plasma membrane of growing pollen tubes (Fig. 3) using an AtSUC1-GFP fusion driven by the native promoter in a wild-type Col-0 background. This construct was the same as was demonstrated to complement the germination defect of the *suc1-2* mutant (Fig. 4A). This is consistent with immunofluorescent detection of AtSUC1 in germinating pollen (Stadler et al., 1999) and a recent report that showed AtSUC1 localization to the plasma membrane by expression in onion (*Allium cepa*) epidermis (Endler et al., 2006). The only indication that AtSUC1 has additional functions in pollen beyond Suc uptake during germination is the observation that pollen germination in Glc medium (lacking Suc) was less efficient for *suc1* mutant pollen compared to wild-type pollen. However, no other functions, aside from proton-coupled glucoside transport, have been demonstrated for plant Suc transporters and the only potential protein interactors that have been identified are other Suc transporters (Reinders et al., 2002). AtSUC1 protein is not detectable by immunofluorescence microscopy in pollen grains until the onset of germination (Stadler et al., 1999); therefore, if AtSUC1 has a direct function in Suc uptake into pollen during development, it must be accomplished by a much lower level of AtSUC1 protein than is found in germinating pollen. Also, antisense repression of extracellular invertase in tobacco (*Nicotiana tabacum*) results in male sterility, indicating that extracellular Suc hydrolysis is essential for pollen development (Goetz et al., 2001). Concerning the lower germination of *suc1* mutant pollen in Glc medium, plant Suc transporters do not transport Glc; the substrate specificity of AtSUC2, which is closely related to AtSUC1, has been analyzed in detail (Chandran et al., 2003; Sivitz et al., 2007). In particular, Suc transport by AtSUC1 is not inhibited by Glc (Sauer and Stolz, 1994), indicating that it is not a transported substrate. Therefore, understanding the cause for reduced pollen germination of *suc1* mutant pollen in medium lacking Suc will require further work.

AtSUC1 is also expressed in roots (Sivitz et al., 2007). Using whole-gene GUS fusions, *AtSUC1* expression was found in elongating and recently elongated parts of the root, including young lateral roots (Fig. 2). Externally applied Suc was found to stimulate *AtSUC1* expression in roots (Figs. 1 and 2). *AtSUC1* promoter-GUS constructs do not confer detectable GUS expression in roots (Stadler et al., 1999; Sivitz et al., 2007). This was also true when *AtSUC1* promoter-GUS plants were grown on Suc medium (data not shown). This indicates that the intragenic element in *AtSUC1* is not simply a transcriptional enhancer and that the intragenic region is necessary for Suc-induced expression of *AtSUC1* in roots.

Symplasmic domains, cells connected by plasmodesmata, which include the phloem in the Arabidopsis root, have been mapped using GFP fusions of various sizes (Stadler et al., 2005). In the region of the root where AtSUC1 is expressed, behind the root tip, the symplasmic domain continuous with sieve elements is limited to only one or two surrounding cell layers. Therefore, it is likely that Suc delivery from the phloem to cells in this area of the root requires transmembrane transport (cellular efflux into the wall space and proton-coupled uptake). AtSUC1 could serve this purpose of proton-coupled uptake. In this context, increased expression of *AtSUC1* due to high concentrations of extracellular Suc makes sense because it would allow plants to utilize any Suc delivered by the phloem. AtSUC1 could also

Figure 5. The reduced anthocyanin phenotype of *suc1-2* is recessive. A to C, Plants were grown for 3 d in liquid 0.5× MS supplemented with 3% Suc. A, Col-0. B, *suc1-2*. C, *suc1-2* heterozygotes. Insets are seedlings grown on liquid 0.5× MS.

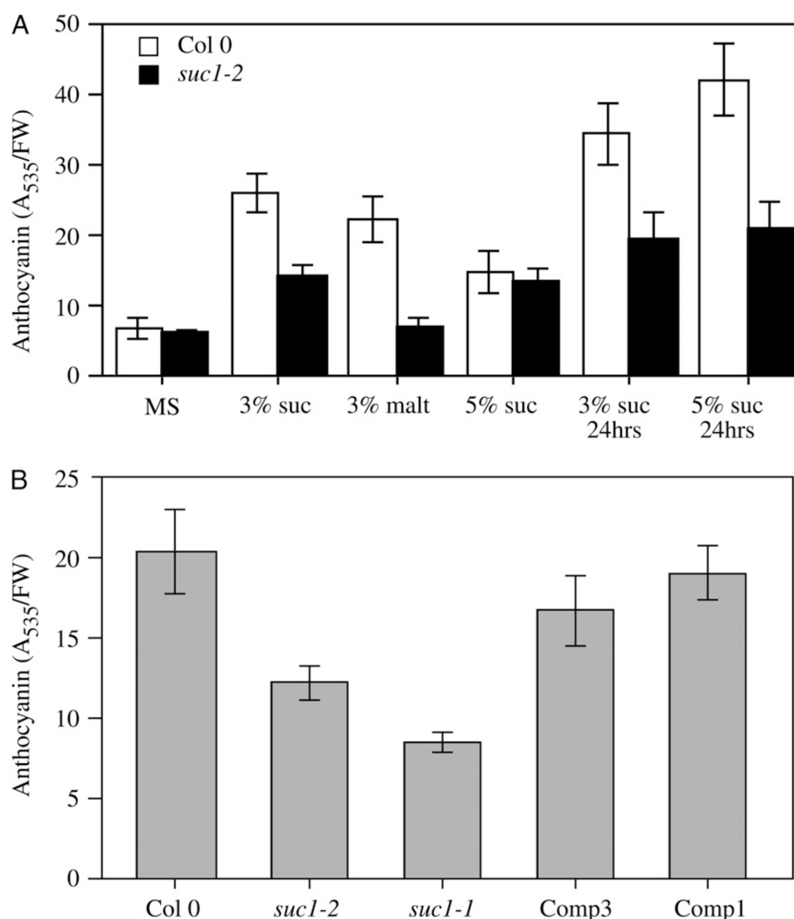

Figure 6. *suc1* mutants accumulate fewer anthocyanins in response to Suc. Anthocyanin content was measured by taking the absorbance of plant extracts at 535 nm over grams of fresh weight (FW). A, Anthocyanin content for 3-d-old seedlings grown continuously in liquid culture (0.5× MS) supplemented with indicated sugar. ''24hrs'' denotes that the 3-d-old seedlings were grown for 2 d in 0.5× MS followed by 1 d of growth on medium supplemented with indicated sugar. B, Anthocyanin content was measured the same as in A. Plants were grown continuously in medium supplemented with 3% Suc. Comp1 and Comp3 are independent transformants of complemented *suc1-2* lines expressing pSUC1::SUC1::$GFP^{+/+}$.

function similarly in lateral roots in the uptake of Suc derived from the phloem.

Suc-induced expression of anthocyanin biosynthetic genes is well known (Solfanelli et al., 2006). However, *suc1* mutants showed an attenuated response (Table IV). This is consistent with the observed reduction in Suc-specific anthocyanin accumulation in *suc1* mutants and highlights a novel role for Suc transporter proteins. Anthocyanins are synthesized via the polypropanoid biosynthetic pathway in the cytosol and then stored in vacuoles (for review, see Koes et al., 2005). Under conditions tested, wild-type seedlings accumulated anthocyanins in the cotyledons, especially around the edges, and at the tip of the hypocotyls (Fig. 5). Homozygous *suc1* mutants accumulated fewer anthocyanins (Figs. 5 and 6) and did not overaccumulate proanthocyanins as evidenced by 4-dimethylaminocinnamaldehyde staining (data not shown). It should be pointed out that *suc1* mutants retained the ability to accumulate anthocyanins in response to Suc especially at high concentrations (5%) of Suc and prolonged exposure. Our hypothesis is that Suc uptake is necessary for Suc-induced anthocyanin production and that other Suc transporters cannot compensate for the loss of AtSUC1 activity.

In summary, *AtSUC1* has important functions in pollen germination and Suc-dependent signal transduction leading to anthocyanin accumulation. Several aspects of *AtSUC1* function will require additional investigation. For example, Suc-induced changes in gene expression (Solfanelli et al., 2006) are strongly inhibited in *suc1* mutants. The mechanism of Suc sensing in plants is not known and it is tempting to speculate that Suc uptake into cells identified as expressing *AtSUC1* are involved.

MATERIALS AND METHODS

Plant Growth Conditions

Plants were grown under long-day conditions (16 h of light) at 23°C unless otherwise noted. For analysis of root growth, plants were grown vertically on 0.5× MS plates supplemented with 2% Suc. Results for root length, silique length, and seeds per silique are presented as mean ± SE.

Pollen Germination Assays (in Vitro and in Vivo)

In vitro pollen germination was determined using liquid medium (5 mM MES-Tris, pH 5.8, 1 mM KCl, 0.8 mM $MgSO_4$, 1.5 mM boric acid, 10 mM $CaCl_2$, 15% [w/v] polyethylene glycol 3350, 5% [w/v] Suc) modified after Fan et al. (2001). For some assays, Suc was replaced with an equimolar amount of Glc (2.5% [w/v]). Anthers were removed from one or two stage 14 flowers. The anthers were briefly agitated in a 30-μL drop of germination medium on a glass slide. The slide was placed in a humidor for 22 h and then a coverslip

Table IV. *Effect of suc1-2 mutation on expression of Suc-responsive genes[a] in the anthocyanin biosynthesis pathway*

Seedlings were grown in liquid culture without Suc and then moved to medium without Suc (MS) for 6 h, or to medium containing 3% Suc for 6 h (Suc) or 24 h (*suc1-2* and Col-0). Normalized signal values are normalized with the MAS 5.0 algorithm and scaled to 100. Each column of signal values represents an independent biological replicate.

AGI Code	Δ	Normalized Signal								Description
		suc1-2		Col-0		MS[a]		Suc[b]		
At5g24530	8.3	50	51	405	434	206	339	96	159	Flavanone 3-hydroxylase-like
At5g07990	3.9	76	40	235	220	33	23	40	213	TT7; flavonoid 3-hydroxylase-like
At4g09820	3.6	12	2	24	26	1	7	7	15	TT8; helix-loop-helix protein
At5g42800	3.6	214	110	557	620	6	10	63	529	TT3; dihydroflavonol 4-reductase
At4g22870	3.5	225	119	609	581	13	3	113	578	Putative anthocyanidin synthase
At3g19010	3.2	75	72	259	218	116	125	73	83	Similar to flavonol synthase (FLS)
At3g51240	3.1	372	312	1,072	1,065	34	3	705	791	TT6; flavanone 3-hydroxylase (FH3)
At1g56650	2.8	28	19	72	57	12	5	194	351	PAP1; MYB transcription factor
At5g35550	2.8	2	2	7	4	6	4	2	4	TT2; R2R3 MYB putative transcription factor
At3g29590	2.7	43	30	102	94	7	8	27	70	Putative anthocyanin 5-aromatic acyltransferase
At5g54060	2.5	134	79	238	298	3	4	95	264	Flavonol 3-*O*-glucosyltransferase-like
At1g66390	2.1	1	1	4	2	3	3	8	6	PAP2; Myb-related transcription factor
At2g30490	1.9	412	430	737	848	350	274	790	635	Cinnamate-4-hydroxylase
At5g08640	1.8	219	301	537	415	39	21	638	147	Flavonol synthase (FLS)
At3g55120	1.8	234	221	445	390	54	47	208	348	Chalcone isomerase
At5g13930	1.7	1,826	2,039	3,131	3,315	185	3	1,667	1,796	TT4; chalcone synthase
At1g65060	1.5	142	115	181	204	29	9	154	100	4-Coumarate:CoA ligase 3
At5g05270	1.5	244	230	410	321	36	2	221	245	TT5; putative chalcone isomerase
At2g37040	1.5	400	368	644	546	146	97	361	322	PAL1; Phe ammonia lyase
At4g38620	1.3	67	60	95	65	117	48	115	144	Putative transcription factor (MYB4)
At4g01070	1.3	83	66	110	88	73	67	240	154	Putative flavonol 3-*O*-glucosyltransferase 5
At5g63590	1.2	20	39	30	40	145	86	217	240	Flavonol synthase
At3g59030	1.0	14	18	13	20	13	5	19	11	TT12; MATE transporter
At4g00730	0.9	155	131	135	133	61	133	113	76	ANL2; homeodomain protein
At1g53520	0.5	396	354	167	172	207	140	205	205	Putative chalcone isomerase

[a]Data values taken from Solfanelli et al. (2006) [b]Δ-Fold change comparing Col-0 to *suc1-2*.

was applied and the number of germinated pollen grains was counted. For each replicate, at least 200 pollen grains were scored.

Insertional Mutant Screening

Insertional mutants were ordered from the Arabidopsis Biological Resource Center. SM_3_20664 (*suc1-2*) and SM_3_19971 (*suc1-1*; Tissier et al., 1999) were screened for homozygous mutants using PCR. The following primers were used: SUC1cdsF2 (5′-agcgttgaagctattctatgggagcctatgaaacagaaa), SUC1_3′-untranslated region (5′-tcgcctaaaccgttctaaactacataaa), and Spm32 (5′-tacgaataagagcgtccattttagagtga).

Anthocyanin Assay

Anthocyanins were extracted as described by Solfanelli et al. (2006), with minor modifications. In brief, plants were grown in liquid culture (0.5× MS supplemented with indicated sugar) for 3 d, and then extracted in 250 μL of 1% HCl in ethanol (v/v). Then 250 μL of water was added. A sample was taken for spectrophotometer readings at 645 and 663 nm. The remaining extract was cleaned with 200 μL of chloroform and another sample was taken for spectrophotometer readings at 535 nm. Anthocyanin content is presented on a fresh-weight basis.

Cloning and Transgenic Arabidopsis

Transgenic Arabidopsis (*Arabidopsis thaliana*) plants with *AtSUC1* whole-gene clones are described by Sivitz et al. (2007). PCR was used to generate a whole-gene *AtSUC1* fragment. The primers SUC1F (5′-cagttaagttttcggttcacaaccaatatg) and SUC1R (5′-gtggaatcctcccatggtcgttgcc) were used with the vector pCR2.1 containing the *AtSUC1* whole gene (Sivitz et al., 2007) as the template. The PCR product was TA cloned into pCR8 (Invitrogen). This plasmid was then recombined with either pMDC99 or pMDC107 (Curtis and Grossniklaus, 2003) using LR recombinase (Invitrogen). Plant transformations were done as by Shigaki et al. (2005). Transformed plants were selected on 30 mg L^{-1} hygromycin.

RT-PCR

RNA was extracted from 5-d-old seedlings of Col-0 and *suc1* mutants using the RNeasy plant mini kit (Qiagen). RT-PCR was performed on 100 μg of RNA using the Qiagen One-Step RT-PCR kit. RT-PCR was performed with 30 cycles each with 1-min, 30-s extension time, and an annealing temperature of 50°C. The following primers were used: SUC1F3 (5′-ctccggtgcaggccaaggactttca), SUC1R1 (5′-aaaggagtactgaaagtaatagctaatggg), and SUC1R3 (5′-ctagtggaatcctcccatggtc).

Quantitative RT-PCR

RNA was extracted using the RNeasy kit (Qiagen) from 5-d-old liquid-grown seedlings. First-strand cDNA was synthesized from 1 μg of total RNA with Superscript III reverse transcriptase (Invitrogen), and quantification on a LightCycler instrument (Roche Diagnostics) with Platinum SYBR Green. Quantitative PCR SuperMix-UDG (Invitrogen) was performed according to the manufacturer's instructions. The quantitative RT-PCR program was as follows: 2 min at 50°C, 2 min at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C, and annealing/extension for 30 s at 60°C. Expression levels were normalized to *Actin-11* expression levels. Quantitative RT-PCR experiments were performed on two independent biological samples and on three technical replicates. The following primers were used: Actin-11F and R (5′-tcgttggtcgtcctcgac and 5′-gggttgagaggagcctca), and SUC1F1 and R1 (5′-agagacacagtcgccgga and 5′-aaaggagtactgaaagtaatagctaatggg).

GUS Staining

Transgenic Arabidopsis plants were vacuum infiltrated with GUS staining solution (1 mM X-GlucA in 50 mM sodium phosphate, pH 7.2, containing 0.5% Triton X-100). Plants were incubated overnight at 37°C and cleared in 70% ethanol.

Radioactive Uptake

Ten seedlings grown for 3 d in liquid 0.5× MS were incubated for 15 min in 0.044 mM ^{14}C Suc, and then washed and incubated for 30 min in 2% SDS. Utlima Gold scintillation cocktail (Packard Biosciences) was added and samples were counted.

Fatty Acid Extraction and Analysis

Pollen was collected by vacuuming open flowers daily and stored at −80°C until extraction. Vacuum collection involved filtering through a 35-μm nylon mesh and collection on a 6-μm nylon filter. The filter and pollen were placed directly into 500 μL of 1 N methanolic HCl containing 25 μg of heptadecanoic acid (17:0) as an internal standard. Samples were heated to 80°C for 1 h and then nylon filters were removed. Equal volumes of 0.9% (w/v) NaCl and hexane were added. The hexane layer was collected and fatty acid methyl esters were analyzed and quantified by gas chromatography. Total protein analysis was done on the aqueous fraction after hexane extraction.

Microarray Analysis

For microarray analysis, seeds stratified for 3 d at 4°C were grown in liquid 0.5× MS for 3 d, then in liquid 0.5× MS medium supplemented with 3% Suc for 24 h. RNA was extracted using the RNeasy plant mini kit (Qiagen). Biotin labeling of the RNA, hybridization to the Affymetrix GeneChip Arabidopsis ATH1 Genome Array, and scanning of the chip were all performed at the University of Minnesota BioMedical Genomics Center Microarray Facility. Data were normalized using the MAS 5.0 algorithm and scaled to 100. Present or absent calls were also made with MAS 5.0. Significant genes, q values, and fold change were generated using *S*-adenosyl-Met.

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