Impaired pH Homeostasis in Arabidopsis Lacking the Vacular Dicarboxylate Transporter and Analysis of Carboxylic Acid Transport across the Tonoplast

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Arabidopsis (Arabidopsis thaliana) mutants lacking the tonoplastic malate transporter AttDT (A. thaliana tonoplast dicarboxylate transporter) and wild-type plants showed no phenotypic differences when grown under standard conditions. To identify putative metabolic changes in AttDT knock-out plants, we provoked a metabolic scenario connected to an increased consumption of dicarboxylates. Acidification of leaf discs stimulated dicarboxylate consumption and led to extremely low levels of dicarboxylates in mutants. To investigate whether reduced dicarboxylate concentrations in mutant leaf cells and, hence, reduced capacity to produce \( \text{OH}^- \) to overcome acidification might affect metabolism, we measured photosynthetic oxygen evolution under conditions where the cytosol is acidified. AtHDT::tDNA protoplasts showed a much stronger inhibition of oxygen evolution at low pH values when compared to wild-type protoplasts. Apparently citrate, which is present in higher amounts in knock-out plants, is not able to replace dicarboxylates to overcome acidification. To raise more information on the cellular level, we performed localization studies of carboxylates. Although the total pool of carboxylates in mutant vacuoles was nearly unaltered, these organelles contained a lower proportion of malate and fumarate and a higher proportion of citrate when compared to wild-type vacuoles. These alterations concur with the observation that radioactively labeled malate and citrate are transported into Arabidopsis vacuoles by different carriers. In addition, wild-type vacuoles and corresponding organelles from AtHDT::tDNA mutants exhibited similar malate channel activities. In conclusion, these results show that Arabidopsis vacuoles contain at least two transporters and a channel for dicarboxylates and citrate and that the activity of AttDT is critical for regulation of pH homeostasis.

In plant cells, excess malate is stored within the large, central vacuole. NMR studies demonstrated that newly synthesized malate first accumulates in the cytoplasm and, after reaching a certain threshold concentration, is subsequently transported into the vacuole, leading to a rapid exchange between the cytosol and the organelle (Gout et al., 1993). This observation, however, does not correspond to the classic storage theory, where a solute is stored within the vacuole until it is utilized by cellular processes, but implies that vacuolar malate accumulation is a complex process comprising synthesis and degradation, energization status of the vacuole, and modulation of transport.

Vacuolar malate transport has already been investigated in detail using flux analysis, membrane potential- and pH-dependent fluorescence probes, and electrophysiological analysis (Martinoia et al., 1985; White and Smith, 1989; Ratajczak et al., 1994; Cerana et al., 1995; Pei et al., 1996; Pantoja and Smith 2002; Hafke et al., 2003). According to these studies, malate uptake is energized by vacuolar proton pumps, and the driving force is the resulting electrochemical potential difference between the cytosol and the vacuole. Depending on the system analyzed, the apparent \( K_m \) values ranged between 1.5 and more than 10 mM, and cross-inhibition studies using radiolabeled malate and citrate suggested that dicarboxylates, as well as citrate, pass the vacuolar membrane by the same transporter (White and Smith, 1989; Rentsch and Martinoia, 1991).

Electrophysiological measurements showed that a malate channel is present in the vacuolar membrane of both C3 and Crassulacean acid metabolism species (Cerana et al., 1995; Pei et al., 1996; Cheffings et al., 1997; Pantoja and Smith, 2002; Hafke et al., 2003). The malate currents observed are strongly inward rectifying, thus favoring the movement of malate from the cytosol into the vacuole. Recently, it was shown that macroscopic currents observed on Kalanchoë
**RESULTS**

**Effect of Acidification on the Level of Various Carboxylates in Leaf Discs**

To gain more information on the implication of carboxylates for pH homeostasis in *Arabidopsis*, we quantified the levels of the three major *Arabidopsis* carboxylic acids (malate, fumarate, and citrate; Chia et al., 2000), dependent upon changing pH. For this, we prepared leaf discs at the end of the light period and subsequently incubated them for 24 h in the dark in MES-buffered medium at various pH values (Fig. 1). Our basic rationale for this approach was that incubation of leaf discs for 24 h at low pH values provokes lowered cytosolic pH values and an increased consumption of malate. We were interested in learning how *AttDT* knock-out mutants, which contain lower malate content (Emmerlich et al., 2003), would behave under such stress conditions and more about regulatory processes.

At the end of the light period, malate levels were 9.4 μmol/g fresh weight (FW) for wild-type and
4.1 μmol/g FW for mutant plants. After 24 h of incubation at pH 7, wild-type leaf discs contained 5.7 μmol/g FW, whereas corresponding knock-out samples contained only 3.0 μmol/g FW (Fig. 1A). A successive decrease of the external pH value correlated in both plant lines with a decrease of leaf malate levels, finally reaching 2.2 μmol/g FW at pH 3.7 in wild-type and less than 0.5 μmol/g FW in knock-out leaf discs (Fig. 1A). Similarly, fumarate levels in wild-type tissue were 17.5 μmol/g FW at pH 7 and decreased by lowering the external pH to about 9 μmol/g FW at pH 3.7 (Fig. 1B). As seen for malate, fumarate was also lower in knock-out leaf discs incubated at pH 7 (2.7 μmol/g FW), and was reduced to 1.3 μmol/g FW at pH 3.7 (Fig. 1B). The corresponding fumarate levels at the end of the light phase were 20.3 (± 3.2) μmol/g FW in wild-type and 6.7 (± 1.1) μmol/g FW in knock-out leaves.

Interestingly, citrate is the sole carboxylic acid measured that is higher in knock-out than in wild-type leaf discs. Wild-type leaf discs incubated at pH 7 contained citrate levels of 9.5 μmol/g FW, whereas in corresponding knock-out tissue, citrate was present at a concentration of 13.5 μmol/g FW (Fig. 1C). The corresponding citrate levels at the end of the light phase were 8.7 (± 0.8) μmol/g FW in wild-type and 13.0 (± 0.3) μmol/g FW in knock-out leaves. It appears remarkable that lowering the pH in the incubation medium did not provoke similar relative citrate changes in both plant lines (Fig. 1C).

**Effect of Acidification on Photosynthetic Oxygen Evolution by Wild-Type or Knock-Out Protoplasts**

Photosynthetic oxygen evolution is a complex process dependent upon a close interaction of a wide number of metabolic processes located in the chloroplasts and cytosol and optimal maintenance of enzyme activity (Walker, 1992). Therefore, the ability to release photosynthetically derived oxygen by protoplasts can be taken as a simple and reliable criterion for controlled cellular pH homeostasis.

To analyze whether knock-out mesophyll cells differ in their ability to cope with increased cytosolic acidification when compared with wild-type cells, we prepared protoplasts from both genotypes and incubated them in MES-buffered medium harboring, in addition, 0.1 mM benzoic acid. Data are the mean ± SE of four individual measurements.

**Subcellular Localization of Carboxylic Acids in Arabidopsis Mesophyll Cells**

As reported above, total levels of carboxylic acids in wild-type and knock-out leaf tissues differ significantly. In addition, the data presented reveal that acidification correlates with an increased consumption of dicarboxylic acids, but the data do not provide information on their subcellular distribution. Assuming that the vacuole accounts for about 80% of the cell volume, it must be suggested that either the AttDT::tDNA mutant exhibits an extremely high cytosolic malate concentration or that, despite the fact that the mutant is lacking the vacuolar malate transporter AttDT, malate is still partially localized within the vacuole.

To answer this question, we isolated Arabidopsis mesophyll protoplasts from wild-type or AttDT::tDNA plants and compared malate, fumarate, and citrate contents in both intact protoplasts and corresponding vacuoles. For better comparison of these metabolite data with the photosynthetic performance of corresponding plant lines (Fig. 2), we isolated protoplasts in the morning and subsequently enriched the vacuoles by a mild hypo-osmotic treatment and flotation of intact vacuoles on a Percoll gradient (for details, see Emmerich et al., 2003; Frangne et al., 2002). The levels of organic acids are given per unit of the vacuolar marker enzyme α-mannosidase. Activities of this enzyme are known to exhibit only minimal fluctuations even under different growth conditions (E. Martinoia, unpublished data) and allow comparison, on one hand, between different plants and, on the
other hand, between the vacuole and the intact protoplast (Boller and Kende, 1979).

As observed for entire leaf discs (Fig. 1), mesophyll protoplasts isolated from wild-type plants and AttDT::tDNA mutants contain more fumarate than malate (Fig. 3A, left). As in entire leaves, citrate content in isolated protoplasts is higher than malate content, and the total malate and fumarate levels in knock-out protoplasts are significantly lower than in corresponding wild-type protoplasts (Fig. 3A, left).

Slight differences in the relative ratios between the individual carboxylic acids in leaf discs and protoplasts probably derive from the fact that the former have been incubated for 24 h in the dark, whereas the latter have been purified in the morning from intact leaves. Alternatively, this ratio could also be slightly different between mesophyll and epidermis cells.

Interestingly, vacuoles prepared from homozygous AttDT knock-out plants contained much lower, but still considerable, amounts of malate and fumarate (Fig. 3A, right). Calculation of the percentage of vacuolar carboxylate revealed that wild-type plant vacuoles contained 56.8% ± 8.8% malate and 35.3% ± 4.6% fumarate (Fig. 3B), whereas AttDT::tDNA vacuoles contained 25.1% ± 6.0% of the cellular malate and 26.8% ± 3.2% of the cellular fumarate (Fig. 3B). It should be mentioned that malate and fumarate content measured in vacuoles from AttDT::tDNA mutants cannot be due to contamination with intact protoplasts or other organelles, which was less than 5% (data not shown).

In contrast to dicarboxylates, the vacuolar proportion of citrate was slightly increased in vacuoles isolated from AttDT::tDNA mutants, namely, 73.2% ± 8.6% and 80.3% ± 14.8% for wild-type and mutant plants, respectively (Fig. 3A, right, and B). However, considering the higher citrate content in AttDT::tDNA protoplasts (Fig. 3A, left), the vacuolar amount of citrate in mutants is about 1.6-fold that detected in wild-type plants (Fig. 3A, right).

Calculation of the cytosolic carboxylic acid concentrations, based on α-mannosidase activity, revealed that mutant plants not only exhibit decreased vacuolar malate and fumarate levels but also, surprisingly, decreased cytosolic concentrations of these dicarboxylates (Fig. 3C). In remarkable contrast to this, the increase in cellular citrate in knock-out lines is mainly due to the higher vacuolar concentration of citrate, whereas the cytosolic citrate concentrations are similar in both plant genotypes (Fig. 3C).

### Respiratory Activities of Arabidopsis Wild-Type and AttDT Knock-Out Leaf Discs

In Figure 3, we show altered subcellular levels of the three main carboxylic acids in knock-out mutants. To reveal whether these alterations might correlate with changing respiratory activities in corresponding tissues, we prepared leaf discs 2 h after the end of the light period and analyzed the rates of CO2 release and oxygen consumption by Warburg manometry.

As shown, knock-out leaf discs released CO2 at a rate that was 33.6% (±5.8%) faster than observed on wild-type leaf discs (Fig. 4A). In combination with the measured rates of oxygen consumption (data not shown), we not only observed increased respiratory activity in knock-out tissues but also calculated an increased respiratory quotient. Respiratory quotients above 1 indicate partial use of carboxylic acids as substrates for mitochondrial ATP synthesis. Wild-type leaf discs exhibited a respiratory quotient of 1.13 (±0.03), whereas knock-out leaf discs exhibited a respiratory quotient of 1.26 (±0.03; Fig. 4B).

### Effect of Acidification on the Expression of the AttDT Gene

To gain insight into the putative interaction between pH control and the expression of the vacuolar dicarboxylate transporter gene AttDT, we prepared leaf discs from Arabidopsis at the end of the light period and incubated these in MES-buffered medium at various pH values for 24 h in the dark. Subsequently, total RNA was isolated and northern-blot analysis was conducted using radioactively labeled AttDT cDNA.

Incubation of leaf discs at pH 7 did not alter the level of AttDT mRNA when compared with the content of this mRNA species at the end of the light period.
By contrast, acidification of the medium below pH 6 strongly induced accumulation of AttDT mRNA in leaf discs, reaching a maximum at pH 4 (Fig. 5). These results clearly reveal a positive correlation between acidification of the incubation medium and accumulation of AttDT mRNA.

Transport of Carboxylates into Isolated Arabidopsis Vacuoles

In conclusion, the data above indicate that vacuoles of AttDT::tDNA plants still possess transport systems for both dicarboxylates and citrate (Fig. 3, A–C) and that mutants are still able to mobilize most of the endogenous dicarboxylic acids upon demand induced by acidification (Fig. 1, A and B). For dicarboxylates, it is still a matter of debate whether the transport activity observed in flux experiments corresponds to the malate channel activity observed by electrophysiological techniques (see introduction).

The high citrate levels in knock-out vacuoles were surprising, since kinetic data raised by use of radioactively labeled carboxylates indicated that both citrate and malate are transported through the same carrier system into the vacuole (Oleski et al., 1987; Rentsch and Martinoia, 1991). The malate channel described also exhibited a citrate permeability that was, however, much lower compared to malate. To explain our unexpected observations of increased citrate and reduced malate levels in knock-out vacuoles (Fig. 1, A and C), we performed (1) transport studies comparing citrate uptake in vacuoles from wild-type and mutant plants; and (2) electrophysiological experiments to reveal whether the vacuolar malate channel described is still present in AttDT::tDNA mutants.

Uptake experiments using 250 μM radioactively labeled malate or citrate showed, similar to our previous publication (Emmerlich et al., 2003), that vacuolar malate uptake capacity is strongly decreased in the AttDT::tDNA mutant (Fig. 6). In contrast to this, citrate transport into vacuoles from knock-out lines and wild-type plants occurred at similar rates (Fig. 6). These results clearly demonstrate that, despite the cross-inhibition of malate and citrate observed in flux experiments (Emmerlich et al., 2003), malate and citrate transport across the tonoplast from Arabidopsis mesophyll cells occurs through separate transporters.

Robust whole-vacuole macroscopic currents could be detected by the patch-clamp technique on vacuoles isolated from Arabidopsis mesophyll cells in the presence of a buffer containing L-(−)-malic acid and 1,3-bis[tris(hydroxymethyl)methylamino] propane (BTP; Fig. 7A). These currents displayed a large time-dependent component that activated at negative transmembrane potentials (please note that the convention proposed by Bertl et al. [1992] is adopted) and a smaller time-independent component present at both positive and negative voltages. While the time-dependent component was very stable (up to 1 h or more), the time-independent current was maximal immediately after the break-in, displayed almost linear current-voltage relationships, and decreased progressively during the registration. In some cases, this component disappeared in a few minutes.

Figure 4. Respiratory activity and respiratory quotient of wild-type and knock-out leaf tissue. Leaf discs from wild-type plants or knock-out lines were prepared from intact leaves 2 h after the end of the light period. CO2 release and oxygen consumption were measured in a Warburg device. A, CO2 release. B, Respiratory quotient (CO2/O2). Data are the mean ± se of four individual measurements.

Figure 5. Northern-blot analysis of AttDT mRNA accumulation in response to decreasing external pH values. Arabidopsis wild-type discs have been prepared from leaves at the end of the light period and incubated for 24 h (in the dark) in MES-buffered water. Subsequently, total RNA was purified and northern-blot analysis was conducted using radioactively labeled AttDT cDNA. Control samples were prepared from leaves at the end of the light period. A representative blot is shown; rRNA is presented as information on loading.
The labile time-independent component did not display any evident ionic selectivity and could not be studied in detail. Therefore, we concentrated our analysis on the time- and voltage-dependent current. The strongly rectifying properties of this component are summarized in the current density plot (pA/pF) obtained by dividing the mean value of the current in the last 20 ms by the tonoplast capacitance (Fig. 7B, white circles).

To gain more information on the nature of this current, we performed a tail analysis in asymmetric ionic solution (malate$^{2-}$/malate$^{2-}$ out/malate$^{2-}$/in $5100/10$), which displayed a reversal potential of about $+10$ mV (Fig. 8, A and B), i.e. close to the theoretical potential of $+23$ mV expected for malate$^{2-}$ at these ionic activities (Hafke et al., 2003) and significantly different from the Nernst potential expected for BTPH$^+$ ($V_{Nernst}$ $= -63$ mV; Hafke et al., 2003). This observation suggests that the time-dependent current originates from inward-rectifying malate channels. Furthermore, after replacing malate by fumarate, we observed that this current was increased by a factor of about 2 and that addition of 10 mM citrate determined an almost complete inhibition of both the malate and the fumarate currents (data not shown).

In sum, these observations correspond to the vacuolar malate channel characteristics described for Arabidopsis cell cultures and other plants and strongly indicate that the currents observed are mediated by malate moving from the cytoplasm into the vacuole. To investigate now whether the malate channel involved corresponds to the malate transporter AtDTD, we additionally patched vacuoles isolated from homozygous AtDTD::tDNA deletion mutants. Interestingly, for unknown reasons, mutant vacuoles appeared to be less stable in this particular experimental setup when compared to wild-type vacuoles. Nevertheless, mutant vacuoles exhibited very similar currents and current density characteristics of the time-dependent component, as observed on wild-type vacuoles (Fig. 7B, black circles). Furthermore, replacement of malate by fumarate results in increased currents as observed for wild-type vacuoles, and citrate, given at a concentration of 10 mM, completely inhibited malate and fumarate currents, as observed in wild-type plants (data not shown). These results show that AtDTD::tDNA plants
still possess the vacuolar malate channel and indicate that  
*At* DT does not correspond to the vacuolar malate  
channel.

**DISCUSSION**

Recently, we identified *At* DT as a vacuolar malate  
transporter and showed that Arabidopsis plants lacking  
in *At* DT exhibited only a small residual malate  
transport activity and significantly reduced total ma-  
late levels in leaf tissues (Emmerlich et al., 2003). Since  
malate homeostasis is thought to be a crucial element  
for maintaining a functional primary metabolism  
in plants, we were surprised that homozygous  
*At* DT::tDNA plants did not exhibit strong phenotypic  
alterations when compared to wild types.

It is known from experiments on other species that  
cellular carboxylate metabolism, especially the malate  
metabolism, is important for regulation of the cytosolic  
*pH* (Smith and Raven, 1979). To reveal whether  
Arabidopsis plants lacking the vacuolar malate trans-  
porter differ in their ability to cope with altered *pH*  
values, we first checked the involvement of carbox-  
ylates in *pH* homeostasis in this species. The observa-  
tion that incubation of leaf discs at low *pH* led to an  
increased consumption of malate and fumarate, but  
not of citrate, in wild-type plants (Fig. 1, A–C) is in full  
accordance with our knowledge of regulation of cyto-  
solic *pH* homeostasis. Acidification activates malate-  
degrading malic enzyme and inhibits PEP carboxylase  
(Davies, 1986). By these opposite effects, the enhanced  
malate degradation correlates with the net production  
of OH⁻, thus compensating for an increased proton  
concentration by acidification. The simultaneous de-  
crease of malate and fumarate (Fig. 1, A and B) is  
probably due to the enzyme connecting both metab-  
olites directly. The enzyme involved, fumarase, cata-  
lyzes a reaction close to thermodynamic equilibrium.  
Interestingly, conversion of malate (and fumarate) to  
pyruvate not only provides the required OH⁻ ions  
(Smith and Raven, 1979), but also supplies mitochon-  
dria with the substrate required for ATP synthesis. By  
doing so, mobilization of dicarboxylates in leaf cells  
also provides the energy for P-type ATPases involved  
in pumping protons against an existing *pH* gradient.  
Therefore, the increased energy consumption at the  
expense of dicarboxylates probably allows the chal-  
lenged cells to arrest the cytosolic *pH* relatively close  
to the optimal value.

In contrast to malate and fumarate, degradation of  
citrate does not occur in low-*pH*-challenged leaf discs  
(Fig. 1C). Although the exact reasons for this discrep-  
ancy are difficult to analyze, we speculate that tight  
control of the metabolic flux within the citric acid  
cycle, and the involvement of several enzymes for  
conversion of citrate to malate (Lehninger et al., 1994),  
might prevent the efficient use of this metabolite for  
regulation of intracellular *pH* homeostasis.

As indicated above, the regulatory action of dicar-  boxylic acids for *pH* homeostasis ultimately depend  
upon the presence of these compounds in the cytosol  
and upon the concentrations of malate and fumarate in  
the vacuole, which can be used if required in the  
cytosol. Therefore, the observation that knock-out  
mesophyll protoplasts are more sensitive to acidifica-  
tion (Fig. 2) concurs with the observation that both the  
vacuolar as well as the cytosolic malate and fumarate  
concentrations were lowered in  
*At* DT::tDNA mutants  
(Fig. 3A, right, and C). Obviously, a disturbed tono-  
plastic malate exchange as present in  
*At* DT knock-out  
plants (Fig. 6; Emmerlich et al., 2003) provokes so far  
unknown regulatory reactions at the expense of cyto-  
solic energy equivalents.

Figure 8. Determination of the reversal potential of the time-dependent  
current in vacuoles from wild-type plants. A, Top, Representative tail  
currents elicited by a tail pulse from −50 to +40 mV (at 10-mV  
increments) following a conditioning prepulse to −120 mV; bottom,  
the same tail currents as in the top image plotted at a larger time and  
current resolution. B, Value of the instantaneous tail currents is plotted  
as a function of the tail potential. The reversal potential (about +10 mV)  
is very close to the theoretical potential for malate and substantially  
different from the Nernst potential for BTP⁺.
This assumption is further reinforced by the demonstration that radioactively labeled malate fed into mutant leaf discs entered the Krebs cycle much faster than in wild-type tissues (Emmerlich et al., 2003). Moreover, the observation that AttDT::tDNA leaf discs exhibited both an increased respiratory activity (Fig. 4A) and an increased respiratory quotient (Fig. 4B) clearly demonstrates the accelerated use of cytosolic carboxylic acids as an energy source in mutant tissue.

We showed that AttDT is able to catalyze malate import into isolated vacuoles (Fig. 6). In addition, the observation that malate feeding into leaf discs promotes expression of this AttDT (Emmerlich et al., 2003) further underscores this function. However, acidification also induces expression of the AttDT gene (Fig. 5) and correlates with a dramatic decrease of total cellular dicarboxylic acid levels (Fig. 1, A and B). AttDT, therefore, may also play a role in malate export. Thus, our observation might explain the rapid cycling of malate between the cytosol and the vacuole observed in NMR studies (Gout et al., 1993). As a consequence of our data, one has to hypothesize that the malate transporter AttDT is less rectifying than the tonoplastic malate channel and that AttDT allows malate to be exported but also to enter the vacuole at lower membrane potential differences. An alternative explanation of the observed up-regulation of AttDT would be that, if flux into or out of the vacuole is increased, the whole vacuolar malate transfer machinery is induced. In any case, the strong decrease of malate and fumarate observed during the acidification experiments indicates AttDT is not the sole vacuolar malate exporter.

Compartmentation analysis revealed that vacuolar concentrations as well as vacuolar proportions of both dicarboxylates were lower in AttDT::tDNA mutants when compared to wild-type plants (Fig. 3A, right, and C). This indicates that both dicarboxylates cross the tonoplast using the same transporter. In addition, this observation may be explained by our finding that a malate transporter (Fig. 6) and a malate channel (Figs. 7 and 8) are present in the tonoplast and exhibit slightly different affinities for both dicarboxylates. However, it must be assumed that the channel does not exhibit sufficient activity to accumulate dicarboxylates at concentrations required for proper metabolic functioning and therefore cannot fully compensate for the absence of AttDT.

The evidence that two dicarboxylate translocating systems exist in the tonoplast may also explain the differences observed concerning the affinity and the relative permeability of the vacuolar transport system when dicarboxylate transport was investigated on different systems (Martinoia and Ratajczak, 1997). Vacuolar vesicles have a much higher surface-to-volume ratio than intact vacuoles. Consequently, due to the different surface-to-volume ratio and a continuous leak of protons and ions through the tonoplast, generation of the electromotive force is faster in vesicles and energy loss by leaking compounds is compensated more efficiently. It is therefore likely that transport experiments using vesicles favor the transfer of dicarboxylates through the channel, which is strongly rectifying and starts to open at membrane potentials higher than 40 to 50 mV. By contrast, intact vacuoles are less energized and the predominant dicarboxylate transport system is the transporter (Emmerlich et al., 2003; Fig. 6).

In contrast to malate and fumarate, citrate content was increased in mutant leaves (Fig. 3A). This result was unexpected since, from flux analysis using radio-labeled malate and citrate, it has been suggested that both carboxylates cross the tonoplast using the same transporter (Oleski et al., 1987; White and Smith, 1989; Rentsch and Martinoia, 1991). By contrast, patch-clamp experiments revealed that citrate permeability was much lower compared to malate permeability. The demonstration that citrate mainly accumulates in the vacuole (Fig. 3A, right), that knock-out vacuoles contain about 1.6-fold more citrate than wild-type vacuoles (Fig. 3A, right), and the flux analysis on isolated wild-type vacuoles (Fig. 6) strongly support our assumption that AttDT is not the main tonoplast citrate carrier. This conclusion does not necessarily contradict our previous observation that malate transport by AtttDT is inhibited by citrate (Emmerlich et al., 2003); it just indicates that kinetic experiments with closely related compounds have to be interpreted carefully. It has to be assumed that vacuolar malate transporters can bind both carboxylates, but transport is restricted to the smaller class of compounds.

CONCLUSION

In total, our observations indicate that AttDT does not correspond to the vacuolar malate channel and explain why substantial amounts of malate and fumarate can be found in AttDT::tDNA vacuoles (Fig. 3B). In addition, we clearly revealed that AttDT is critical for pH homeostasis under the conditions tested. The two dicarboxylate transport systems (transporter and channel) in concert with the so far unknown citrate transporter allow the plant cell to regulate the storage and possibly the release of citrate and malate independently. Furthermore, regulation of energization across the tonoplast could also lead to a preferential import or export of malate or fumarate. This gives the plant an important flexibility to adapt the metabolism to particular situations.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Wild-type plants and AttDT::tDNA plants were grown under short-day conditions in a growth chamber as described previously (Emmerlich et al., 2003). All experiments have been conducted with the mutant line AttDT::tDNA. We already showed that effects provoked by deletion of the vacuolar dicarboxylate transporter are specifically linked to the t-DNA insertion into the AttDT gene (Emmerlich et al., 2003). Plants used for patch-clamp studies have been grown in an 8-h light phase.
Isolation of Protoplasts and Quantification of Photosynthetic Oxygen Evolution

Protoplasts from wild-type and knock-out leaves were isolated as in Stitt et al. (1982). Photosynthesis was measured in a Clark oxygen electrode as in Herold et al. (1981). Incubation media were buffered with MES (20 mM) at various pH values; benzoic acid was present at a concentration of 0.1 mM.

Warburg Manometry

CO₂ release and oxygen consumption were measured in a Warburg device (Braun-GmbH, Melsungen, Germany). Leaf discs were prepared 2 h after the end of the light period from intact plants and respiratory activity (CO₂ release and oxygen consumption) was quantified for the next 2 h.

Extraction of Total RNA and Northern Blotting

RNA isolation and northern-blot analysis were performed using standard procedures (Sambrook et al., 1989) from the kits and materials described by Emmerlich et al. (2003).

Quantification of Carboxylates

Fifty to 100 mg of leaf material were rapidly frozen in liquid nitrogen. One milliliter of water (about 80°C) was added and the probes were heated for 8 min at 95°C in an Eppendorf thermo-incubator. After centrifugation (5 min, 12,000g), the supernatant was used to determine the carboxylates. Malate, fumurate, and citrate were quantified spectrophotometrically in a combined assay according to Passonneau and Lowry (1993). Recovery experiments revealed that about 95% of each carboxylate was recovered during the extraction.

α-Mannosidase Assay

α-Mannosidase was measured using p-nitrophenyl mannospyranoside as substrate (Boller and Kende, 1979).

Isolation of Vacuoles, Localization Studies, and [14C]Carboxylate Flux Experiments

For flux analysis and localization experiments, protoplasts and vacuoles were isolated as described (Frangne et al., 2002). For localization experiments, α-mannosidase and carbohydrates were determined in the protoplast and vacuole fractions. Vacular proportions were measured assuming that α-mannosidase is completely localized in the vacuole (Boller and Kende, 1979).

For patch-clamp experiments, protoplasts were loaded on a cover slide. After the cells were attached to the bottom, vacuoles were released by incubating the protoplasts in 160 mM BTP, 100 mM malic acid, 3 mM MgCl₂, and 0.1 mM CaCl₂ (pH 7.4 with BTP). The osmolarity solution was adjusted to 440 mosmol kg⁻¹ with sorbitol.

Patch Clamping and Data Acquisition

Solutions for patch clamping were prepared according to Hafke et al. (2003). Osmolarity of the bath (cytosolic side) solution contained 160 mM BTP, 100 mM malate, 3 mM MgCl₂, 1 mM CaCl₂, and 0.1 mM EGTA/2 mM TRIS (pH 7.4 with BTP). Osmolarity of the patch solution was adjusted to 440 mosmol kg⁻¹ with sorbitol.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AJ223445.

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LITERATURE CITED


