

Diurnal and Light-Regulated Expression of AtSTP1 in Guard Cells of Arabidopsis¹

Ruth Stadler, Michael Büttner, Peter Ache, Rainer Hedrich, Natalya Ivashikina, Michael Melzer, Sarah M. Shearson, Steven M. Smith, and Norbert Sauer*

Molekulare Pflanzenphysiologie, Universität Erlangen-Nürnberg, Staudtstrasse 5, D-91058 Erlangen, Germany (R.S., M.B., N.S.); Julius-von-Sachs-Institut für Biowissenschaften, Lehrstuhl für Molekulare Pflanzenphysiologie und Biophysik (Botanik I), Julius-von-Sachs-Platz 2, D-97082 Würzburg, Germany (P.A., R.H., N.I.); Institut für Pflanzengenetik und Kulturpflanzenforschung, Corrensstrasse 3, D-06466 Gatersleben, Germany (M.M.); and Institute of Cell and Molecular Biology, University of Edinburgh, Mayfield Road, Edinburgh EH9 3JH, Scotland (S.M. Shearson, S.M. Smith)

Guard cell chloroplasts are unable to perform significant photosynthetic CO₂ fixation via Rubisco. Therefore, guard cells depend on carbon supply from adjacent cells even during the light period. Due to their reversible turgor changes, this import cannot be mediated by plasmodesmata. Nevertheless, guard cells of several plants were shown to use extracellular sugars or to accumulate sucrose as an osmoticum that drives water influx to increase stomatal aperture. This paper describes the first localization of a guard cell-specific Arabidopsis sugar transporter involved in carbon acquisition of these symplastically isolated cells. Expression of the *AtSTP1* H⁺-monosaccharide symporter gene in guard cells was demonstrated by in situ hybridization and by immunolocalization with an AtSTP1-specific antiserum. Additional RNase protection analyses revealed a strong increase of *AtSTP1* expression in the dark and a transient, diurnally regulated increase during the photoperiod around midday. This transient increase in *AtSTP1* expression correlates in time with the described guard cell-specific accumulation of sucrose. Our data suggest a function of AtSTP1 in monosaccharide import into guard cells during the night and a possible role in osmoregulation during the day.

The main purpose of guard cells is the minimization of water loss and the simultaneous optimization of CO₂ uptake. This regulation of stomatal conductance is triggered by several environmental stimuli, such as light, humidity, temperature, or CO₂ concentration. Changes in guard cell water potential and subsequent influx or efflux of water modulate shape and aperture of the guard cells (MacRobbie, 1998). This unique property depends on their symplastic isolation, which is mediated by a lack of functional plasmodesmata in mature guard cells (Palevitz and Hepler, 1985).

Analyses of the products obtained after ¹⁴CO₂-labeling of guard cells identified ¹⁴C-labeled malate, the primary fixation product of phosphoenolpyruvate carboxylase, but hardly any Calvin cycle metabolites (Willmer and Ditttrich, 1974; Raschke and Ditttrich, 1977). This was shown to result from an extremely low activity of Rubisco and other Calvin cycle enzymes in guard cell chloroplasts (Willmer and Ditttrich, 1974; Raschke and Ditttrich, 1977; Outlaw et al., 1979, 1982; Reckmann et al., 1990).

Due to this lack of significant photosynthetic CO₂ fixation and of functional plasmodesmata, guard cells depend on carbohydrate import from the apoplast. It was observed very early that guard cells are able to incorporate externally supplied Glc or Suc (Pallas, 1964; Ditttrich and Raschke, 1977), and a correlation between transpiration and apoplastic as well as cytosolic Suc concentrations was shown for broad bean (*Vicia faba*) guard cells (Lu et al., 1995). Pulse labeling of intact broad bean leaves with ¹⁴CO₂ (Lu et al., 1997) showed that ¹⁴C-labeled Suc identified in guard cells resulted from fixation products synthesized in and secreted from the palisade parenchyma. However, the identity of the transported molecules (Glc, Fru, or Suc) had not been determined. The first detailed analyses of guard cell-specific sugar transport (Ritte et al., 1999) identified and characterized a monosaccharide-H⁺ symporter activity in pea (*Pisum sativum*). Moreover, in the same paper, the existence of an additional Suc transporter was shown to be very likely.

Sugars imported into guard cells are necessary for the formation of starch. During stomatal opening, starch molecules are degraded to phosphoenolpyruvate and eventually into malate, a counter ion for accumulated K⁺ (Willmer and Ditttrich, 1974; Raschke and Ditttrich, 1977; Outlaw and Manchester, 1979; Schnabl et al., 1982; Tarczynski and Outlaw, 1990). More than a century ago (Kohl, 1886) it was postulated that sugars accumulating in guard cells

¹ This work was supported by the Deutsche Forschungsgemeinschaft (grant nos. Sa382/5 and Sa382/8 to N.S. and grant no. Bu973/4 to M.B.).

* Corresponding author; e-mail nsauer@biologie.uni-erlangen.de; fax 49-9131-85-28751.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.103.024240.

might also be used as osmotica themselves and thus directly influence stomatal aperture. However, due to the extensive analyses of potassium ions as guard cell osmoticum (Assmann, 1993; Maathuis et al., 1997; MacRobbie, 1998; Dietrich et al., 2001), this suggested role of sugars has almost been forgotten. Nevertheless, several authors reported Suc accumulation in guard cells of different plant species (Reddy and Rama Das, 1986; Poffenroth et al., 1992; Talbott and Zeiger, 1993; Lu et al., 1997), and Talbott and Zeiger (1996, 1998) provided evidence that K^+ and Suc are used as osmotica at different day times. Their data suggested that Suc replaces K^+ as the primary guard cell osmoticum around midday (Talbott and Zeiger, 1996, 1998).

The *AtSTP1* gene from *Arabidopsis* was the first cloned gene of a higher plant plasma membrane transporter (Sauer et al., 1990). In contrast to the extensive functional analyses of the *AtSTP1* protein (Sauer et al., 1990; Boorer et al., 1994; Stolz et al., 1994), surprisingly little is known about the localization of the *AtSTP1* protein in planta and about its physiological properties. Northern analyses suggested strong expression of *AtSTP1* in leaves (Sauer et al., 1990), but in these experiments, cross reactions of the *AtSTP1* probe with mRNAs from other *AtSTP* genes that were identified since then (Büttner and Sauer, 2000) could not be excluded. In addition, seedlings of an *Atstp1* T-DNA insertion line have reduced sensitivity to toxic concentrations of D-Gal and D-Man (Shearson et al., 2000), but the precise site of *AtSTP1* expression remained obscure.

This paper demonstrates that the *AtSTP1* monosaccharide- H^+ symporter gene (Sauer et al., 1990) is strongly expressed in guard cells and that this expression is strongly regulated. This is the first carbohydrate transporter gene shown to be expressed in guard cells. Circadian changes in *AtSTP1* expression during the day and its modulation by light suggest two independent physiological functions of *AtSTP1* in carbohydrate acquisition of guard cells. An *Atstp1* T-DNA insertion line is analyzed.

RESULTS

Localization in Guard Cells of *AtSTP1* mRNA by in Situ Hybridization and of *AtSTP1* Protein by Immunohistochemistry

Northern analyses suggested a strong expression of *AtSTP1* in leaves (Sauer et al., 1990), but at that time, cross reactions of the probe with mRNAs of other, only recently identified *AtSTP* gene family members (Büttner and Sauer, 2000) could not be excluded. In a recent paper by Shearson and coworkers (2003), this expression was confirmed by independently performed northern blots and by analyses of *AtSTP1*-promoter:luciferase plants. This paper clearly showed that *AtSTP1* is strongly expressed in leaves of *Arabidopsis* seedlings and in leaf and stem tissue

of mature *Arabidopsis* plants. The strong *AtSTP1* expression in roots of *Arabidopsis* seedlings deduced from transport studies with an *Atstp1* T-DNA insertion mutant (Shearson et al., 2000) has not been confirmed by these analyses.

For a detailed analysis of this observed *AtSTP1* expression in leaf and stem tissue (Sauer et al., 1990; Shearson et al., 2003) on the cellular level, in situ hybridizations were performed with radiolabeled *AtSTP1* sense or antisense mRNA. In cross sections of rosette leaves (Fig. 1, A–D) or cotyledons (Fig. 1C) treated with a radiolabeled antisense probe, signals were observed only in guard cells. No signals were obtained in any other cell type or in sections treated with the sense probe (Fig. 1, D and E).

This result was confirmed by immunohistochemical analyses of *AtSTP1* localization (Fig. 2) using anti-*AtSTP1* antiserum from rabbits immunized with recombinant *AtSTP1* protein (Stolz et al., 1994). Binding of anti-*AtSTP1* antibodies was visualized by indirect immunofluorescence with anti-rabbit IgG decorated with FITC-isomer 1-conjugate. *AtSTP1*-specific and FITC-IgG-dependent fluorescence was confined to guard cells of various *Arabidopsis* tissues, such as rosette leaves (Fig. 2, A–D), sepals (Fig. 2, G–K), cotyledons (Fig. 2, L and M), ovaries (Fig. 2, N and O), or stems (data not shown). The fluorescence is clearly visible and specific for this single cell type. No fluorescence was detected in leaves, when the second antibody was omitted (data not shown) or when an antiserum was used that had been raised against the pollen-specific *AtSTP2* protein and not against *AtSTP1* (Fig. 2, E and F; Truernit et al., 1999).

Immunohistochemical analyses with sepals revealed a clear difference in the amount of *AtSTP1*

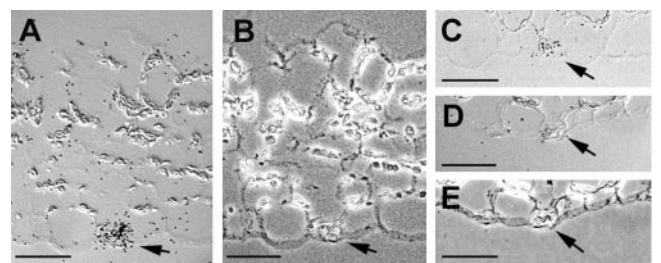
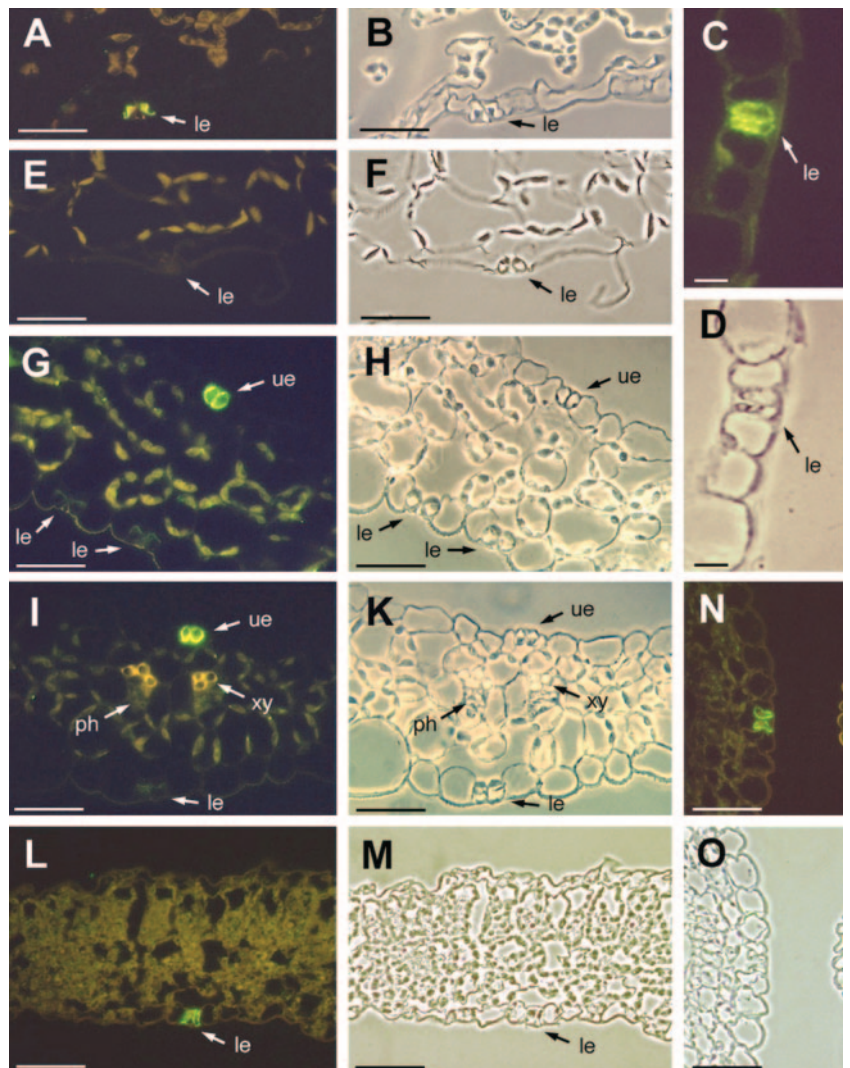


Figure 1. Localization of *AtSTP1* mRNA in guard cells of *Arabidopsis* leaves and seedlings by in situ hybridization. A, Cross section through a rosette leaf hybridized to *AtSTP1* antisense RNA and photographed with differential interference contrast for optimal visualization of the hybridization signals. Accumulation of label is seen in the guard cell in the lower epidermis. B, Same section as in A photographed with phase contrast for better visualization of the guard cell and the epidermal cell walls. C, Cross section through a cotyledon hybridized to *AtSTP1* antisense RNA and photographed with differential interference contrast. Accumulation of label is seen in the guard cell in the lower epidermis. D, Cross section through a rosette leaf hybridized to *AtSTP1* sense RNA and photographed with differential interference contrast. No accumulation of label is seen in the guard cells. E, Same section as in D photographed with phase contrast. Guard cells are marked with an arrow. All space bars = 25 μ m.

Figure 2. Immunolocalization of AtSTP1 protein in guard cells from different Arabidopsis tissues. A, Cross section through a rosette leaf stained with anti-AtSTP1 antiserum/fluorescein isothiocyanate (FITC). B, Same section as in A photographed under white light. C, Guard cell from an Arabidopsis rosette leaf stained with anti-AtSTP1 antiserum/FITC shown at higher magnification. D, Same section as in C photographed under white light. E, Cross section through a rosette leaf treated with an anti-AtSTP2 control serum antiserum/FITC. F, Same section as in E photographed under white light. G, Cross section through a sepal stained with anti-AtSTP1 antiserum/FITC. H, Same section as in G photographed under white light. I, Cross section through a sepal stained with anti-AtSTP1 antiserum/FITC. K, Same section as in I photographed under white light. L, Cross section through a 4-d-old cotyledon showing anti-AtSTP1 antiserum/FITC-specific labeling only in the guard cells. M, Same section as in L photographed under white light. N, Cross section through the ovary stained with anti-AtSTP1 antiserum/FITC. O, Same section as in N photographed under white light. Arrows show guard cells of the lower (le) or upper epidermis (ue), the xylem (xy) and the phloem (ph). Scale bars = 10 μm in C and D; all other scale bars = 25 μm .



protein found in guard cells of the upper (inner) and the lower (outer) epidermis (Fig. 2, G–K). Reproducibly stronger AtSTP1-specific fluorescence was detected in guard cells of the upper epidermis (facing the petals) than in guard cells of the lower epidermis.

AtSTP1 Protein Is Absent from Guard Cells of *Atstp1* Knockout Plants

We analyzed thin sections of the above-mentioned *Atstp1* mutant (Shearson et al., 2000) for the presence or absence of AtSTP1 protein using the identical antibodies and the same technique as described above. Figure 3 shows that the AtSTP1 protein is absent from guard cells of the *Atstp1* mutant plants. This result confirms the disruption of the *AtSTP1* gene in the mutant line. It also confirms the data obtained by in situ hybridization in Figure 1 and the specificity of the antibody used for the immunohistochemical localization of AtSTP1 in Figure 2.

AtSTP1 Expression Is Induced/Derepressed in the Absence of Light and Is Transiently Increased at Midday

Guard cell chloroplasts contain only marginal amounts of Calvin cycle enzymes. This allows the light-dependent formation of ATP, but little or no photosynthetic CO₂ fixation. Thus, AtSTP1 may import monosaccharides for guard cell metabolism, and an increased expression of *AtSTP1* might be expected in the dark, when light-dependent ATP formation is zero. Therefore, we isolated total RNA from rosette leaves harvested at different times of the day and analyzed *AtSTP1* mRNA levels. In Figure 4, one of three independently performed RNase protection analyses is presented. In all experiments, *AtSTP1* mRNA levels increased significantly after dusk. During the night, *AtSTP1* mRNA levels decreased slightly reaching a minimal level in the morning after the end of the dark phase.

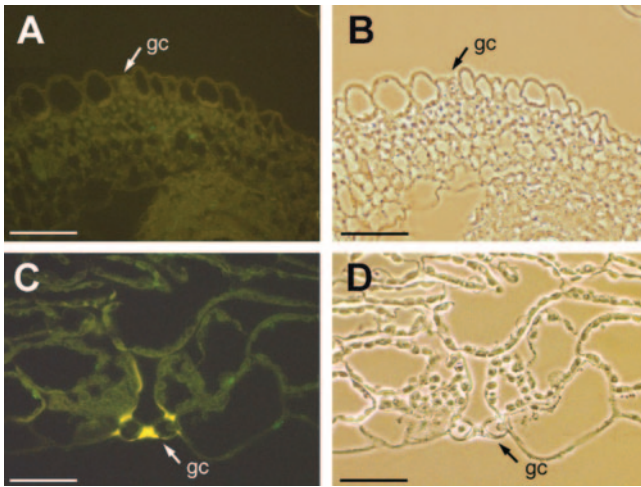


Figure 3. Lack of AtSTP1 protein in *Atstp1* knockout plants. Sections were prepared from T-DNA-tagged *Atstp1*-knockout plants and treated with anti-AtSTP1 antiserum and FITC-conjugated second antiserum simultaneously with the sections presented in Figure 2. A, Cross section through the ovary of an *Atstp1* mutant plant treated with an anti-AtSTP2 control serum antiserum/FITC. No green FITC-fluorescence is seen in the guard cells. The yellow color results from phenolics deposited in the guard cell wall. B, Same section as in A photographed under white light. C, Cross section through the cotyledon of an *stp1* mutant plant stained with anti-AtSTP1 antiserum/FITC. No green FITC fluorescence is detected in the guard cells. D, Same section as in C photographed under white light. Arrows show guard cells (gc). All scale bars = 25 μm .

A less strong, but reproducible increase in *AtSTP1* mRNA was also observed at midday (Fig. 4). This increased expression extended toward late afternoon and disappeared before dusk. The time course of this increase correlates well with the time of maximal Suc accumulation in guard cells described, e.g. for broad bean (Talbot and Zeiger, 1996). Recent high-density microarray analyses of more than 8,000 *Arabidopsis* genes for potential circadian clock regulation identified 453 cycling genes (Harmer et al., 2000). One of these genes was *AtSTP1*, which showed a 2.5-fold induction of mRNA levels peaking 8 h after subjective dawn. Both level and time of this circadian *AtSTP1* induction correlate perfectly with the observed increase of *AtSTP1* expression around midday (Fig. 4), suggesting that this peak results from circadian clock regulation.

In contrast, the observed increase of *AtSTP1* mRNA levels at the onset of the dark period (Fig. 4) was not seen in these microarray analyses (Harmer et al., 2000), suggesting that this enhanced expression is not due to diurnal regulation. Further RNase protection analyses (Fig. 5A) support this conclusion and demonstrate that these changes are due to a rapid induction of *AtSTP1* expression in the absence of light. Dark incubation of *Arabidopsis* plants for 1 h during the photoperiod caused a strong increase in *AtSTP1* mRNA levels similar to that seen at the end of the photoperiod in Figure 4. *AtSTP1* mRNA levels

in control plants kept for 1 h in light showed no change.

To confirm that the observed induction/derepression results from *AtSTP1* gene expression in guard cells and not from other cells of the leaf, a quantitative reverse transcriptase (RT)-PCR was performed with a LightCycler using mRNAs from guard cell-enriched preparations (Becker et al., 1993). This analysis (Fig. 5B) showed that mRNA preparations obtained from strongly enriched, intact guard cells of dark-treated plants (1.5 h) had about 9-fold increased *AtSTP1* mRNA levels compared with guard cells from plants grown under constant light. This corresponds perfectly with the described induction/derepression at the end of the light phase in Figure 5 confirming that enhanced *AtSTP1* expression is restricted to guard cells.

Lack of AtSTP1 Does Not Cause a Detectable Change in Guard Cell Phenotype

A lack of a monosaccharide transporter in the plasma of the guard cells might cause phenotypic changes due to the suboptimal or missing supply of organic carbon. This might result in the inability to perform volume changes or to respond to environ-

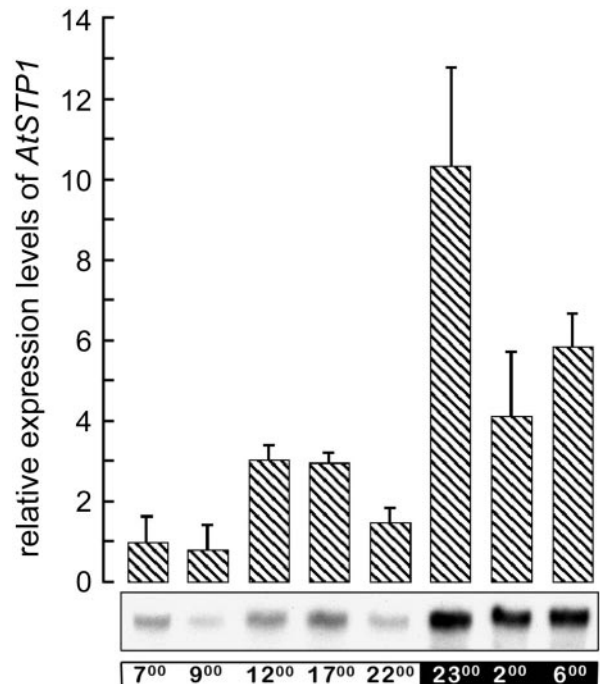


Figure 4. Diurnal changes of *AtSTP1* expression in rosette leaves. RNase protection analyses (10 μg total RNA per lane) were performed with total RNA isolated at the indicated time points from *Arabidopsis* plants grown in a growth chamber under a 16-h-light/8-h-dark regime (three independent experiments). Gels were exposed to x-ray films (one typical autoradiogram is shown) and counts in *AtSTP1* mRNA bands were quantified with a phosphor imager. The relative amounts of *AtSTP1* mRNA determined in three experiments are presented (mean \pm SD).

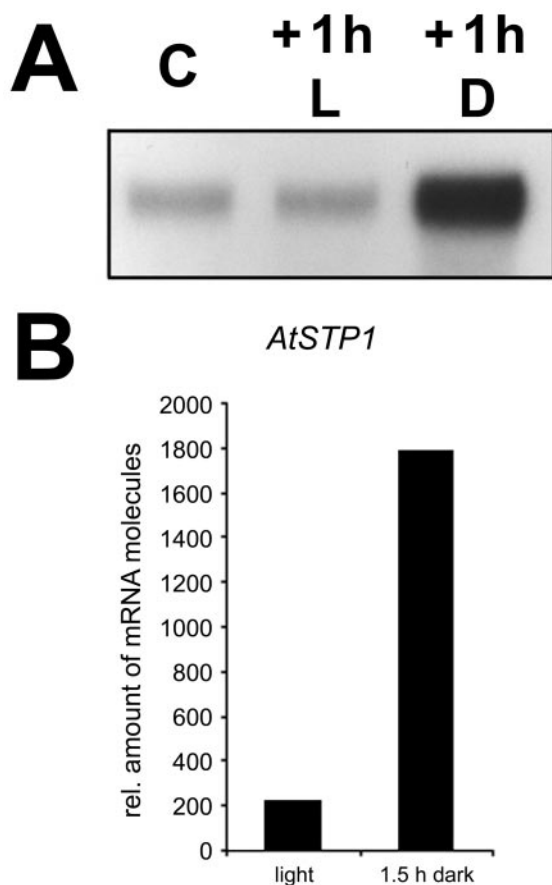


Figure 5. Dark induction of *AtSTP1* gene expression. A, RNase protection analyses (8 μ g per lane) were performed with total RNA isolated from rosette leaves of plants grown under the conditions described in Figure 4. C, Control leaves were harvested in the early afternoon. At this time several plants were put in the dark. Rosette leaves were harvested 1 h later from plants kept in the light (+1 h L) or put in the dark (+1 h D). B, Quantitative RT-PCR on mRNA from enriched guard cells. Plants were darkened for 1.5 h in the early afternoon while controls were kept illuminated. As in A, *AtSTP1* expression was strongly induced (9-fold) upon darkening.

mental stimuli. However, number, size, and morphology of guard cells in *Atstp1* mutants were the same as in *AtSTP1* wild-type (WT) plants. Moreover, mutant plants grew equally well under all conditions tested, and detached mutant leaves showed no altered water loss compared with detached leaves from WT plants (data not shown).

Reduced import of carbohydrates might also influence the starch content of the guard cells. Therefore, rosette leaves were harvested from Arabidopsis WT and mutant plants at the end of the dark phase and at the end of the light phase. Sections of fixed and embedded tissue were analyzed for starch granules in guard cells under the electron microscope. Figure 6 demonstrates that guard cell chloroplasts of both lines contained large amounts of starch at the end of the 8-h light period and little or no starch at the end of the 16-h dark phase. The variation in starch con-

tent of individual guard cells of WT or mutant plants at the end of the dark phase (no starch or tiny starch granules) was absolutely comparable. This suggests that carbon import into Arabidopsis guard is mediated not only by the exclusive transport activity of *AtSTP1*.

The lack of phenotypic differences between WT and *Atstp1*-mutant plants might result from a compensation of this genetic defect, e.g. by up-regulation of another member of the *AtSTP* gene family or of a K^+ channel gene. Alternatively, the transport rates of already present sugar transporters or K^+ channels could be increased. Therefore, K^+ currents across the plasma membrane of guard cell protoplasts from WT and mutant plants (Fig. 7) and the potential induction of K^+ channel genes or of *AtSTP* genes (Fig. 8) known to be expressed in guard cells were analyzed (*AtSTP5* and *AtSTP13*; M. Büttner and N. Sauer, unpublished data).

The observed inward K^+ currents were identical in protoplasts isolated from leaves of WT and mutant plants (Fig. 7), which indicates that the lack of *AtSTP1* is not compensated by an increased potassium influx into guard cells of the *Atstp1* mutant. This was supported by quantitative PCR analyses with mRNAs obtained from guard cell protoplasts of *AtSTP1* WT and *Atstp1* mutant plants. Analyses of Arabidopsis guard cell K^+ channels (Szyroki et al., 2001) revealed no differences in the mRNA levels of the analyzed channels (Fig. 8). In parallel, the relative mRNA levels of *AtSTP5* and *AtSTP13* were determined, which also turned out to be unchanged in WT and mutant lines (Fig. 8). These data suggest that none of the analyzed candidate genes is up-regulated in response to the knockout mutation in the *AtSTP1* gene. However, it cannot be excluded that Glc trans-

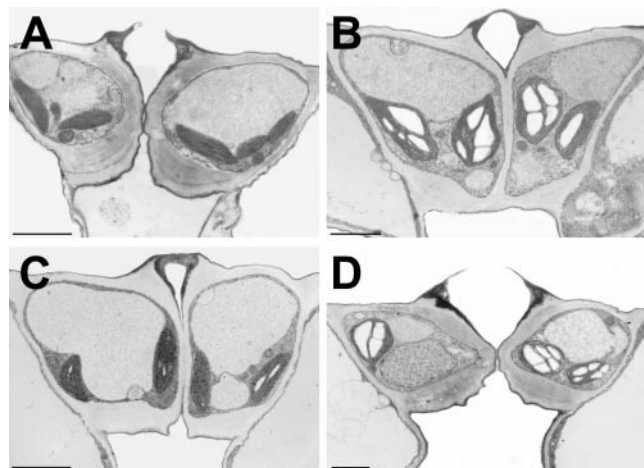


Figure 6. Accumulation of starch in guard cells of Arabidopsis WT and *AtSTP1* mutant plants. Rosette leaves were harvested from *Arabidopsis Atstp1* mutant plants (A and B) or from WT plants (C and D) either at the end of the dark phase (A and C) or at the end of the 8-h-light phase (B and D). Leaf sections were fixed, embedded, and sectioned for electron microscopic analyses. Space bars = 2 μ m.

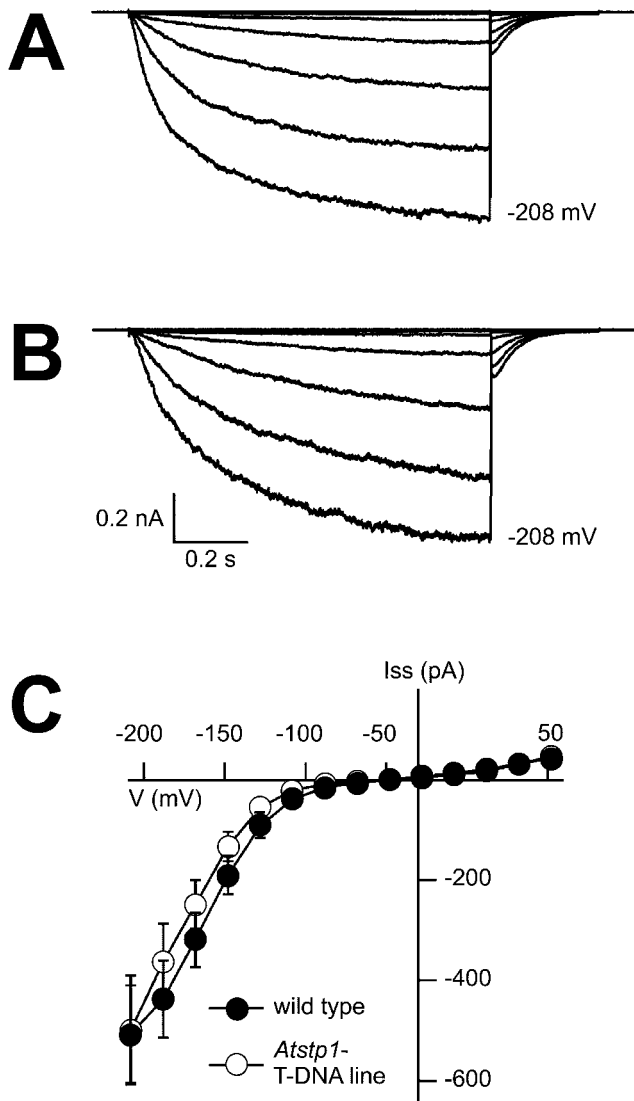


Figure 7. Electrophysiological analyses of macroscopic K^+ currents in Arabidopsis guard cell protoplasts. Voltage- and time-dependent properties of inward K^+ currents in guard cell protoplasts from an *AtSTP1* WT plant (A) and from the *Atstp1* T-DNA insertion mutant (B). Voltage pulses were applied to the protoplasts in the whole-cell configuration starting from a holding potential -48 mV in 20-mV decrements from $+52$ to -208 mV. Pipette solution contained 150 mM potassium gluconate, 2 mM $MgCl_2$, 10 mM EGTA, 2 mM Mg-ATP, and 10 mM HEPES/Tris (pH 7.4). External solution contained 30 mM potassium gluconate, 1 mM $CaCl_2$, and 10 mM MES/Tris (pH 5.6). C, Voltage dependence of inward K^+ currents in guard cell protoplasts from WT ($n = 13$) and *AtSTP1* knockout plants ($n = 14$). Current amplitudes were sampled at the end of 1-s pulses to voltages in the range from $+52$ to -208 mV. External solution contained 20 mM $CaCl_2$, 30 mM potassium gluconate, and 10 mM MES/Tris (pH 5.6). Data points represent means \pm SE.

port by *AtSTP5* and/or *AtSTP13* is enhanced by an increased stability of these proteins or by altered translation rates for the corresponding mRNAs.

Eventually, stomatal movement and photosynthesis were followed by measuring water loss and CO_2

assimilation with infrared gas analyzers (Hedrich et al., 2001) to examine the possibility that a loss of *AtSTP1* affects guard cell performance. However, in *AtSTP1* WT and in *Atstp1* mutant plants, the observed transpiration was comparable (Fig. 9). Predarkened leaves opened and closed their stomata identically in response to light and darkness under ambient CO_2 ($300 \mu L L^{-1}$). Neither the amplitude nor the rate of stomatal movement was significantly altered in mutant plants. CO_2 -uptake rates were also comparable in mutant and WT plants (data not shown). Thus, the lack of *AtSTP1* does not seem to result in a modification of stomatal properties.

DISCUSSION

It is known that guard cells can import Glc from the apoplast and that this transport can be inhibited by other monosaccharides, such as Man or Fru (Ritte et al., 1999). Glc transport is energy dependent, enhanced in the presence of fusicoccin, an activator of plasma membrane H^+ -ATPases (Marrè, 1979), and inhibited by uncouplers. Our present paper provides evidence that in Arabidopsis, the H^+ -monosaccharide symporter *AtSTP1* is responsible at least for part of this transport. Moreover, it shows that guard cell-specific *AtSTP1* expression is strongly regulated by the diurnal clock and by light and that *AtSTP1* expression varies between guard cells from both sides of a leaf. Analysis of an *Atstp1* T-DNA insertion mutant showed that *AtSTP1* protein and mRNA are in fact absent in guard cells of this line. However, phenotypic alterations could not be detected in mutant plants and guard cells.

Our analyses revealed an interesting regulation of *AtSTP1* expression in guard cells with *AtSTP1* transcript levels being significantly increased at the be-

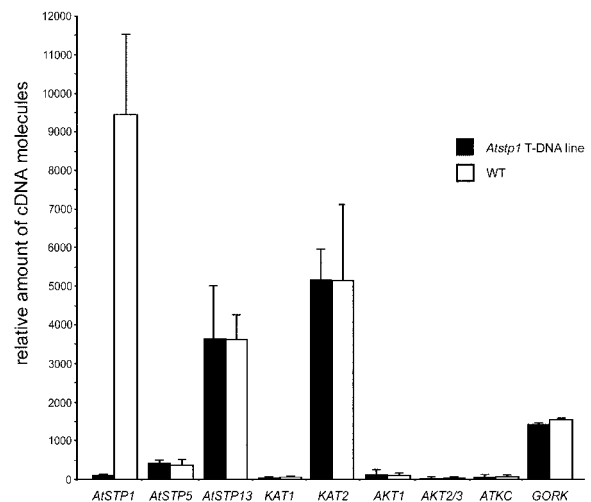


Figure 8. Loss of *AtSTP1* is not compensated by enhanced expression of *AtSTP5*, *AtSTP13*, or a K^+ channel gene. Amounts of cDNA molecules in guard cell protoplasts were determined by quantitative RT-PCR on WT and the *Atstp1* T-DNA insertion mutant (mean \pm SD).

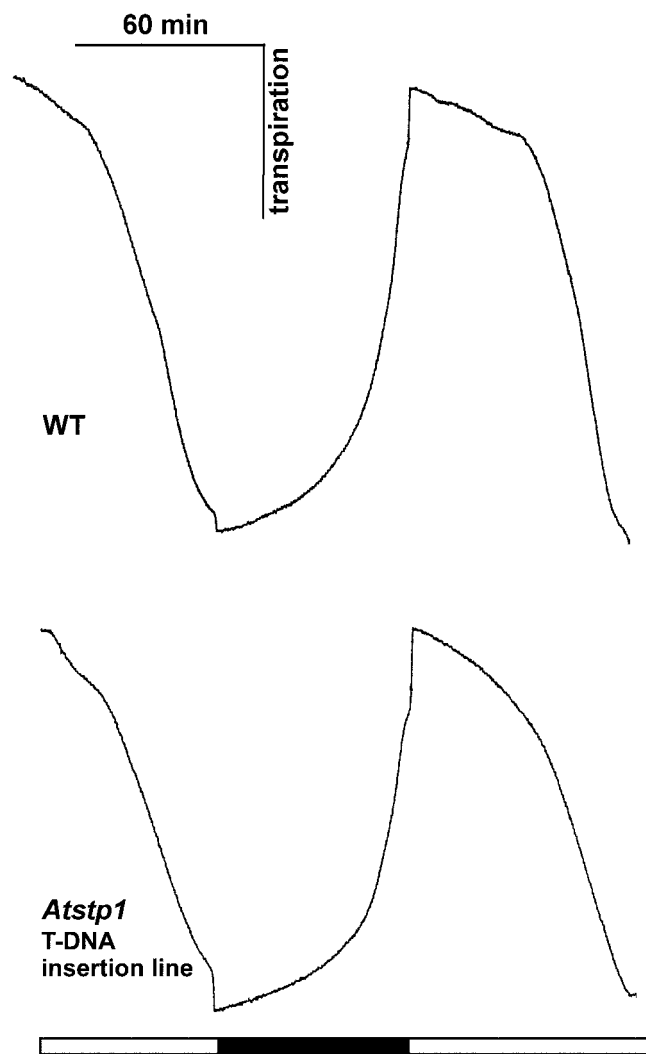


Figure 9. Gas exchange measurements with *AtSTP1* WT and *Atstp1* mutant plants. Transpiration of 8-week-old rosette leaves from *Arabidopsis* plants grown in a climate chamber under short-day conditions (8 h). Leaves from *AtSTP1* WT (top panel) and *Atstp1* knockout plants (bottom panel) were incubated in $300 \mu\text{L L}^{-1} \text{CO}_2$ and alternating light and dark conditions for stomatal opening and closure. Black box, Darkness; white box, light ($700 \mu\text{mol photons m}^{-2} \text{s}^{-1}$); representative results of four independent experiments are given.

gining of the dark phase (Fig. 4). This enhanced expression is due to dark induction or light repression of *AtSTP1* and is also observed after a 1-h dark treatment of WT plants during the day (Fig. 5). The induction does not result from an increased *AtSTP1* expression in other leaf cells, because it is also seen in strongly enriched preparations of intact guard cells (Fig. 5B; Becker et al., 1993). This suggests an increased import of carbohydrates into guard cells after the end of the photoperiod. This is surprising, because guard cell chloroplasts are packed with starch at this time (Fig. 6). Starch is known to be converted to malate, which acts as counter ion to K^+ imported in the light during stomatal opening (Schnabl et al., 1982; Outlaw and Tarczynski, 1984) and might there-

fore be stored for the start of the next photoperiod. However, our results demonstrate that more or less all starch is metabolized during the dark phase in both *AtSTP1* WT and mutant plants (Fig. 6). This suggests that the primary role of starch in guard cells is to drive the energy supply of the guard cells in the dark. Induction of *AtSTP1* after sunset may support this carbon metabolism with carbohydrates imported from the apoplast. Alternatively, *AtSTP1* may act as a retrieval transporter to avoid loss of Glc released from the apoplast during starch breakdown in the night.

The observation that *Arabidopsis* guard cells are practically free of starch in the morning makes it necessary to rethink the generally accepted model of starch breakdown for malate formation (Schnabl et al., 1982; Outlaw and Tarczynski, 1984). Our data suggest that malate might rather be formed from sugars imported by *AtSTP1* or another guard cell monosaccharide transporter (*AtSTP5* or *AtSTP13*). Alternatively, carbohydrates might be imported as Suc. Transport analyses performed by Ritte et al. (1999) on guard cells from broad bean with radiolabeled Suc provided evidence that these cells may possess Suc transport proteins. Recent analyses from our lab support this observation, because *AtSUC2* mRNA (Sauer and Stolz, 1994) was shown to be present in guard cells by RT-PCR performed on guard cell-specific RNA preparations (P. Ache, R. Hedrich, and N. Sauer, unpublished data). Moreover, the *AtSUC3* Suc transporter gene (Meyer et al., 2000) also seems to be expressed in *Arabidopsis* guard cells, because plants expressing the green fluorescent protein under the control of the *AtSUC3*-promoter show strong fluorescence in these cells (S. Meyer, C. Lauterbach, and N. Sauer, unpublished data). Finally, *Arabidopsis* may use Cl^- as counter ion for K^+ rather than malate. This has been shown for broad bean, where Cl^- ions replace malate as counter ion depending on the growth conditions (Talbot and Zeiger, 1998).

The transient increase in *AtSTP1* expression around the mid-point of the photoperiod is unlikely to reflect an increased demand of carbohydrates for ATP synthesis. Photophosphorylation is maximal at this time of the day. The increase in *AtSTP1* expression may rather reflect an increased demand of carbon for the production of organic compounds, e.g. Suc. It is known that at this time of the day, guard cells start to replace K^+ , their major osmoticum during the morning hours (Raschke et al., 1988; Hedrich and Becker, 1994), by Suc (Lu et al., 1995; Talbot and Zeiger, 1996, 1998). Moreover, a clear correlation between Suc concentration and guard cell aperture in the afternoon has been shown (Talbot and Zeiger, 1996). Guard cell content of Glc or Fru did not change significantly over the course of the light period (Talbot and Zeiger, 1996), suggesting a rapid conversion of most of the imported monosaccharide into Suc.

AtSTP1 protein and *AtSTP1* mRNA were localized in guard cells of different leaf types and of stems (Figs. 1 and 2). In sepals, higher levels of AtSTP1 protein were found reproducibly in the upper (facing the petals) than in the lower epidermis. Sometimes, but far less reproducibly and less obviously, we found also slight differences in the amount of AtSTP1 protein between the upper and lower epidermis of rosette leaves. In that case, however, the amount of AtSTP1 protein was higher in the guard cells of the lower epidermis. These differences in *AtSTP1* expression between the upper and the lower epidermis layers may result from differences in the light intensity on both sides of the respective leaves. The upper epidermis of rosette leaves and the lower epidermis of the sepals in a budding flower are exposed to direct sunlight, and *AtSTP1* expression may be repressed to some extent. Alternatively, the observed differences in the amount of *AtSTP1* protein might be a measure for differences in the guard cell activities on both sides of these leaves.

We were not able to identify phenotypic differences between *AtSTP1* WT and *Atstp1* mutant plants with respect to their stomatal aperture, their water loss from detached leaves, or their growth and development. *Atstp1* mutant plants were previously shown to be less sensitive to monosaccharides, such as Man or Gal, which inhibit germination and seedling development in WT Arabidopsis (Shearson et al., 2000). This reduced uptake capacity of mutant seedlings for radiolabeled monosaccharides can certainly not be explained by the lack of AtSTP1 protein in guard cells of cotyledons. Obviously, it cannot be excluded that besides the strong expression of the *AtSTP1* gene in guard cells that can be visualized by the techniques used in this paper, there may be an additional, but weaker *AtSTP1* expression in other cell types of Arabidopsis, e.g. in certain cells of the roots (Shearson et al., 2000). All attempts to identify AtSTP1 protein or *AtSTP1* mRNA in roots by immunolocalization or in situ hybridization failed (data not shown). This observation is in perfect agreement with recent analyses of *AtSTP1*-promoter:luciferase plants (Shearson et al., 2003) showing strong *AtSTP1* expression only in the leaves. In contrast to earlier reports (Shearson et al., 2000) *AtSTP1* expression in roots of Arabidopsis seedlings was extremely weak (Shearson et al., 2003).

The lack of a detectable *Atstp1* mutant phenotype in guard cells, in leaf development, or in plant growth (not shown) is not due to the enhanced expression of one of the other guard cell-specific *AtSTP* genes (Fig. 8; M. Büttner and N. Sauer, unpublished data). Moreover, we were not able to observe complementation of the midday induction of *AtSTP1* (Fig. 4) by an increased inflow of K⁺ ions (Fig. 7) or by enhanced expression of a K⁺ channel gene (Fig. 8), which might replace the possibly lacking osmoticum Suc by K⁺.

Our data clearly support a physiological function of AtSTP1 in guard cells of all epidermis layers. However, the lack of detectable phenotypic differences between mutant and WT plants suggests that other transporters (or channels) can compensate this defect in *Atstp1* plants. Therefore, the generation of multiple knockout plants will be necessary to get an answer to this question.

MATERIALS AND METHODS

Strains and Growth Conditions

If not otherwise indicated, plants of Arabidopsis ecotype Wassilewskija and plants of the corresponding *Atstp1* mutant line (Shearson et al., 2000) were grown in potting soil in the greenhouse or on agar medium in plastic containers under a 8-h-light/16-h-dark regime at 22°C and 60% relative humidity.

Immunohistochemical Techniques

The anti-AtSTP1 antiserum used in this paper has been described (Stolz et al., 1994). Crude antiserum was purified according to the procedure of Sauer and Stadler (1993).

Semithin sections (3–6 μm) were prepared from methacrylate embedded Arabidopsis tissue, treated with affinity-purified anti-AtSTP1 antiserum, and stained with anti-rabbit IgG-FITC-isomer 1-conjugate as published (Stadler and Sauer, 1996).

In Situ Hybridization with *AtSTP1* Antisense Probes

For generation of radiolabeled *AtSTP1* sense and antisense RNA probes, a 1,600-bp *Hind*III fragment of the *AtSTP1* cDNA clone pHEX313H (Stolz et al., 1994) was cloned in both orientations into *Hind*III-digested pBluescript II SK⁻ (Stratagene, La Jolla, CA). ³⁵S-labeled antisense and sense RNAs were synthesized with T7 RNA polymerase from the *Bam*HI-linearized constructs.

For preparation of tissue sections, Arabidopsis tissue was fixed in 3.7% (v/v) formaldehyde, 50% (v/v) ethanol, and 10% (v/v) acetic acid at room temperature for 1 h and embedded in methacrylate as described (Stadler et al., 1996). Sections were hybridized and washed as published (Truernit et al., 1999).

RNA Isolation and RNase Protection Analyses

Arabidopsis tissue was collected, and RNA was isolated as described previously (Sauer et al., 1990). RNase protection analyses were performed as published (Ratcliffe et al., 1990; Gahrz et al., 1996). For generation of the *AtSTP1* antisense RNA probe, a 161-bp *Hinc*II/*Sac*I fragment (bp 209–370) of the *AtSTP1* cDNA clone pTF414A (Sauer et al., 1990) was cloned into pBluescript II SK⁻ (Stratagene). ³⁵S-labeled antisense RNA was synthesized with T3 RNA polymerase from the *Hinc*II-linearized construct yielding a 178-bp probe. The hybridizing portion of this probe was 164 bp long.

RNAs isolated from eight tissue samples withdrawn during a photoperiod were separated on 10% (w/v) polyacrylamide gels, and the counts per *AtSTP1* band were determined with a phosphor imager. Counts of each time points were expressed as percentage of the whole day and used to determine the relative expression levels.

Quantitative RT-PCR

For RT-PCR experiments, guard cell protoplasts were isolated as described below. Poly(A⁺) RNA was purified, first-strand cDNA was prepared, and PCR was performed in a LightCycler (Roche Diagnostics, Basel) as described (Szyroki et al., 2001). The K⁺ channel-specific primers were identical to those used by Szyroki et al. (2001). For *AtSTP1*, we used the primers STP1LC2fwd (5'-GCA GCT TGC ATA GGG-3') and STP1LC2rev (5'-TCT CAA GCG CAT TCC-3'); for *AtSTP5*, the primers STP15LCfwd

(5'-AGC CGA ACT GTT TAT-3') and STP15LCrev (5'-AAG AAG CGA ACC AAG-3'); and for *AtSTP13*, the primers STP13LC2fwd (5'-CCG TCG CTT TAC AGA-3') and STP13LC2rev (5'-ACG CCA AGG ATA ATG-3').

The GenBank accession numbers for K⁺ channels are as given by Szyroki et al. (2001), the Munich Information Center for Protein Sequences numbers for *AtSTPs* are as follows: *AtSTP1*, At1g11260; *AtSTP5*, At1g34580; and *AtSTP13*, At5g26340. All quantifications were normalized to 10,000 molecules of the actin 2/8 gene fragments of 435 bp amplified by ACT2/8fwd (5'-GGT GAT GGT GTG TCT-3') and ACT2/8rev (5'-ACT GAG CAC AAT GTT AC-3'). Fragment lengths are: *AtSTP1* = 400 bp, *AtSTP5* = 208 bp, and *AtSTP13* = 334 bp.

Isolation of Guard Cells and Protoplast Isolation

Enriched guard cell fractions were isolated from *Arabidopsis* leaves by the Blender method described by Becker et al. (1993). Where indicated, guard cell protoplasts were isolated from this enriched fraction as described (Hedrich et al., 1990). The enzyme solution contained 0.8% (w/v) cellulase (Onozuka R-10), 0.1% (w/v) pectolyase, 0.5% (w/v) bovine serum albumin, 0.5% (w/v) polyvinylpyrrolidone, 1 mM CaCl₂, and 8 mM MES/Tris, pH 5.6. The osmolarity of the enzyme solution was adjusted to 540 mosmol kg⁻¹ using D-sorbitol.

Patch-Clamp Recordings

Measurements were performed as described (Szyroki et al., 2001). Pipette solutions (cytoplasmic side) contained: 150 mM potassium-gluconate, 2 mM MgCl₂, 10 mM EGTA, 2 mM Mg-ATP, and 10 mM HEPES/Tris (pH 7.4). The standard external solutions contained: 30 mM potassium-gluconate, 1 mM CaCl₂, and 10 mM MES/Tris (pH 5.6). Osmolarity of the solutions was adjusted to 540 mosmol kg⁻¹ using D-sorbitol. Data were digitized (ITC-16, Instrutech, Elmont, NY), stored on hard disc, and analyzed using PULSE and PULSEFIT software (HEKA Elektronik, Lambrecht, Germany) and IGOR Pro software (WaveMetrics, Lake Oswego, OR).

Gas Exchange Measurements

Transpiration and CO₂ exchange were determined as previously described (Hedrich et al., 2001; Szyroki et al., 2001) using an infrared gas analyzer in the differential mode (Binos, Leybold-Heraeus, Hanau, Germany).

Electron Microscopic Analyses

Arabidopsis plants were grown under short day conditions (8 h light/16 h dark), and rosette leaves were harvested from plants at different developmental stages either at the end of the dark phase or at the end of the light phase. For primary fixation, sections were incubated for 3 h at room temperature in 50 mM cacodylate buffer, pH 7.2, containing 2.5% (v/v) glutaraldehyde and 2.0% (v/v) formaldehyde. All further treatments and electron microscopic analyses were performed as described (Meyer et al., 2000).

Received March 25, 2003; returned for revision April 21, 2003; accepted May 15, 2003.

LITERATURE CITED

- Assmann SM (1993) Signal transduction in guard cells. *Annu Rev Cell Biol* 9: 345–375
- Becker D, Zeilinger C, Lohse G, Depta H, Hedrich R (1993) Identification and biochemical characterization of the plasma-membrane proton ATPase in guard cells of *Vicia faba* L. *Planta* 190: 44–50
- Boorer KJ, Loo DDF, Wright EM (1994) Steady-state and pre-steady-state kinetics of the H⁺/hexose cotransporter (STP1) from *Arabidopsis thaliana* expressed in *Xenopus* oocytes. *J Biol Chem* 269: 20417–20424
- Büttner M, Sauer N (2000) Monosaccharide transporters in plants: structure, function and physiology. *Biochim Biophys Acta* 1465: 263–274
- Dietrich P, Sanders D, Hedrich R (2001) The role of ion channels in light-dependent stomatal opening. *J Exp Bot* 52: 1959–1967
- Dittrich P, Raschke K (1977) Uptake and metabolism of carbohydrates by epidermal tissue. *Planta* 134: 83–90
- Gahrtz M, Schmelzer E, Stolz J, Sauer N (1996) Expression of the *PmSUC1* sucrose carrier gene from *Plantago major* L. is induced during seed development. *Plant J* 9: 93–100
- Harmer SL, Hogenesch JB, Straume M, Chang H-S, Han B, Zhu T, Wang X, Kreps JA, Kay SA (2000) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. *Science* 290: 2110–2113
- Hedrich R, Becker D (1994) Green circuits: the potential of plant specific ion channels. *Plant Mol Biol* 26: 1637–1650
- Hedrich R, Busch H, Raschke K (1990) Ca²⁺ and nucleotide dependent regulation of voltage dependent anion channels in the plasma membrane of guard cells. *EMBO J* 9: 3889–3892
- Hedrich R, Neimanis S, Savchenko G, Felle HH, Kaiser WM, Heber U (2001) Changes in apoplastic pH and membrane potential in leaves in relation to stomatal responses to CO₂, malate, abscisic acid or interruption of water supply. *Planta* 213: 594–601
- Kohl FG (1886) Die Transpiration der Pflanzen und ihre Einwirkung auf die Ausbildung pflanzlicher Gewebe. Harald Bruhn, Braunschweig, Germany
- Lu P, Outlaw WH Jr, Smith BG, Freed GA (1997) A new mechanism for the regulation of stomatal aperture size in intact leaves: accumulation of mesophyll-derived sucrose in the guard-cell wall of *Vicia faba*. *Plant Physiol* 114: 109–118
- Lu P, Zhang SQ, Outlaw WH Jr, Riddle KA (1995) Sucrose: a solute that accumulates in the guard-cell apoplast and guard-cell symplast of open stomata. *FEBS Lett* 362: 180–184
- Maathuis FJ, Ichida AM, Sanders D, Schroeder JI (1997) Of higher plant K⁺ channels. *Plant Physiol* 114: 1141–1149
- MacRobbie EA (1998) Signal transduction and ion channels in guard cells. *Philos Trans R Soc Lond* 353: 1475–1488
- Marrè E (1979) Fusicoccin: a tool in plant physiology. *Annu Rev Plant Physiol* 30: 273–288
- Meyer S, Melzer M, Truernit E, Hümmer C, Besenbeck R, Stadler R, Sauer N (2000) AtSUC3, a gene encoding a new *Arabidopsis* sucrose transporter, is expressed in cells adjacent to the vascular tissue and in a carpel cell layer. *Plant J* 24: 869–882
- Outlaw WH Jr, Manchester J (1979) Guard cell starch concentration quantitatively related to stomatal aperture. *Plant Physiol* 64: 79–82
- Outlaw WH Jr, Manchester J, Dicamelli CA, Randall DD, Rapp BR, Veith GM (1979) Photosynthetic carbon reduction pathway is absent in chloroplasts of *Vicia faba* guard cells. *Proc Natl Acad Sci USA* 76: 6371–6375
- Outlaw WH Jr, Tarczynski MC (1984) Guard cell starch biosynthesis is regulated by effectors of ADP-glucose pyrophosphorylase. *Plant Physiol* 74: 424–429
- Outlaw WH Jr, Tarczynski MC, Anderson LC (1982) Taxonomic survey for the presence of ribulose-1, 5-bisphosphate carboxylase activity in guard cells. *Plant Physiol* 70: 1218–1220
- Palevitz BA, Hepler PK (1985) Changes in dye coupling of stomatal cells of *Allium cepa* and *Commelina* demonstrated by microinjection of Lucifer yellow. *Planta* 164: 473–479
- Pallas JE (1964) Guard cell starch retention and accumulation in the dark. *Bot Gaz* 125: 102–107
- Poffenroth M, Green DB, Tallman G (1992) Sugar concentrations in guard cells of *Vicia faba* illuminated with red or blue light: analysis by high performance liquid chromatography. *Plant Physiol* 98: 1460–1471
- Raschke K, Dittrich P (1977) [¹⁴C]Carbon-dioxide fixation by isolated leaf epidermis with stomata closed or open. *Planta* 134: 69–75
- Raschke K, Hedrich R, Reckmann U, Schroeder JI (1988) Exploring biophysical and biochemical components of the osmotic motor that drives stomatal movement. *Bot Acta* 4: 283–294
- Ratcliffe PJ, Jones RW, Phillips RE, Nicholls LG, Bell JI (1990) Oxygen-dependent modulation of erythropoietin mRNA levels in isolated rat kidney studied by RNase protection. *J Exp Med* 172: 657–660
- Reckmann U, Scheibe R, Raschke K (1990) Rubisco activity in guard cells compared with the solute requirement for stomatal opening. *Plant Physiol* 92: 246–253
- Reddy AR, Rama Das VS (1986) Stomatal movement and sucrose uptake by guard cell protoplasts of *Commelina benghalensis* L. *Plant Cell Physiol* 27: 1565–1570
- Ritte G, Rosenfeld J, Rohrig K, Raschke K (1999) Rates of sugar uptake by guard cell protoplasts of *Pisum sativum* L. related to the solute requirement for stomatal opening. *Plant Physiol* 121: 647–656

- Sauer N, Friedländer K, Gräml-Wicke U (1990) Primary structure, genomic organization and heterologous expression of a glucose transporter from *Arabidopsis thaliana*. *EMBO J* **9**: 3045–3050
- Sauer N, Stadler R (1993) A sink specific H⁺/monosaccharide cotransporter from *Nicotiana tabacum*: cloning and heterologous expression in baker's yeast. *Plant J* **4**: 601–610
- Sauer N, Stolz J (1994) SUC1 and SUC2: two sucrose transporters from *Arabidopsis thaliana*. Expression and characterization in baker's yeast and identification of the histidine tagged protein. *Plant J* **6**: 67–77
- Schnabl H, Elbert C, Krämer G (1982) The regulation of strach-malate balances during volume changes of guard cell protoplasts. *J Exp Bot* **33**: 996–1003
- Shearson SM, Alford HL, Forbes SM, Wallace G, Smith SM (2003) Roles of cell-wall invertases and monosaccharide transporters in the growth and development of *Arabidopsis*. *J Exp Bot* **54**: 525–531
- Shearson SM, Hemmann G, Wallace G, Forbes S, Germain V, Stadler R, Bechtold N, Sauer N, Smith SM (2000) Monosaccharide/proton symporter AtSTP1 plays a major role in uptake and response of *Arabidopsis* seeds and seedlings to sugars. *Plant J* **24**: 849–857
- Stadler R, Brandner J, Schulz A, Gahrtz M, Sauer N (1996) Phloem loading by the PmSUC2 sucrose carrier from *Plantago major* occurs into companion cells. *Plant Cell* **7**: 1545–1554
- Stadler R, Sauer N (1996) The *Arabidopsis thaliana* AtSUC2 gene is specifically expressed in companion cells. *Bot Acta* **109**: 299–306
- Stolz J, Stadler R, Opekarová M, Sauer N (1994) Functional reconstitution of the solubilized *Arabidopsis thaliana* STP1 monosaccharide-H⁺ symporter in lipid vesicles and purification of the histidine tagged protein from transgenic *Saccharomyces cerevisiae*. *Plant J* **6**: 225–233
- Szyroki A, Ivashikina N, Dietrich P, Roelfsma MRG, Ache P, Reintanz B, Deeken R, Godde M, Felle H, Steinmeyer R et al. (2001) KAT1 is not essential for stomatal opening. *Proc Natl Acad Sci USA* **98**: 2917–2921
- Talbott TD, Zeiger E (1993) Sugar and organic acid accumulation in guard cells of *Vicia faba* in response to red and blue light. *Plant Physiol* **102**: 1163–1169
- Talbott TD, Zeiger E (1996) Central roles for potassium and sucrose in guard-cell osmoregulation. *Plant Physiol* **111**: 1051–1057
- Talbott TD, Zeiger E (1998) The role of sucrose in guard cell osmoregulation. *J Exp Bot* **49**: 329–337
- Tarczynski MC, Outlaw WH Jr (1990) Partial characterization of guard-cell phosphoenolpyruvate carboxylase: kinetic datum collection in real time from single-cell activities. *Arch Biochem Biophys* **280**: 153–158
- Truernit E, Stadler R, Baier K, Sauer N (1999) A male gametophyte-specific monosaccharide transporter in *Arabidopsis*. *Plant J* **17**: 191–201
- Willmer CM, Dittrich P (1974) Carbon dioxide fixation by epidermal and mesophyll tissue of *Tulipa* and *Commelina*. *Planta* **117**: 123–132