Cloning, localization and expression analysis of vacuolar sugar transporters in the CAM plant *Ananas comosus* (pineapple)

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Abstract

In photosynthetic tissues of the CAM plant pineapple (*Ananas comosus*), storage of soluble sugars in the central vacuole during the daytime and their remobilization at night is required to provide carbon skeletons for nocturnal CO₂ fixation. However, soluble sugars produced photosynthetically must also be exported to support growth processes in heterotrophic tissues. To begin to address how vacuolar sugar storage and assimilate partitioning are regulated in *A. comosus*, degenerate PCR and cDNA library screening were used to clone three candidate sugar transporters from the leaves of this species. Subcellular localization of the three transporters was investigated via expression of YFP-fusion proteins in tobacco epidermal cells and their co-localization with subcellular markers by confocal microscopy. Using this strategy, a putative hexose transporter (*AcMST1*) and a putative inositol transporter (*AcINT1*) were identified that both localized to the tonoplast, whereas a putative sucrose transporter (*AcSUT1*) was found to localize to prevacuolar compartments. A cDNA (*AcMST2*) with high similarity to a recently characterized tonoplast hexose transporter in *Arabidopsis* was also identified from an *A. comosus* fruit EST database. Analyses of transcript abundance indicated that *AcMST1* was more highly expressed in fruits compared to leaves of *A. comosus*, whilst transcripts of *AcINT1*, *AcSUT1*, and *AcMST2* were more abundant in leaves. Transcript abundance of *AcINT1*, the putative inositol transporter, showed day–night changes comparable to those of other CAM-related transcripts described in *Mesembryanthemum crystallinum*. The results are discussed in terms of the role of vacuolar sugar transporters in regulating carbon flow during the diel cycle in CAM plants.

Key words: Crassulacean acid metabolism, hexose, sucrose, sugar transporters, tonoplast, vacuole.

Introduction

In the green, photosynthetically active cells of plants that perform crassulacean acid metabolism (CAM), a huge central vacuole dominates about 95% of the cell’s volume and plays a key role in regulating carbon metabolism over the 24 h cycle. By acting as a temporary repository for malic acid synthesized as a result of CO₂ fixed at night via phosphoenolpyruvate carboxylase (PEPC), the vacuole helps to maintain the temporal separation of carboxylation processes that distinguishes CAM from C₃ and C₄ photosynthesis. In many CAM plants, including the crop species pineapple (*Ananas comosus*), the vacuole also acts as a daytime store for considerable amounts of sugars, which can represent up to 20% of leaf dry biomass (Holtum *et al.*, 2005). The sugars are subsequently degraded at night to generate the 3-carbon acceptor phosphoenolpyruvate (PEP) for nocturnal carboxylation. The capacity of the vacuole as a sink for carbohydrate may be an important determinant of CAM expression in soluble-sugar-storers like pineapple, and also has...
important implications for plant growth and productivity. CAM plants must retain sufficient carbohydrate in the vacuole during the day to support the substrate requirements of PEPC in the subsequent night, yet must also continue to export carbohydrate to maintain the growth of sink tissues (Borland and Dodd, 2002). Understanding the mechanisms that regulate carbohydrate partitioning between the competing sinks is of particular interest in pineapple, which is the most agronomically important CAM species, producing up to 86 tonnes ha\(^{-1}\) (Morton, 1987) with an international trade value of US$1.9 billion year\(^{-1}\) in 2003 (FAOSTAT, 2005).

The role of the vacuole in carbohydrate partitioning in CAM plants remains almost wholly unexplored, although it has been reported that isolated vacuoles of pineapple contain mainly glucose and fructose (Kenyon et al., 1985; Christopher and Holtum, 1998), whilst whole-leaf extracts contain substantial amounts of sucrose (Kenyon et al., 1985). Given the very high extractable activities of acid invertase reported for pineapple leaves (Black et al., 1996), it can be proposed that sucrose is synthesized in the cytoplasm during the day, transported across the tonoplast into the vacuole, and hydrolysed in the vacuolar lumen by acid invertase (Smith and Bryce, 1992). To avoid futile cycling, the hexoses thus produced would need to be stored in the vacuole and not released to the cytoplasm until the following dark period, when they would be metabolized by glycolysis to provide the C\(_3\) substrate (PEP) for nocturnal malate synthesis. This model is supported by the finding that the tonoplast of pineapple possesses a sucrose-transport system capable of catalysing sucrose fluxes of the required magnitude (McRae et al., 2002). A specific hexose-transport system has also been observed recently in isolated pineapple tonoplast vesicles (D Haines and JAM Holtum, personal communication). Thus, sucrose and hexose transporters located on the tonoplast could play a strategic role in controlling the supply and demand for carbon in pineapple over the day–night cycle.

Sugar transporters have been recognized previously as key targets for regulatory roles in the distribution and partitioning of assimilates in plants (Williams et al., 2000). To date, most work has focused on the role of sucrose transporters in long-distance transport of carbohydrate via the phloem, a process that is highly regulated at multiple levels and in response to changing sucrose concentration (Vaughn et al., 2002; Ransom-Hodgkins et al., 2003). The role of vacuolar sucrose and hexose transporters in regulating assimilate partitioning has received relatively little attention due to limited information on the molecular characterization of these proteins. Sucrose transporters typically possess 12 predicted transmembrane \(a\)-helices and are comprised of two modules of six helices connected by a central loop which varies in length (Lemoine, 2000). Three clades of plant sucrose transporters have been distinguished on the basis of physiological properties and molecular structure (Kühn, 2003; Lalonde et al., 2004). Clade I contains transporters that exhibit a high affinity (i.e. \(K_m\) between 139 \(\mu\)M and 1.5 mM), but relatively low transport capacity for sucrose, and are found exclusively in eudicotyledonous plants (Kühn, 2003). All Clade I sucrose transporters investigated to date localize to the plasma membrane, and many have been implicated in phloem loading (Sauer, 2007). In contrast, the genes contained within Clade II encode transporters with a low affinity (\(K_m\)=12 mM) but high transport capacity for sucrose and are found in both monocots and eudicots (Kühn, 2003). Some members of this clade have been localized to the plasma membrane; however, HvSUT2 from barley was immunolocalized to the plasma membrane by Wang (2003), but to the tonoplast using fluorescence protein fusion (Endler et al., 2006). Clade III contains transporters from monocots and eudicots that have a low affinity and high transport capacity for sucrose. Some Clade III transporters possess an extended cytoplasmic loop between transmembrane spans VI and VII, but few have been localized to date at the subcellular level. The physiological characteristics described for sucrose transport across pineapple tonoplast vesicles are indicative of a low-affinity (\(K_m\)=50 mM), high-capacity vacuolar sucrose transporter (McRae et al., 2002).

The Arabidopsis genome also contains a monosaccharide transporter-like (MST) gene family with 53 members clustered in nine clades (Lalonde et al., 2004; see also Büttner, 2007). The MST transporters possess 12 predicted transmembrane spans, and ten or more of these proteins have been characterized as plasma membrane-localized monosaccharide importers (Lalonde et al., 2004; Büttner, 2007). Chiou and Bush (1996) first reported the cloning of an MST from sugar beet that immunolocalized to the tonoplast, but the protein did not show transport activity when expressed in yeast. The Arabidopsis orthologue (At1g75220) of this putative vacuolar transporter is found in Clade IV of the MST gene family (Lalonde et al., 2004). Recently, the first tonoplast monosaccharide transporters (AtTMT) were functionally identified from Arabidopsis; these form a subfamily of three members with an extended middle loop between predicted transmembrane helices six and seven (Wormit et al., 2006). The phylogenetic tree for MST genes devised by Lalonde et al. (2004) placed the AtTMT homologues in Clade III. A further and distinct subfamily of three MST genes from Arabidopsis (belonging to Clade VII of MST genes) has recently been identified, two of which encode proteins that localize to the vacuolar membrane and catalyse transport of glucose and fructose (Aluri and Büttner, 2007; Büttner, 2007). It remains to be established if these various subfamilies of vacuolar hexose transporters differ in terms of substrate specificity and/or
function, and indeed whether there are other undiscovered vacuolar hexose transporters within the Arabidopsis genome.

Phylogenetic analysis of the sucrose and monosaccharide transporters described so far therefore suggests that homology could reflect, at least in part, subcellular localization. This would serve as a useful starting point for identifying candidate vacuolar sugar transporters in a plant species like pineapple for which limited gene sequence information is available. The relatively high degree of amino acid sequence conservation in many membrane proteins makes it possible to identify orthologues across quite considerable phylogenetic distances, as for example between the sequenced genomes of Arabidopsis and rice (Aoki et al., 2003). Moreover, pineapple (A. comosus) is a member of the monocot family Bromeliaceae, which belongs to the order Poales and is thus reasonably closely related to the grass family Poaceae (Angiosperm Phylogeny Group II, 2003). This relationship between pineapple and rice can potentially be exploited to identify candidate tonoplast-localized sugar transporters in the sequenced CAM species.

In the present work, rice sequences belonging to Clades IV, VII, and VIII of the MST gene family and to Clade III of the sucrose transporter family were identified and these were used to design degenerate oligonucleotide primers to amplify fragments of pineapple sugar transporter cDNAs. Degenerate PCR and cDNA library screening resulted in the cloning of three full-length candidate sugar transporter cDNAs from the leaves of pineapple. Subcellular localization of these three transporters was investigated by designing constructs encoding translational fusions with yellow fluorescent protein (YFP) and examining the colocalization of these fusion proteins with subcellular markers by transient expression in tobacco epidermal cells. We also queried an expressed sequence tag (EST) database for pineapple containing 5500 ESTs from fruit and root tissue for candidate sugar transporters (Moyle et al., 2005). The transcript abundance of the identified sugar transporters was compared in leaves and fruits and in two cultivars of pineapple differing in their level of CAM expression.

Materials and methods

Plant material and growth conditions

Plants of A. comosus (L.) Merr. were cultivated for at least 4 months under natural illumination in heated glasshouses at Close House Field Station and at Moorbank Botanic Gardens (Newcastle University). Experimental plants were grown in 20-cm-diameter pots in peat- and sand-based potting mixture with a complete slow-release fertilizer and were watered every 2 d. Approximately 4–6 weeks before experimentation, plants were transferred to a climate-controlled growth chamber under a 12 h photoperiod, photon flux density (PFD) of 350 μmol m⁻² s⁻¹ at plant height, day/night temperature regime of 29/18 °C and day/night relative humidity of 60–75%. Sampling for RNA, metabolites, and measurements of gas exchange were conducted on the D leaves (Bartholomew and Kadzimin, 1977), the youngest fully-mature leaves on a pineapple plant.

cDNA cloning

cDNAs of candidate sugar transporters from A. comosus were amplified from total RNA extracted from leaves. Powdered leaf samples from material frozen in liquid nitrogen were extracted in a buffer containing 100 mM TRIS (pH 8.0), 25 mM EDTA, 1% (v/v) 2-mercaptoethanol, 2% (w/v) PVP-40 (polyvinylpyrrolidone), average molecular mass 40 000), 2% (w/v) CTAB (cetyltrimethylammonium bromide), and 2% (v/v) Nonidet-P40 and spun at 10 000 g for 10 min. The supernatant was added to an equal volume of Tri- Reagent (Helena Biosciences, UK) and RNA subsequently extracted as described in Taybi and Cushman (1999). RNA was subsequently treated with DNase I (Invitrogen, UK) to prevent amplification of genomic DNA.

To clone pineapple sugar transporters, degenerate primers were designed using three separate approaches. (i) The sequence of a putative vacuolar sugar transporter from Beta vulgaris (GenBank accession number U43629; Chiou and Bush, 1996), which belongs to Clade IV of the monosaccharide transporter family (Lalonde et al., 2004), was used to search the TIGR databases (http://www.tigr.org) of a number of grass species that included rice, wheat, and barley using the BLAST algorithm (Altschul et al., 1997). Homologous sequences thereby obtained were aligned using ClustalX (Thompson et al., 1997), and conserved regions were identified for the design of degenerate primers. (ii) Nucleotide sequences of transporters belonging to Clades VII and VIII of the monosaccharide transporter family (Lalonde et al., 2004) were identified from a number of monocotyledonous species. Sequences were aligned and degenerate primers were designed from conserved regions. (iii) Nucleotide sequences belonging to Clade III of the sucrose transporter family (Lalonde et al., 2004) were identified from the TIGR databases for rice, barley, and wheat. Sequences were aligned and areas of high similarity were identified for primer design.

Degenerate primers were used to amplify cDNA fragments using RNA extracted from pineapple leaves as template or, in the case of the sucrose transporter, using a cDNA phage library prepared from pineapple leaves. The fragments obtained were subsequently used as probes to screen the pineapple cDNA library to obtain full-length sequences. The sequences obtained were deposited in GenBank with accession numbers EF460876 (AcMST1), EF460877 (AcINT1), and EF460878 (AcSUT1).

Phylogenetic analysis

Phylogenetic trees showing the relationships between AcMST1, AcINT1, and AcSUT1 with sugar transporters from A. thaliana and rice were constructed by aligning amino acid sequences with the program ClustalX and edited using Bioedit and Gblocks using the protocols described in Harrison and Langdale (2006). Unrooted trees were constructed and viewed using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

AcMST1–YFP, AcINT1–YFP and AcSUT1–YFP constructs and Agrobacterium transformation

Three sugar transporters (AcMST1, AcINT1, AcSUT1) cloned from A. comosus were each inserted in the modified binary vector pVKHEn6-HA Venus (Zheng et al., 2005) containing sequences for an HA epitope-tagged version of the Venus variant of enhanced
YFP (Nagai et al., 2002) and kanamycin- and hygromycin-resistance genes. The sugar transporters were cloned upstream of the YFP gene using the XhoI site using the following primers: AcMST1 forward primer (5'-TATATCTAGAATGAGCTTGAGG-GAGGAC-3') and AcMST1 reverse primer (5'-TATATCTA-GTCCAGCTCTCCTGCA-3'). AcINT1 forward primer (5'-TATATCTAGAATGAGCTTGAGGAC-3') and AcINT1 reverse primer (5'-TATATCTAGAATGAGCTTGAGGAC-3'). AcSUT1 forward primer (5'-TATATCTAGAATGAGCTTGAGGAC-3') and AcSUT1 reverse primer (5'-TATATCTAGAATGAGCTTGAGGAC-3').

**Analyses of transcript abundance**

*Confocal microscopy*

All specimens were viewed with a Zeiss CLSM-510 confocal microscope. Small sections of transfected leaves were floated with the lower epidermis uppermost on water on a microscope slide and observed for fluorescence. The excitation wavelength depended on the fluorescent protein (513 nm for YFP, 488 nm for GFP, and 558 nm for the fluorescent protein (513 nm for YFP, 488 nm for GFP, and 558 nm for the fluorescent protein). Emission filters (527 nm YFP, 507 nm GFP, and 583 nm for RFP). Emission filters (527 nm YFP, 507 nm GFP, and 583 nm for RFP). Emission filters (527 nm YFP, 507 nm GFP, and 583 nm for RFP). Emission filters (527 nm YFP, 507 nm GFP, and 583 nm for RFP). Emission filters (527 nm YFP, 507 nm GFP, and 583 nm for RFP). Emission filters (527 nm YFP, 507 nm GFP, and 583 nm for RFP).

**Metabolite measurements**

D leaves of pineapple plants were collected at the start (06:00 h) and end (18:00 h) of the photoperiod, frozen in liquid nitrogen, powdered and stored at -80 °C for further analysis. Fruits were also sampled at the start and end of the photoperiod and stored as described above. Soluble metabolites, including titratable acids and soluble sugars, were extracted in methanol by heating 0.5 g of frozen, powdered plant sample in 5 ml methanol for 45 min. The magnitude of CAM expression was determined by monitoring the change in leaf titratable acid content at the start and end of the dark period. Exactly 1 ml of methanol extract was added to 2 ml of sterile water and titrated against 0.05 M NaOH to a neutral endpoint, as indicated by phenolphthalein.

Soluble sugar content in methanol extracts was measured using the colorimetric phenol–sulfuric acid method (Dubois et al., 1956). Leaf material that remained after methanol extraction was washed with distilled water and subjected to enzymatic hydrolysis via amylase and amyloglucosidase to liberate glucose equivalents from starch (Borland, 1996), which were subsequently analysed as described above for soluble sugars.

**Results**

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**AcMST1: sequence analysis and localization**

Conserved regions of monocot sequences showing high similarity to the Clade IV subgroup of the monosaccharide transporter(-like) (MST) family were used to design degenerate primers that resulted in the cloning of AcMST1 from *A. comosus* (GenBank accession number EF460876). *AcMST1* showed 74–81% identity with a number of nucleotide sequences from rice, Beta vulgaris, and A. thaliana belonging to Clade IV of the MST gene family (Lalonde et al., 2004). The predicted open reading frame (ORF) of *AcMST1* translates into a polypeptide of 496 amino acids in length, with a predicted mass of 53.6 kDa and a pI of 8.6. The transmembrane topology prediction server HMMPred (http://www.enzim.hu/hmmpred) predicted that this polypeptide would contain 12 α-helical transmembrane spans, with the N- and C-termini located in the cytoplasm. Sugar transporter signatures that include Gly-Arg-Lys (GRK) in loop 2, Asp-Arg-Ala-Gly-Arg (DRAGR) in loop 8, and in particular the Pro-Glu-Thr-Lys-Gly-Arg (PETKGR) motif after the 12th transmembrane span, are present in the predicted amino acid sequence for AcMST1. Another sugar transporter signature, Gly-Trp-Gly-Pro-Ile-Pro-Trp (GWGPIPWP) found in human GLUT1 (Joost and Thorens, 2001), was also detected, but with a change in the amino acid sequence in plants to GMGAIPW. The motif QLS in transmembrane helix 7 was consistent with the predicted sugar transporter activity of the AcMST1 protein (Doerge et al., 2000).

An *AcMST1–YFP* construct was transiently expressed in tobacco epidermal cells and was found to co-localize with the tonoplast intrinsic protein marker BobTIP-GFP (Fig. 1). This is evident in the co-localization of YFP and GFP fluorescence on the transvacuolar strands that cross the cell interior (arrow 1). Figure 1D also suggested that

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**Analyses of transcript abundance**

The diel transcript abundance of *AcMST1, AcMST2, AcINT1,* and *AcSUT1* was studied in leaves and fruits of *A. comosus* using single-step semi-quantitative RT-PCR with the following gene-specific primers: AC1F (5'-TCTTGTGATGTGATGACGAGG-3') and AC1R (5'-CGCGCATAATTTGTACATGC-3') for amplification of *AcMST1, FASUT4* (5'-ACAATTCCGATCTTGATAGC-3') and *RASUT4* (5'-TCTCAGATTTGACTATGTCG-3') for amplification of *AcMST2, AC3F* (5'-TTCTGTCTTCCTCGCTTCCTTTG-3') and *AC3R* (5'-CCGGCATAATTTGTACATGC-3') for amplification of *AcINT1, and AC6F* (5'-GAATGGACGGCGCCGCCGCCGC-3') and AC6R (5'-AGTCTTAGCGACCGGCTTGCG-3') for amplification of *AcSUT1*. The *RBS* gene was used as the control and was amplified using primer AC18SF (5'-AGTCTTAGCGACCGGCTTGCG-3') and AC18SR (5'-TGCTAACCTTTGGCTAT- GTCGTG-3').
AcMST1–YFP might be retained in another organelle (arrow 3), most probably the endoplasmic reticulum. To investigate this further, tobacco epidermal cells were co-infiltrated with the AcMST1–YFP construct and an endoplasmic reticulum marker, GFP-HDEL (Fig. 2). HDEL is a C-terminal His-Asp-Glu-Leu tetrapeptide that participates in the retrieval of soluble proteins from the Golgi to the ER (Brandizzi et al., 2002). Figure 2D shows the invagination of the tonoplast (arrow 1), a feature commonly observed for this membrane and thought to accommodate small organelles like the Golgi or mitochondria in the cytosol. In Fig. 2D, the tonoplast is seen to be separated from the ER, which is appressed to the tonoplast in several places, although in a few places co-localization was noticed. The ER is a general route for the sorting of membrane proteins, so there is a possibility of some retention of AcMST1–YFP in the ER en route to the tonoplast, or when expressed at high levels under control of the 35S promoter.

The finding that AcMST1 appears to localize to the tonoplast, prompted a phylogenetic analysis of AcMST1 in relation to recently identified tonoplast sugar transporters described for Arabidopsis (At3g1490, At4g35300; Wormit et al., 2006) and barley (HvSTP1, GenBank accession number CAD58598; Endler et al., 2006). A cladogram was constructed using the amino acid sequences of these tonoplast sugar transporters together with AcMST1 and homologues from rice and Arabidopsis (Fig. 3). This analysis indicated that AcMST1 belongs to a separate subgroup (designated Clade IV after Lalonde et al., 2004) when compared with the previously described tonoplast transporters in Arabidopsis and barley (Clade III). HvSTP1 and homologues are characterized by the presence of a predicted cytoplasmic loop of approximately 200 amino acids in length between transmembrane

![Fig. 1. Localization of AcMST1 in relation to a tonoplast marker in tobacco epidermal cells.](image)

![Fig. 2. Localization of AcMST1 in relation to an endoplasmic reticulum marker in tobacco epidermal cells.](image)
helices 6 and 7 that is absent in AcMST1. The Clade III transporters lack the extended N-terminus present in AcMST1, which contains several predicted phosphorylation sites. Several of the sugar transporter signature sequences described above for AcMST1 (i.e. GRK, DRAGRR, and QLS) are absent from HvSTP1.

**AcINT1: sequence analysis and localization**

Conserved regions of monocot sequences belonging to Clades VII and VIII of the MST gene family were used to design a second set of degenerate primers. These primers resulted in the cloning of a full-length cDNA from pineapple (GenBank accession number EF460877) that showed 65–77% identity at the nucleotide level with monosaccharide transporters from Arabidopsis, Medicago, and rice. The closest homologue in Arabidopsis, AtINT1 (At2g43330), was a member of the inositol transporter family, so the pineapple cDNA was designated AcINT1. The ORF of AcINT1 translates into a 511 amino acid protein with a theoretical mass of 54.4 kDa, a calculated pI of 5.7, and 12 predicted transmembrane spans with N- and C-termini in the cytoplasm. The AcINT1 transporter belongs to the Major Facilitator Superfamily possessing conserved sugar transporter domains (Lalonde et al., 2004; Büttner, 2007). Sugar transporter signatures that include Gly-Arg-Lys (GRK) in loop 2, Asp-Arg-Ala-Gly-Arg-Arg (DRAGRR) in loop 8, and in particular the PETKGR motif following the 12th transmembrane span, GWGIPPW in the 10th transmembrane span, and PESPR in the cytoplasmic loop between helix 6 and helix 7 are present in the predicted amino acid sequence of AcINT1.

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Fig. 3. Unrooted phylogenetic tree constructed with AcMST1, HvSTP1, and homologues in *Arabidopsis* and rice. The sequences were retrieved from NCBI and the rice TIGR database and were aligned using ClustalX. Unrooted trees were constructed using the Neighbor–Joining method and viewed using TreeView software.
A translational fusion of \textit{AcINT1} with YFP at the C-terminus was constructed and its localization investigated by co-infiltrating tobacco epidermis cells with \textit{AcINT1}–YFP and the tonoplast intrinsic protein BobTIP-GFP. Figure 4 shows the co-localization of the two proteins. Also observed were transvacuolar membrane strands (arrow 2), a previously reported characteristic of plant vacuoles (Uemura \textit{et al.}, 2002), thereby supporting the assertion that \textit{AcINT1} is a tonoplast-localized sugar transporter.

A phylogenetic analysis was performed to establish the position of \textit{AcINT1} within the clades of MST-gene families, particularly with regard to previously reported tonoplast sugar transporters. The closest homologues of \textit{AcINT1} in rice and \textit{Arabidopsis} were found to be Os04g41460 and At2g43330, respectively. The latter, \textit{ArINT1}, belongs to a small family in \textit{Arabidopsis} containing four highly similar proteins, of which \textit{AtINT4} (At4g16480) was recently shown to be a high-affinity H\textsuperscript{+}-myo-inositol symporter located on the plasma membrane (Schneider \textit{et al.}, 2006). \textit{AcINT1} also shares homology with MITR1 (accession number AF280431) and MITR2 (accession number AF280432), two putative tonoplast myo-inositol transporters in \textit{M. crystallinum} (Chauhan \textit{et al.}, 2000). A third member of this group from \textit{M. crystallinum}, MITR3, has also been identified at the sequence level (accession number AY233386).

Another group of inositol and polyol transporters reported for \textit{Arabidopsis} belongs to Clade II of the MST-gene family (Klepek \textit{et al.}, 2005). An unrooted phylogenetic tree was reconstructed to reveal the relationship between \textit{AcINT1} and the polyol and inositol transporters described above, together with homologues from rice (Fig. 5). \textit{AcINT1} was found to belong to Clade V, which includes the \textit{Arabidopsis} and \textit{M. crystallinum} myo-inositol transporters. However, \textit{AcINT1} together with MITR3, Os04g41460, and At2g43330 (\textit{ArINT1}) formed a subbranch within Clade V. The transporters found within this subgroup lack several amino acids compared to the myo-inositol transporters MITR1 and MITR2. Seven amino acids in the 6th loop positioned inside the cytoplasm, including four uncharged amino acids flanked by two charged amino acids (one positive and the other negative), are also missing in the sequences belonging to the \textit{AcINT1} subbranch. Two glycosylation consensus sequences located in MITR1 and homologues, i.e. NKT (Asn-Lys-Thr) and NAT (Asn-Ala-Thr), are also missing in the \textit{AcINT1} subbranch.

\textbf{\textit{AcSUT1}: sequence analysis and localization}

Conserved regions of monocot sequences belonging to Clade III of the sucrose transporter tree (Kühn, 2003; Lalonde \textit{et al.}, 2004) were identified for the design of degenerate primers, which resulted in the cloning of \textit{AcSUT1} from pineapple (GenBank accession number EF460878). \textit{AcSUT1} was found to show 62–68\% identity at the nucleotide level with SUT2-type sucrose transporters (Clade III) from potato, \textit{Arabidopsis}, rice, and maize. The predicted open reading frame (ORF) of \textit{AcSUT1} translated into a 617 amino acid polypeptide with a theoretical mass of 66.5 kDa and 12 predicted transmembrane spans, with N- and C-termini in the cytoplasm. Conserved regions known from other sucrose transporters are present in \textit{AcSUT1}, including LGV in the second transmembrane span and CGNLK and DTDWM in loops 6 and 7, respectively. The cytoplasmic loop between the 6th and 7th transmembrane spans in \textit{AcSUT1} appeared to be longer than comparable domains in other transporters belonging to Clade III of the sucrose transporter tree (Kühn, 2003; Lalonde \textit{et al.}, 2004).

\textit{AcSUT1} was tagged with YFP at the C-terminus and transiently expressed in tobacco epidermal cells together with tonoplast intrinsic protein fused with GFP (Fig. 6). BobTIP marked the tonoplast (Fig. 6B), but \textit{AcSUT1}–YFP was observed as multivesicular bodies (Fig. 6C). A few of these bodies were seen on transvacuolar strands extending across the cell (Fig. 6D as indicated by the arrow). The small vesicular bodies containing the
AcSUT1–YFP fusion protein could represent a number of small organelles, including Golgi bodies, mitochondria, or prevacuolar bodies. To elucidate the exact subcellular localization of AcSUT1–YFP, the construct was co-infiltrated with a number of other markers that included sialyltransferase (ST–GFP, a Golgi marker), mitochondrial β-ATPase (mit-GFP), and the prevacuolar compartment marker (BP80-GFP). There was no co-localization of AcSUT1 with either the Golgi or mitochondrial markers (data not shown). However, AcSUT1–YFP co-localized with the prevacuolar body marker BP80-GFP (Fig. 7). BP80 is a vacuolar sorting receptor that selects the cargo protein at the trans-Golgi network (TGN), from where the proteins pass to vacuoles via prevacuolar compartments (PVC; Tse et al., 2004) along with the sorting receptor BP80. The BP80 protein not co-localizing with AcSUT1 (Fig. 7D) is seen as green vesicles and probably represents vesicles recycling back to the TGN (Oliviusson et al., 2006).

An unrooted phylogenetic tree was reconstructed to establish the relationship between AcSUT1 and other homologues within Clade III of the sucrose transporter family (Fig. 8). Clade III contains sucrose transporters that are distinguished from other sucrose transporters in having extended domains at the N-terminus and longer cytoplasmic loops than those in Clades I and II (Kühn, 2003; Lalonde et al., 2004). The phylogenetic tree shown in Fig. 8 can be divided into two subgroups based on the presence (within the highlighted region) or absence of an extended loop between transmembrane spans 6 and 7. In the highlighted subgroup with an extended cytoplasmic domain, the eudicot sequences (CsSUT2, AtSUT3, VvSUC12, LeSUT2, StSUT2, and PmSUC3) and monocot sequences (AcSUT1, OsSUT4, and ZmSUT2) form two separate clades.

**CAM expression, carbohydrate turnover, and transcript abundance of sugar transporters**

The transcript abundances of AcMST1, AcINT1, and AcSUT1 were compared in leaves of two cultivars of *A. comosus* that differed in the level of CAM expression.
The cultivar referred to as Spike accumulated over twice as much malate overnight (110 mmol malate kg\(^{-1}\) f wt) as the cultivar referred to as Smooth (50 mmol malate kg\(^{-1}\) f wt). Assuming that each mol of malate required 1 mol of PEP for its formation, and that each mol of hexose can potentially generate 2 mol of PEP, the hexose requirements for nocturnal malate accumulation in the Spike and Smooth cultivars were calculated as shown in Table 1. The hexose requirements were then compared with the measured depletion of soluble sugars and starch in leaves of the two cultivars. The results indicated that in the high-CAM Spike cultivar, soluble sugars alone were unable to provide all the PEP required for nocturnal malate accumulation. In contrast, starch depletion could, in principle, have provided adequate PEP for nocturnal malate accumulation in either cultivar (Table 1).

Transcript abundances of AcSUT1 (putative tonoplast monosaccharide transporter) and AcSUT1 (prevacuolar sucrose transporter) were measured at the start (06.00 h) and end (18.00 h) of the photoperiod in leaves of the two A. comosus cultivars and in fruits from the Smooth cultivar (Fig. 9). In addition, the transcript abundance of a cDNA (AcMST2) identified from an A. comosus fruit EST database was examined that showed similarity to three isoforms of a recently identified tonoplast hexose transporter in Arabidopsis (At1g20840, At3g51490, At4g35300; Wormit et al., 2006). There was no day/night difference in transcript abundance of AcMST1, AcMST2, or AcSUT1, nor were there any differences in transcript abundance between leaves of the Smooth and Spike cultivars (Fig. 9). Whilst transcript abundance of AcMST1 was higher in fruit compared to leaf tissue, there was no difference between leaves and fruit in terms of transcript abundance of AcMST2. Leaves showed higher transcript abundance of the sucrose transporter AcSUT1 compared to fruit (Fig. 9).

Transcript abundance of the putative tonoplast myoinositol transporter AcINT1 was higher in leaves of the high-CAM Spike cultivar; a day/night difference in transcript abundance was also apparent in leaves of this cultivar (Fig. 10). Transcripts of AcINT1 were present in very low abundance in fruit (data not shown). The transcript abundance of MITR3, a homologue of AcINT1 in M. crystallinum, was found to increase after the imposition of salinity (300 mM NaCl for 14 d), a treatment that induces CAM in this species (Fig. 10). After salinity treatment, the CAM-performing leaves of M. crystallinum showed a day/night difference in transcript abundance of AcINT1.
night difference in transcript abundance of MITR3, with more transcript apparent at the start of the photoperiod, a pattern that was consistent with the day/night transcript abundance of AcINT1 in leaves of the high-CAM Spike cultivar of A. comosus (Fig. 10).

Table 1. Calculated hexose requirements (1 hexose = 2 PEP) for measured nocturnal malate accumulation in two varieties of pineapple and a comparison with measured overnight depletion of starch and soluble sugars in leaves of the two varieties

<table>
<thead>
<tr>
<th>Pineapple variety</th>
<th>mmol hexose equivalents kg⁻¹ f wt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nocturnal malate accumulation</td>
</tr>
<tr>
<td>Spike</td>
<td>55</td>
</tr>
<tr>
<td>Smooth</td>
<td>25</td>
</tr>
</tbody>
</table>

Discussion

Identification of putative tonoplast-localized monosaccharide transporters in A. comosus

To begin to address how vacuolar sugar storage and assimilate partitioning is regulated in the CAM species A. comosus, a strategy was devised for cloning and identifying tonoplast-localized sugar transporters in this species for which little genetic sequence information is available. A monosaccharide transporter designated AcMST1 that localized to the tonoplast and the ER in tobacco leaf epidermis was isolated from pineapple leaves on the basis of homology with monocot sequences belonging to Clade IV of the MST-gene family. It is currently not known if this dual localization is a consequence of placing the YFP-constructs under control of the strong constitutive 35S promoter or is due to the partial retention of AcMST1 in the ER en route to the final target of the tonoplast.
However, Clade IV has previously been found to include a putative hexose transporter from sugar beet for which transport activity could not be demonstrated, but which was shown via immunoblotting to localize to the vacuole (Chiou and Bush, 1996). The *Arabidopsis* sequences with greatest sequence similarity to *AcMST1* (i.e. At1g75220 and At1g19450) have also previously been proposed as candidate vacuolar sugar transporters on the basis of a tonoplast proteomics approach (Endler et al., 2006), although localization to the tonoplast has not been verified for these proteins. In a recent phylogenetic review of the MST family, Büttner (2007) placed the Clade IV *Arabidopsis* homologues described above within a subfamily designated as AtERD6-like genes. Although the 19 genes within the AtERD6-like family are the least investigated subgroup within the MST family, the results described in the present work imply that some of these genes could encode tonoplast-localized sugar transporters. The AtERD6-like genes are named after the ERD6 (early-responsive to dehydration) gene that codes for a putative sugar transporter (Kiyosue et al., 1998), and other gene members of this group are induced/up-regulated in response to senescence, drought and wounding (Büttner, 2007). Transport activity has not been demonstrated for any of the AtERD6-like genes but, as found for *AcMST1*, the members of this group show considerable sequence similarity to the mammalian GLUT family of proteins that transport glucose via facilitated diffusion (Lalonde et al., 2004; Büttner, 2007). To date, no energy-independent monosaccharide transporters have been identified at the molecular level in plants, and it has been suggested that AtERD6 homologues could play a role in the transport of sugars out of the vacuole (Büttner, 2007). The model proposed for vacuolar sugar transport in the leaves of *A. comosus* (McRae et al., 2002) implies the existence of a tonoplast-localized hexose transporter to permit efflux of glucose and fructose at night to provide substrates for dark CO₂ uptake. It remains to be established if *AcMST1* fulfils this role in the leaves of *A. comosus*. Indeed, the mechanism that restricts efflux of vacuolar hexose to the dark period and avoids futile cycling during the light period is still unknown. It should also be noted that the transcript abundance of *AcMST1* was higher in the fruit compared to leaves. The fruit tissue does not perform CAM or turn over soluble sugars on a day/night basis, but contains twice as much hexose on a fresh weight basis as the leaves. Further work is required to establish the physiological function of *AcMST1* and to determine in which cell type the gene products occur.

A homologue of the *Arabidopsis* tonoplast monosaccharide transporter (*AtTMT*) subgroup was also identified from an *A. comosus* fruit and root EST database that was designated *AcMST2*. In contrast to *AcMST1*, which was more highly expressed in fruits compared to leaves, the transcript abundance of *AcMST2* was similar in both leaves and fruits, implying that these two vacuolar transporters differ in terms of physiological function. The *AtTMT* transporters are believed to operate via a proton-coupled antiport mechanism and to allow the active transport and accumulation of hexoses (glucose and
fructose) in the vacuole, particularly in response to stimuli (i.e. cold, drought, salinity) that promote sugar accumulation in Arabidopsis (Wormit et al., 2006). Whilst it is interesting to note that both the AtTMT and AtERD6-like genes respond to osmotic stressors, it should be noted that under ambient growing conditions the day/night turnover of vacuolar sugars in the leaves of Arabidopsis (a starch accumulator) is considerably lower than that in many grasses/cereals, as well as in A. comosus. Thus, although it would appear that the Arabidopsis vacuolar sugar transporters belonging to AtTMT and AtERD6-like gene families have tonoplast-localized homologues in barley and A. comosus, it will be of future interest to examine if and how transcriptional and post-translational regulation of these vacuolar transporters differs between starch and soluble sugar accumulators, and indeed between C₃ and CAM species.

A tonoplast-localized putative inositol transporter in A. comosus

A third tonoplast-localized sugar transporter isolated from A. comosus, designated AcINT1, showed homology to the families of myoinositol transporters in Arabidopsis (AtINT) and M. crystallinum (MITR). Myoinositol and its phosphorylated derivatives play important roles as osmolytes and in cell wall biosynthesis, ascorbate biosynthesis, mineral storage, cellular energy currency, and in several signalling pathways. In M. crystallinum, MITR1 and MITR2 have been proposed to facilitate Na⁺/myoinositol symport across the tonoplast membrane (Chauhan et al., 2000). By contrast, AtINT4, the closest Arabidopsis homologue of MITR1 and MITR2, has been shown to be a high-affinity H⁺/myoinositol symporter localized to the plasma membrane. Such apparent discrepancy in subcellular localization between these homologues could reflect the different approaches used for localization. Since MITR1 and MITR2 were immunolocalized to the tonoplast, it is possible that the antibodies used were not completely specific to these two transport proteins and recognized a further member of the MITR family. The phylogenetic position of MITR3 on a branch of the inositol transporter tree that also contains the tonoplast localized AcINT1 from A. comosus lends support to this argument and implies that AtINT1 and Os04g41460 could also be vacuolar inositol transporters. The function of vacuolar inositol transporters in species like pineapple, rice, and Arabidopsis, which do not accumulate significant amounts of myoinositol, remains unclear. In M. crystallinum the concentration of myoinositol increases substantially in response to salinity in line with its proposed role as an osmolyte (Nelson et al., 1998). As shown in the present work, salinity also elicited a substantial increase in the transcript abundance of MITR3 in M. crystallinum, and a clear day/night difference in transcript abundance was apparent in salt-treated plants, which also performed CAM. A clear day/night difference in transcript abundance of AcINT1 was observed in the high-CAM expressing cultivar of pineapple. An interrogation of Arabidopsis microarray data (www.genevestigator.ethz.ch) collected over the day/night cycle indicated that AtINT1 does not show appreciable day/night differences in transcript abundance. Given that inositol is implicated in very diverse aspects of metabolism, including calcium signalling, auxin conjugation, and ascorbate biosynthesis, the significance of a day/night shift in transcript abundance of a vacuolar inositol transporter for the operation of CAM remains to be established. Conceivably this might be involved in transport of inositols to help maintain a balance of cytoplasmic and vacuolar osmotic pressures in the course of the day/night cycle. However, further work is needed to determine the substrate specificity of AcINT1 and to establish tissue specificity of this transporter.

A sucrose transporter from A. comosus localized to prevacuoles

A sucrose transporter designated AcSUT1 was isolated from A. comosus and was found to localize to prevacuoles when transiently expressed in tobacco leaf epidermis. A recent phylogenetic analysis of confirmed and predicted sucrose transporters revealed four distinct groups in plants (Sauer, 2007). AcSUT1 can be assigned to group 3 (also referred to as AtSUC3/SUT2-type transporters), which have higher \( K_m \) values (lower affinity) for sucrose transport than the other groups. The sucrose transport characteristics of isolated tonoplast vesicles of A. comosus are consistent with those of a low-affinity/high capacity sucrose transporter (McRae et al., 2002). The group 3 transporters also possess 15–20% more amino acids than the other groups of plant sugar transporters due to the presence of an extended cytoplasmic loop between transmembrane helices 6 and 7. This extended cytoplasmic loop appears to have no significant function in controlling substrate affinity or transport (Schulze et al., 2000), and a proposed function in sucrose-sensing, based on the similarity of extended domains of yeast sensors RGT2 and SNF3 (Kühn, 2003; Lalonde et al., 2004; Sauer, 2007), has yet to be proven. To date, the functions and localization of group 3 (SUT2) sucrose transporters (of which there is one in Arabidopsis) have only been studied in eudicots, in which they appear to localize to the plasma membrane of sieve elements and are particularly highly expressed in sink tissues. Thus, in eudicots, a role for group 3 sucrose transporters as efflux carriers implicated in phloem unloading has been proposed. In A. comosus, AcSUT1 was more highly expressed in leaves compared to fruits, and a comparison of chlorophyllous and achlorophyllous leaf tissue of a variegated cultivar of Ananas

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also indicated higher expression of \textit{AcSUT1} in the photosynthetic tissue (data not shown). The monocot sucrose transporters formed a separate clade from the eudicot sequences in this group (see also Sauer, 2007), perhaps reflecting differences in vascular bundle anatomy/venation between eudicots and monocots, with the implication that group 3 sucrose transporters may have different localization patterns and functions in these two groups of plants. In the unrooted tree constructed for the implication that group 3 sucrose transporters may have venation between eudicots and monocots, with the eudicot sequences in this group (see also Sauer, 2007), sucrose transporters formed a separate clade from the photosynthetic tissue (data not shown). The monocot prevacuolar compartments (PVC) mediate transport of prevacuolar compartments has not been reported previously for any sucrose transporter. In plant cells, prevacuolar compartments (PVC) mediate transport of proteins from the trans-Golgi network to the vacuole (Tse \textit{et al.}, 2004; Miao \textit{et al.}, 2006). The localization of \textit{AcSUT1} in PVC could facilitate vesicle-mediated transport of sucrose into the apoplast for subsequent loading into the phloem (Echeverria, 2000) or could be involved in regulation of turnover of membrane sucrose transporters. To date, the only putative vacuolar sucrose transporters to be identified at the molecular level via a tonoplast proteomics approach (\textit{HvSUT2} from barley and \textit{ArSUC4} from \textit{Arabidopsis}) belong to group 4 of the sucrose transporter phylogenetic tree (Sauer, 2007). However, \textit{HvSUT2} has also been assigned to the purified plasma membrane fraction of barley plants (Wang, 2003). Fractionation of different membranes and isolation of purified tonoplast membrane is required to establish if \textit{HvSUT2} (and \textit{ArSUC4}) are indeed localized to the tonoplast. It is possible that the sucrose transporter(s) that catalyse the import of sucrose to the vacuole (cf. McRae \textit{et al.}, 2002) have not yet been identified at the molecular level.

Conclusions

By exploiting the phylogenetic relatedness of rice and \textit{A. comosus}, it was possible to identify three putative vacuolar sugar transporters in the agronomically important CAM plant pineapple. At the level of transcript abundance, there was no relationship between the magnitude of CAM and either of the two putative hexose transporters (\textit{AcMST1}, \textit{AcMST2}) or the sucrose transporter (\textit{AcSUT1}), although the putative inositol transporter (\textit{AcANT1}) did show a positive relationship with CAM expression. The existence of phosphorylation motifs in the sequences of the transporters identified from \textit{A. comosus} suggest the possibility of post-translational regulation of sugar fluxes across the vacuolar membrane. Although \textit{A. comosus} is known to turn over large amounts of soluble sugars during the day/night cycle, starch degraded at night could furnish all the substrate required for nocturnal carboxylation in this species. Further work is required to establish the substrate specificities, tissue localization, and physiological function of the pineapple sugar transporters described here.

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