

# Identification of a Vacuolar Sucrose Transporter in Barley and Arabidopsis Mesophyll Cells by a Tonoplast Proteomic Approach<sup>1</sup>

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The vacuole is the main cellular storage pool, where sucrose (Suc) accumulates to high concentrations. While a limited number of vacuolar membrane proteins, such as V-type H<sup>+</sup>-ATPases and H<sup>+</sup>-pyrophosphatases, are well characterized, the majority of vacuolar transporters are still unidentified, among them the transporter(s) responsible for vacuolar Suc uptake and release. In search of novel tonoplast transporters, we used a proteomic approach, analyzing the tonoplast fraction of highly purified mesophyll vacuoles of the crop plant barley (*Hordeum vulgare*). We identified 101 proteins, including 88 vacuolar and putative vacuolar proteins. The Suc transporter (SUT) HvSUT2 was discovered among the 40 vacuolar proteins, which were previously not reported in Arabidopsis (*Arabidopsis thaliana*) vacuolar proteomic studies. To confirm the tonoplast localization of this Suc transporter, we constructed and expressed green fluorescent protein (GFP) fusion proteins with HvSUT2 and its closest Arabidopsis homolog, AtSUT4. Transient expression of HvSUT2-GFP and AtSUT4-GFP in Arabidopsis leaves and onion (*Allium cepa*) epidermal cells resulted in green fluorescence at the tonoplast, indicating that these Suc transporters are indeed located at the vacuolar membrane. Using a microcapillary, we selected mesophyll protoplasts from a leaf protoplast preparation and demonstrated unequivocally that, in contrast to the companion cell-specific AtSUC2, HvSUT2 and AtSUT4 are expressed in mesophyll protoplasts, suggesting that HvSUT2 and AtSUT4 are involved in transport and vacuolar storage of photosynthetically derived Suc.

In mature plant cells, the central vacuole occupies 80% to 90% of the cell volume. Vacuoles contain a large number of hydrolytic and biosynthetic enzymes, inorganic ions, soluble carbohydrates, organic acids, amino acids, secondary compounds, and modified xenobiotics (Maeshima, 2001; Martinoia et al., 2002). Based on the potential toxicity of many of these compounds, Matile (1984) suggested that the distance between life and death is 7.5 nm, the thickness of the vacuolar membrane. Plants have only a limited capacity to excrete potentially toxic compounds; therefore, the term internal excretion has also been used (Martinoia et al., 1993) to indicate that, for some classes of compounds, the vacuolar membrane mimics the function and con-

tains homolog transporters of the liver plasma membrane (Kreuz et al., 1996). However, the function of the vacuole is not restricted to the storage of potentially toxic compounds. For optimal function of the metabolic pathways, the concentration of metabolites and ions has to be tightly regulated in the cytoplasm. Metabolites produced in excess are transported into the vacuole, which serves as a temporary storage pool, and released to the cytoplasm when required for metabolism.

Increasing evidence shows that impaired vacuolar deposition or retrieval affects plant metabolism. Catala et al. (2003) showed that the vacuolar calcium-proton exchanger CAX1 is induced during cold treatment and is involved in the regulation of genes responsible for cold tolerance. In the case of the vacuolar AtNRAMP3, Thomine et al. (2003) demonstrated that knockout plants exhibited an increased cadmium tolerance. It was postulated that AtNRAMP3 is an iron exporter and that under iron deficiency the plant is no longer able to mobilize vacuolar iron. The vacuolar malate carrier has recently been identified (Emmerlich et al., 2003). Cellular and vacuolar malate and fumarate contents were strongly reduced in deletion mutants of this transporter, despite the fact that a functional malate channel was still present in the tonoplast (Hurth et al., 2005). Knock-out plants also exhibited an increased respiratory

<sup>1</sup> This work was supported by the Plant Science Center Zurich-Basel (graduate research fellowship), by the project Novel Ion Channels in Plants (grant no. EU HPRN-CT-00245), and by the Deutsche Forschungsgemeinschaft (project no. ME 1955/2).

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Article, publication date, and citation information can be found at [www.plantphysiol.org/cgi/doi/10.1104/pp.106.079533](http://www.plantphysiol.org/cgi/doi/10.1104/pp.106.079533).

coefficient, indicating a shift of the substrates respired from carbohydrates in wild-type plants to mainly organic acids in the mutant. Recently, the  $\text{Ca}^{2+}$ -dependent  $\text{Ca}^{2+}$ -release channel TCP1 has been identified and functionally characterized. Deletion mutants lack functional slow channel activity, and are defective in abscisic acid-induced repression of germination and in the response of stomata to extracellular calcium (Peiter et al., 2005). These examples show that a detailed knowledge of vacuolar transporter function and regulation is required to better understand mechanisms regulating cytosolic homeostasis. However, despite some progress in identifying novel vacuolar transporters, our present knowledge of the intrinsic transporters in the tonoplast remains vague. A case in point is that to date one of the most important vacuolar transporters, the Suc transporter (SUT), has not been identified. In leaves, a large proportion of the Suc produced by photosynthesis is transported into the vacuole during the light period for storage. At night, Suc is released and loaded into the phloem for transport to the sink tissues (Kaiser and Heber, 1984; Martinoia et al., 1987). Inhibition of vacuolar Suc transport will increase cytoplasmic Suc concentrations and inhibit photosynthesis. Furthermore, many plants, such as barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*), synthesize fructans in leaves (Pollock and Cairns, 1991). Fructan synthesis occurs within the vacuole and requires Suc (Wagner et al., 1983). Fructan accumulation allows such plants to store carbohydrates within the large central vacuole, additionally limiting the increase of osmolarity. Furthermore, fructans may play an important role as cold, drought, and salt stress protectants (Ritsema and Smeekens, 2003).

One possibility to identify new vacuolar transporters is by a proteomic approach. Thus far, vacuolar proteomic approaches have been described using *Arabidopsis* (*Arabidopsis thaliana*) vacuoles (Carter et al., 2004; Sazuka et al., 2004; Shimaoka et al., 2004; Szponarski et al., 2004). Currently, no data are available for monocotyledonous cereal plants.

In this work, we present data on a vacuolar membrane proteomic approach using highly purified vacuolar membranes isolated from mesophyll cells of the crop plant barley. We identified 101 unique proteins; 45.5% of the detected barley vacuolar proteins have not been reported in the *Arabidopsis* vacuolar proteome. Among them is the Suc transporter HvSUT2, previously shown to catalyze Suc uptake when heterologously expressed in yeast (*Saccharomyces cerevisiae*; Weschke et al., 2000). HvSUT2, as well as its *Arabidopsis* homolog AtSUT4, were localized at the tonoplast as the first vacuolar Suc transporter using green fluorescent protein (GFP) fusion constructs.

## RESULTS AND DISCUSSION

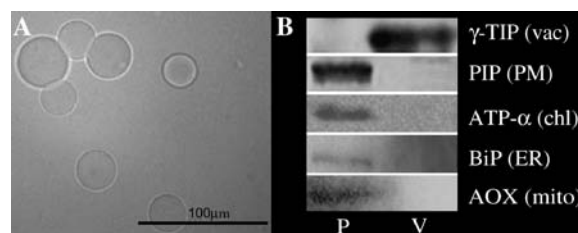
To understand the function of the vacuole and the interactions between the vacuole and the metabolic

pathways occurring in the cytoplasm, it is important to identify vacuolar transporters that are involved in the export of solutes from the cytosol and the reimport in case of metabolically relevant compounds, such as sugars, organic acids, nitrate, or phosphate. To date, a range of transporters (Maeshima, 2001; Martinoia et al., 2002) have been identified, but, as can be deduced from vacuolar localization and transport studies, a large number remain uncharacterized. To identify novel tonoplast proteins, we isolated highly purified vacuolar membranes from barley mesophyll cells. Barley is an important crop plant closely related to wheat and rice (*Oryza sativa*). It contains only one type of mesophyll cells, whereas *Arabidopsis* has two types of mesophyll cells, spongy and palisade cells.

### Purification of Barley Vacuoles

We could overcome the limiting factor of tonoplast proteomic projects, the requirement of highly purified vacuolar membranes in sufficient amounts, by improving a previously described method (Rentsch and Martinoia, 1991) for the isolation of vacuoles from barley leaves. Intact vacuoles were stained with neutral red and the purity was visually checked by bright-field microscopy. No obvious chloroplast or protoplast contaminations were observed (Fig. 1A). As described by Kaiser et al. (1986), the purity of the vacuole preparations was analyzed by the measurement of marker enzymes found in cytoplasm, mitochondria, endoplasmic reticulum (ER), and chloroplast (Table I). Contamination with these organelles was less than 1%, except for the ER (8%).

The purity of the tonoplast fractions was further confirmed by western blots. The mitochondrial marker alternative oxidase (AOX), the  $\alpha$ -subunit of the chloroplastic ATPase (ATP- $\alpha$ ), the endoplasmic marker luminal binding protein (BiP), and the plasma membrane intrinsic protein (PIP) were only detectable in the total membrane protein fraction of protoplasts and not in the tonoplast fraction (Fig. 1B), providing a reliable indicator of the purity of the extraction. The



**Figure 1.** Bright-field microscopy of purified vacuoles from barley mesophyll protoplasts (A). Western-blot analysis (B) with compartment-specific antibodies: TIP (dimer 45 kD), PIP (dimer 50 kD), ATP- $\alpha$  (55 kD), BiP (73 kD), and AOX (36 kD). Aliquots (5  $\mu$ g) of vacuolar membrane proteins (V) and total membrane proteins of protoplasts (P) were loaded on each lane, respectively. vac, Vacuole; PM, plasma membrane; chl, chloroplast; ER, endoplasmic reticulum; mito, mitochondria.

**Table I.** Activities of marker enzymes in the vacuolar preparations of barley mesophyll protoplasts

Marker enzyme activities for mitochondria (NAD-malic dehydrogenase), chloroplasts (NADP-gyceraldehydphosphate dehydrogenase, Glc-phosphate isomerase), ER (NADPH-cytochrome-*c*-reductase), and cytosol (Glc-phosphate isomerase, NAD-malic dehydrogenase) were measured in protoplast and vacuolar fractions. The percentage of contamination was calculated assuming an exclusive localization of  $\alpha$ -Mannosidase in the vacuole (Martinoia et al., 1981).

Marker Enzyme	Activities per 10 <sup>6</sup> Protoplasts	Activities per 10 <sup>6</sup> Vacuoles	% in Vacuole Preparations
	<i>nkat</i>	<i>nkat</i>	
$\alpha$ -Mannosidase	0.15	0.147	98
NADP-gyceraldehydphosphate dehydrogenase (chloroplast)	5.7	0.009	0.16
Glc-phosphate isomerase (cytosol, chloroplast)	0.35	0.003	0.96
NAD-malic dehydrogenase (mitochondria, chloroplast, cytosol)	12.8	0.069	0.54
NADPH-cytochrome- <i>c</i> -reductase (ER)	0.32	0.27	8.4

$\gamma$ -tonoplast intrinsic protein (TIP) was highly concentrated in the tonoplast fraction (Fig. 1B).

#### Analysis of Tonoplast Proteins by Liquid Chromatography-Tandem Mass Spectrometry

An untreated vacuolar membrane fraction as well as vacuolar membrane fractions washed with either 0.5 M NaOH or 0.3 M KI were analyzed by liquid chromatography-tandem mass spectrometry (MS/MS). Alkaline (NaOH) and saline (KI) treatment reduced the proportion of bound peripheral proteins from 53% in the untreated fraction to 38% in the KI-treated fraction and 34% in the NaOH-treated fraction. Cumulatively, we identified 101 proteins, 49 proteins with at least one transmembrane domain and 52 proteins with no transmembrane domain (Tables II–IV).

#### Known Vacuolar Membrane Proteins

All eight subunits of the V<sub>1</sub> sector (A–H) and three subunits of the V<sub>0</sub> sector (a, c, and d) of the well-characterized V-type H<sup>+</sup>-ATPase were detected, as well as two isoforms of the V-type H<sup>+</sup>-pyrophosphatase, indicating the high coverage of vacuolar membrane proteins. Only one aquaporin, a  $\gamma$ -TIP-like protein, was identified. We additionally identified the vacuolar membrane proteins HvTAP2 (Yamaguchi et al., 2002) and TaMRP1 (Theodoulou et al., 2003), homologs of the TCP1 (Peiter et al., 2005), the sodium/proton antiporter NHX (Xia et al., 2002), and the ATP-binding cassette (ABC) transporter MRP2 (Liu et al., 2001), which have been previously shown to reside in the tonoplast (Table II).

We identified 11 of 12 subunits of the V-type H<sup>+</sup>-ATPase (Sze et al., 2002). In comparison with published Arabidopsis vacuolar proteomic projects, only Carter et al. (2004) and Shimaoka et al. (2004) obtained such a complete set of subunits for the V-type H<sup>+</sup>-ATPase. Despite this, our proteomic analysis resulted in a limited number of known vacuolar proteins. This discrepancy may be explained by the

absence of some known vacuolar transporters in barley mesophyll cells, or barley proteins are not similar enough to permit an unequivocal attribution of peptides during the database searches.

#### Soluble Proteins and Nonvacuolar Proteins

In our investigation of the tonoplast, we found 52 proteins with no transmembrane domain. Most of these putative membrane-associated proteins are involved in stress response or in membrane fusion and remodeling (Table II).

We identified a few known nonvacuolar proteins, including four membrane proteins (Table III) and nine soluble proteins (Table IV). Soluble proteins can associate with membranes, which might explain their detection in the tonoplast preparation. The view that these proteins were peripherally associated with the membrane is supported by the fact that the proportion of soluble known nonvacuolar proteins was the highest in the nontreated vacuolar membrane fraction (8.8%). However, since the vacuole is a lytic organelle, a proportion of these apparent contaminants may be a result of the ongoing degradation processes. Interestingly, only one plasma membrane protein, aquaporin 1, was detected, demonstrating the high purity of our preparations. Most of the known nonvacuolar proteins (61.5%) were previously reported in one of the Arabidopsis vacuolar proteomic studies (Tables III and IV).

#### Novel Vacuolar Membrane Proteins

Besides well-known vacuolar membrane proteins, a large number of annotated membrane proteins with an unknown subcellular localization were identified, including several sugar transporters (gi|7024413, gi|26986186, gi|49388943, gi|51854311, and gi|31433313) and ABC proteins (gi|55773917, gi|34912536, and gi|34915038), an amino acid transporter (gi|47497044), a peptide transporter (gi|15076661), an organic anion transporter (gi|34898286), a putative calcium translocating P-type ATPase (gi|14275750), and a putative chloride channel (gi|34015349) of the CLC family

**Table II.** *Characterization of the identified vacuolar proteins*

Accession numbers are from NCBI (<http://www.ncbi.nlm.nih.gov/>). Fractions are indicated by "x" in which each identified protein was present. The number of transmembrane domains (TMDs) is according to the prediction by SOSUI (<http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html>). MW, Predicted M<sub>r</sub>; % Cov., sequence coverage in percent; % Seq., sequence similarity of the closest Arabidopsis homologs; Ref., Arabidopsis homolog(s) found by Carter et al. (2004; a), Shimaoka et al. (2004; b), Szponarski et al. (2004; c), and Sazuka et al. (2004; d).

Accession No.	Description	Non-treated	KI <sup>a</sup>	NaOH <sup>b</sup>	TMDs	MW	pI	% Cov.	pp <sup>c</sup>	Arabidopsis Homologs	% Seq.	Ref.
Known vacuolar proteins												
gi 1051258	V-ATPase A-subunit	x	x	x	0	64	5.3	77.8	1	At1g78900	90	a, b, c, d
gi 167108	V-ATPase B-subunit (isoform 1)	x	x	x	0	54	5.1	84.5	1	At1g76030	96	a, b, c
										At4g38510	95	a, b
										At1g20260	94	a, b
gi 167110	V-ATPase B-subunit (isoform 2)	x	x	x	0	53	5.1	81.6	1	At4g38510	97	a, b
										At1g76030	96	a, b
										At1g20260	95	a, b
gi 12585487	V-ATPase C-subunit	x	x	x	0	40	6.0	78.0	1	At1g12840	67	a, b, c
gi 50929105	V-ATPase D-subunit	x	x	x	0	29	9.8	33.1	1	At3g58730	76	a, b, c
gi 4099148	V-ATPase E-subunit	x	x	x	0	26	6.5	92.5	1	At4g11150	75	a, b, c
										At1g64200	74	a, b
gi 50916028	V-ATPase F-subunit	x	x	x	0	14	5.6	16.9	1	At4g02620	84	a, b
gi 50928483	V-ATPase G-subunit	x			0	12	8.0	12.7	1	At3g01390	72	a, b
										At4g23710	67	a
gi 34898596	V-ATPase H-subunit	x	x	x	1	51	7.1	11	1	At3g42050	71	a, b, c
gi 18657017	V-ATPase subunit-a	x	x	x	7	89	5.6	7.2	1	At2g21410	76	a, b, d
										At4g39080	76	a, b, c, d
gi 52353608	V-ATPase subunit-c	x	x	x	4	16	8.6	10.8	1	At1g19910	96	d
										At2g16510	96	d
										At4g34720	96	b, d
										At4g38920	96	a
										At1g75630	95	d
gi 20160977	V-ATPase subunit-d	x		x	0	40	4.8	27.6	1	At3g28715	90	a, b
										At3g28710	89	a, b
gi 11527561	Pyrophosphatase VP1	x	x	x	12	80	5.0	27.5	1	At1g15690	86	a, b, c, d
gi 18274925	Pyrophosphatase AVP3	x	x	x	12	79	5.2	30.6	1	At1g15690	86	a, b, c, d
gi 520936	γ-TIP-like protein	x	x	x	6	25	6.0	10.4	1	At2g36830	74	
										At3g26520	74	a
										At4g01470	68	
gi 12278525	Putative ABC transporter TAP2	x	x	x	5	69	8.5	9.3	1	At5g39040	77	a, b
gi 22022396	ABC transporter TaMRP1			x	6	85	5.5	10.5	1	At3g62700	74	a, b
										At2g47800	72	a, b, c
gi 27263148	Putative ABC transporter MRP2	x	x	x	14	183	6.3	11.8	1	At2g34660	72	b
										At1g30400	70	a, b, c, d
										At1g30410	69	
gi 39545849	Two-pore calcium channel	x		x	12	85	5.4	10.4	1	At4g03560	59	a, c
gi 28201131	Na <sup>+</sup> /H <sup>+</sup> antiporter HvNHX1	x			9	59	7.6	8.2	0.98	At3g05030	75	
										At5g27150	73	
gi 50911128	Putative cytochrome b-561	x			6	27.6	6.6	9.1	0.97	At4g25570	60	a, b
Putative vacuolar transport proteins												
gi 7024413	Suc transporter HvSUT2	x	x	x	11	54	8.9	7.4	1	At1g09960	62	
gi 26986186	Hexose transporter HvSTP1	x			12	79	4.8	2.0	1	At4g35300	71	a
										At1g20840	62	a
gi 55773917	Putative ABC transporter	x			10	149	6.0	1.9	0.93	At2g07680	59	
gi 34912536	Putative ABC transporter (PDR-like)	x			14	162	8.0	1.5	1	At1g15520	68	
										At2g36380	60	
										At1g15210	58	
gi 34915038	Putative ABC transporter	x	x		9	162	10.7	1.7	1	At2g47800	50	a, b, c
										At3g62700	49	a, b

(Table continues on following page.)

**Table II.** (Continued from previous page.)

Accession No.	Description	Non-treated	KI <sup>a</sup>	NaOH <sup>b</sup>	TMDs	MW	pI	% Cov.	pp <sup>c</sup>	Arabidopsis Homologs	% Seq.	Ref.
gi 34898286	Canalicular multispecific organic anion transporter 2-like protein	x			15	164	7.8	1.7	0.91	At3g59140	65	
gi 15076661	Putative peptide transporter	x	x	x	11	65	5.1	11.3	1	At2g02040 At2g02020 At3g54140	69 68 62	a, b
gi 34015349	Putative chloride channel (CLC family)	x			8	86	8.8	3.0	0.97	At5g33280 At5g49890	65 59	
gi 47497044	Amino acid-like transporter	x			8	31	9.1	4.5	0.98	At2g41190	66	
gi 49388943	Putative hexose transporter			x	11	80	5.2	2.1	1	At4g35300	72	a
gi 51854311	Putative sugar transporter	x	x	x	10	53	9.1	4.4	1	At1g75220 At1g19450	73 71	a
gi 31433313	Putative sugar transporter			x	12	79	4.9	2.2	0.96	At4g35300	72	a
gi 14275750	Putative calcium translocating P-type ATPase	x	x	x	10	63	4.9	8.3	1	At3g57330 At2g41560 At4g37640	68 66 64	a, b, c a
Putative vacuolar membrane proteins												
gi 50726573	Putative protein kinase Xa21	x			2	124	5.9	3.3	0.93	At3g47110 At3g47570 At3g47090	41 41 39	
gi 8886326	Vacuolar targeting receptor bp-80			x	2	69	5.4	4.8	0.97	At3g52850 At2g14740 At2g14720	70 68 68	a
gi 50943395	Putative syntaxin of plants 52			x	1	26	8.6	10.3	1	At1g79590 At1g16240	63 62	a a
gi 37651973	Blue copper-binding protein	x		x	2	17	5.5	13	0.99	At3g27200 At2g32300 At2g44790	40 37 36	
gi 34913062	Putative calcium sensor protein	x			1	31	4.9	2.9	1	At4g33000	77	
gi 41529149	Putative acid phosphatases	x	x	x	1	29	8.6	18.4	1	At4g29270 At4g29260 At4g25150	45 44 41	a
gi 41019551	Putative Cys protease	x			1	41	5.8	5.0	0.99	At4g39090 At2g21430 At4g16190	67 64 62	a
gi 34904294	Putative bark storage protein	x		x	1	36	5.6	5.7	1	At4g24340	38	
gi 53792725	EREBP-4-like protein	x		x	1	23	5.3	12.5	1	At1g47740 At4g17486 At5g47310	62 53 54	
gi 544242	Endoplasmic reticulum precursor	x			1	92	4.8	6.7	1	At4g24190	77	a
gi 7288255	RNase S-like protein precursor	x	x	x	1	27	8.8	52	1	At1g14220 At2g02990 At1g26820	36 34 34	a
gi 23345048	Hypersensitive-induced reaction protein 4	x			1	32	5.2	27.8	1	At5g51570	76	a, b
gi 50252990	SPX domain-containing-like protein	x	x		10	78	6.0	2.4	0.99	At1g63010 At4g22990 At4g11810	68 68 67	
gi 50726593	Putative nodulin-like protein	x		x	10	58	8.3	9.2	1	At4g34950 At2g16660	75 65	
gi 51854288	Putative integral membrane protein	x	x	x	10	54	8.6	2.0	0.99	At1g75220 At1g19450	71 70	a

(Table continues on following page.)

**Table II.** (Continued from previous page.)

Accession No.	Description	Non-treated	KI <sup>a</sup>	NaOH <sup>b</sup>	TMDs	MW	pI	% Cov.	pp <sup>c</sup>	Arabidopsis Homologs	% Seq.	Ref.
gi 51536186	Translocation related protein	x			4	42	9.1	3.8	0.98	At3g20920	59	
gi 50948653	Type 1 membrane-like protein	x		x	3	12	4.8	8.2	1	At3g24160	41	
gi 50929895	Unknown protein		x		2	113	9.3	1.8	0.91	At1g22610 At4g11610	58 52	
gi 55771327	Unknown protein	x			5	152	6.0	1.7	1	At5g11700	66	a, b
gi 50926173	Unknown protein	x			2	47	5.2	5.4	1	At1g16010 At1g80900	78 74	a
gi 50399936	Expressed protein	x			1	36	6.0	4.5	1	At5g06300 At3g53450	31 31	
Putative vacuolar membrane-associated proteins												
Stress response												
gi 53791853	Putative propyzamide-hypersensitive 1	x			0	97	5.77	4.1	0.91	At5g23720	59	
gi 5822249	Zymogen of a barley vacuolar aspartic proteinase	x			0	51	5.2	5.4	1	At1g11910 At1g62290 At4g04460	72 68 64	a, b a, b
gi 23345042	Hypersensitive-induced reaction protein 1	x		x	0	31	5.3	45.8	1	At5g62740 At1g69840	87 85	a, b b
gi 18650668	Temperature stress-induced lipocalin	x			0	22	5.5	5.3	0.99	At5g58070	74	d
gi 50912867	Putative elicitor-inducible protein EIG-J7	x			0	20	6.1	4.7	0.98	At4g39730 At2g22170	55 52	a a
gi 28555904	NBS-LRR disease-resistance protein homolog	x			0	98	7.0	2.9	0.94	At5g43470 At1g50180 At5g48620	31 30 30	
gi 124037	Bowman-Birk-type trypsin inhibitor	x			0	14	8.77	10.5	0.99	At5g43060 At1g32190 At1g74670	31 30 30	b
gi 50911973	Ethylene-responsive-like protein	x			0	20	5.9	6.2	0.98	At5g14680 At3g01520	73 72	
Membrane fusion and remodeling												
gi 32308080	$\alpha$ -SNAP	x	x		0	12	4.3	87.5	1	At3g56190 At3g56450	63 54	a, b
gi 47834379	Cycloartenol synthase	x	x	x	0	86	6.1	1.8	1	At2g07050	74	a, b
gi 50912455	Putative ADP-ribosylation factor	x		x	0	20	8.6	12.5	1	At5g37680 At5g67560	90 80	a
gi 50943481	Putative $\alpha$ -soluble NSF attachment protein	x			0	32	5.0	20.4	1	At3g56190	63	a, b
gi 52077324	Putative GTP-binding protein	x			0	26	5.2	3.8	0.93	At1g01200	65	
Other												
gi 51035342	Putative carotenoid cleavage dioxygenase 1		x	x	0	7	4.3	28.4	0.99	At3g63520	77	a
gi 1167957	O-Methyltransferase		x		0	30	6.4	6.6	0.99	At5g54160 At1g33030 At1g51990	44 39 38	
gi 50900950	Putative gag-pol precursor	x			0	206	8.9	3.0	1			
gi 34901992	Putative sulfolipid synthase	x			0	46	9.0	4.6	1	At5g01220	71	
gi 37718792	Calcineurin B protein	x			0	26	4.7	12.9	1	At4g26570 At5g55990	88 88	a a
gi 34904770	Putative esterase	x			0	38	6.0	2.5	0.99	At5g23530 At5g27320 At3g05120	47 42 39	

(Table continues on following page.)

**Table II.** (Continued from previous page.)

Accession No.	Description	Non-treated	KI <sup>a</sup>	NaOH <sup>b</sup>	TMDs	MW	pI	% Cov.	pp <sup>c</sup>	Arabidopsis Homologs	% Seq.	Ref.
gi 11360993	Gly dehydrogenase	x			0	111	6.3	1.66	0.99	At4g33010	81	
gi 21954110	RNase S-like protein	x			0	28	6.5	21.2	1	At2g26080	80	
										At1g26820	39	a
										At2g02990	38	
										At1g14220	38	
gi 7576892	Fatty acid $\alpha$ -oxidase	x			0	71	7.7	5.2	0.96	At3g01420	62	
										At1g73680	58	
gi 51592190	Nucleotide pyrophosphatase/ phosphodiesterase	x			0	39.9	5.5	3.4	0.92	At4g24890	73	
										At5g50400	73	
										At1g13750	66	
gi 21742152	Unknown protein	x			0	35	9.3	11.0	1	At4g13010	64	a
gi 38345613	Unknown protein	x			0	33	5.0	4.4	1	At4g20410	67	
gi 55771325	Unknown protein	x			0	47	5.77	1.8	0.99	At5g12010	59	
										At4g29780	55	
gi 42408418	Hypothetical protein	x			0	16	11.5	10.8	0.99			
gi 49389235	Hypothetical protein	x			0	22	11.6	13.7	0.98			
gi 47847977	Hypothetical protein	x			0	14	5.0	16.5	0.97			
gi 50928857	Unknown protein	x			0	78	6.8	6.3	0.95	At3g11590	44	
										At3g20350	42	
										At1g50660	39	
gi 27497206	Unknown protein	x			0	66	5.6	1.0	0.94	At2g20370	67	
gi 52353607	Unknown protein		x		0	45	8.4	4.2	1			
gi 37533500	Hypothetical protein	x			0	71	6.0	3.7	0.92			

<sup>a</sup>Tonoplast fraction washed with 0.3 M KI. <sup>b</sup>Tonoplast fraction washed with 0.5 M NaOH. <sup>c</sup>Protein-Prophet scores (pp) above 0.9 were accepted; at this cutoff, the rate of false positive protein identifications is less than 10%.

(Nakamura et al., 2006). Moreover, there were also nine proteins with at least one transmembrane helix for which a function has yet not been elucidated (Table II).

The closest Arabidopsis homolog of the identified peptide transporter (gi|15076661) is AtPTR2-B (At2g02040), which transports dipeptides and tripeptides and is constitutively expressed in all plant organs (Song et al., 1996). AtPTR2-B was also found by Carter et al. (2004) and Shimaoka et al. (2004) during their Arabidopsis vacuolar proteome analysis. AtPTR1, another peptide transporter of the gene family, has been localized in the plasma membrane (Dietrich et al., 2004). These results indicate that in a multigene family, different members may not be localized at the same membrane. This has also been demonstrated in studies

on the subfamily of the MRP-type ABC transporters (Geisler et al., 2004; Klein et al., 2004).

One of the identified sugar transporters (gi|51854311) is a close homolog of a hexose transporter (U43629) in sugar beet (*Beta vulgaris*), which has previously been postulated to catalyze facilitated diffusion of Glc across the vacuolar membrane of sugar beet (Chiou and Bush, 1996). Facilitated diffusion of Glc has also been reported to occur in barley vacuoles (Martinoia et al., 1987). Therefore, it is highly likely that this hexose transporter is indeed localized at the vacuolar membrane.

Interestingly, an additional sugar transporter identified in our proteomic approach was HvSUT2. HvSUT2 was previously expressed in yeast and shown

**Table III.** Known nonvacuolar membrane proteins

Accession numbers are from NCBI. The number of transmembrane domains (TMDs) is according to the prediction by SOSUI. % Seq., Sequence similarity of the closest Arabidopsis homologs; Ref., Arabidopsis homolog(s) found by Carter et al. (2004; a), Shimaoka et al. (2004; b), Szponarski et al. (2004; c), and Sazuka et al. (2004; d).

Accession No.	Description	TMDs	Arabidopsis Homologs	% Seq.	Ref.
gi 7649310	Aquaporin 1	4	At2g37170	82	d
			At3g53420	82	a, d
			At5g60660	80	
gi 439586	Calreticulin	1	At1g56340	72	
			At1g09210	72	a
gi 19093	PSI polypeptide	1	At1g52230	69	a
			At3g16140	66	
gi 37534052	Putative cell elongation protein DIMINUTO	1	At3g19820	80	a, b

**Table IV.** Soluble known nonvacuolar proteins

Accession numbers are from NCBI. Fractions are indicated by "x" in which each identified protein was present. % Seq., Sequence similarity of the closest Arabidopsis homologs; Ref., indicates Arabidopsis homolog(s) found by Carter et al. (2004; a), Shimaoka et al. (2004; b), Szponarski et al. (2004; c), and Sazuka et al. (2004; d).

Accession No.	Description	Nontreated	KI <sup>a</sup>	NaOH <sup>b</sup>	Arabidopsis Homologs	% Seq.	Ref.
gi 1224079	Rubisco	x		x	rbcl	91	
gi 57471706	Ribosomal protein L13a			x	At5g48760	83	
					At4g13170	81	
					At3g24830	81	
gi 729003	Carbonic anhydrase precursor	x			At5g14740	59	
					At1g70410	56	
					At1g23730	54	
gi 41052969	Putative 60S ribosomal protein L12	x			At2g37190	90	a
					At3g53430	89	
					At5g60670	87	
gi 1785948	Cytosolic triosephosphate isomerase	x			At3g55440	78	a
gi 50917247	Putative translation elongation factor	x			At3g22980	57	
gi 493591	Disulfide isomerase	x			At1g21750	57	a
					At1g77510	56	
gi 50913205	Constitutive photomorphogenesis 1 (COP1)	x			At2g32950	70	
gi 7452979	Allene oxide synthase	x	x	x	At5g42650	53	a

<sup>a</sup>Tonoplast fraction washed with 0.3 M KI.

<sup>b</sup>Tonoplast fraction washed with 0.5 M NaOH.

to catalyze Suc uptake in intact yeast cells (Weschke et al., 2000). The facilitated diffusion of Suc across the vacuolar membrane of barley has already been demonstrated in earlier studies (Martinoia et al., 1987). Although Suc accumulation is one of the key functions of mesophyll vacuoles, the vacuolar transporters responsible for Suc uptake and release are as yet unidentified.

#### A Comparison of the Arabidopsis and Barley Tonoplast Proteome

The vacuole possesses specialized functions in different cell types and in different plant species. For example, in Arabidopsis, vacuoles accumulate mainly monosaccharides, whereas in barley, fructans may be synthesized and stored in vacuoles. To identify proteins that are particular for the vacuolar function in barley, we compared the vacuolar proteins found in barley to proteins identified in the four Arabidopsis vacuolar proteomic approaches (Carter et al., 2004; Sazuka et al., 2004; Shimaoka et al., 2004; Szponarski et al., 2004). We searched the 88 identified vacuolar proteins for the closest homologs in the Arabidopsis genome. If a similarity of 30% is used as a cutoff, six proteins, including the putative gag-pol precursor and five hypothetical proteins, have no homolog in Arabidopsis (Table II).

Although 34 to 402 vacuolar proteins were detected in the four Arabidopsis proteomic studies, only 48 (54.5%) identified barley vacuolar proteins have also been identified in at least one of the Arabidopsis studies (Table II; Fig. 2). The highest overlap was found with Carter et al. (2004; 48 proteins) and Shimaoka et al. (2004; 29 proteins). However, 40 proteins (45.5%) of 88

detected barley vacuolar proteins were not reported in the Arabidopsis vacuolar proteomic studies, suggesting a higher relative abundance of these proteins in barley leaf mesophyll vacuoles compared to Arabidopsis leaf mesophyll and cell culture vacuoles. Eighteen (43%) of these 40 proteins are membrane proteins, including three ABC transporters (gi|55773917, gi|34912536, and gi|34898286), one amino acid transporter (gi|47497044), one putative CLC-type chloride channel (gi|34015349), six membrane proteins with an unknown function (gi|50252990, gi|50726593, gi|51536186, gi|50948653, gi|50929895, and gi|50399936), and the Suc transporter HvSUT2 (gi|7024413). The detection of HvSUT2 in the vacuolar membrane fraction indicates a high expression of this Suc transporter in barley mesophyll cells. This can be explained by the fact that barley mesophyll cells accumulate large amounts of Suc during the day (Kaiser and Heber, 1984) and that fructan synthesis in barley mesophyll vacuoles requires Suc.

#### Subcellular Localization of HvSUT2 and AtSUT4

The small number of nonvacuolar proteins that we detected in comparison to the published proteomic approaches using Arabidopsis vacuoles reveals that the purity of our barley tonoplast fractions is clearly higher. However, since the protein content of the vacuolar membrane constitutes less than 1% of the total cellular protein, small contaminations in the range of 2% to 4% have a strong impact on the detected proteins. It should also be taken into account that the plasma membrane contains about double the amount of protein compared to the vacuolar membrane. Furthermore, in mesophyll cells, the surface area of chloroplast membranes by far exceeds that of the

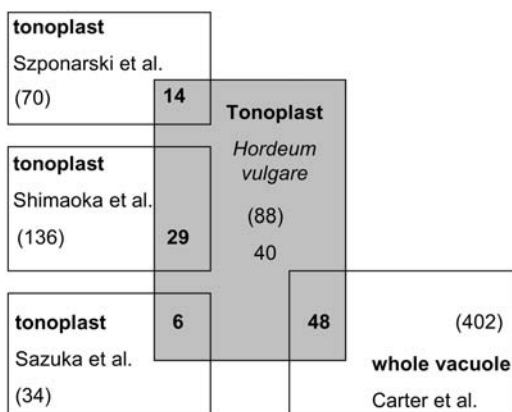


vacuole. Consequently, a contamination of 2% with chloroplasts would result in about 20% plastid proteins in the vacuolar fraction. GFP localization of the newly identified membrane proteins is therefore a prerequisite to confirm their putative localization obtained with a proteomic approach. This effort has not been undertaken in the vacuolar proteomic reports published so far.

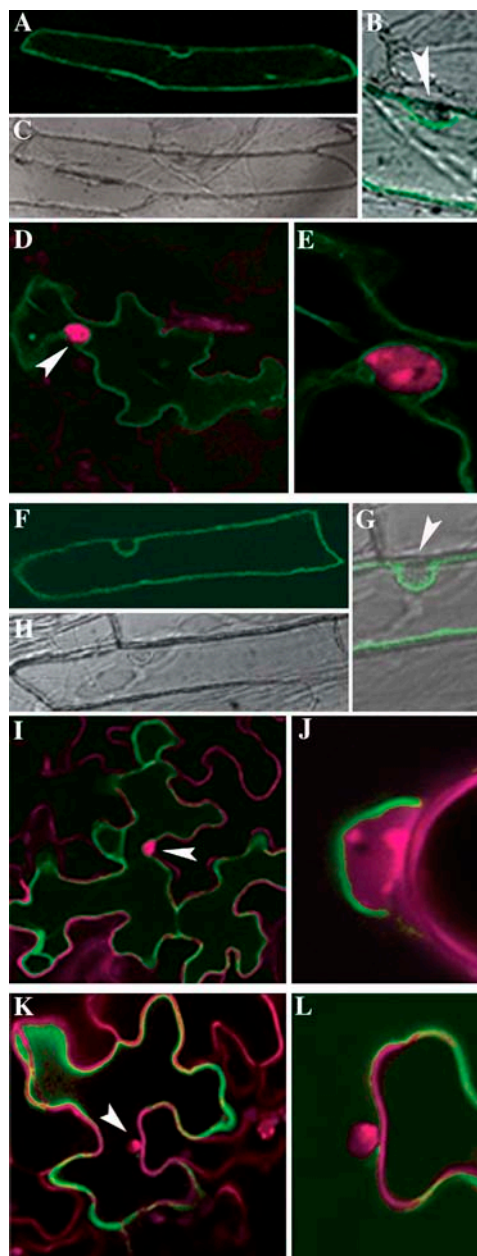
Among the potentially novel vacuolar transporters, we were particularly interested in HvSUT2, since the vacuolar Suc transporter, which plays a central role in plant metabolism, still awaits identification. As aforementioned, HvSUT2 exhibited Suc transport activity over the plasma membrane when expressed in yeast (Weschke et al., 2000). However, heterologous expression in yeast does not necessarily provide information about the intracellular localization of the protein in planta.

The high purity of our vacuole preparation encouraged us to test whether HvSUT2 and its Arabidopsis counterpart, AtSUT4, are vacuolar Suc transporters, despite the fact that the closest homologs of HvSUT2 found in tomato (*Lycopersicon esculentum*; LeSUT4) and potato (*Solanum tuberosum*; StSUT4) have been immunolocalized in the plasma membrane of sieve elements (Weise et al., 2000).

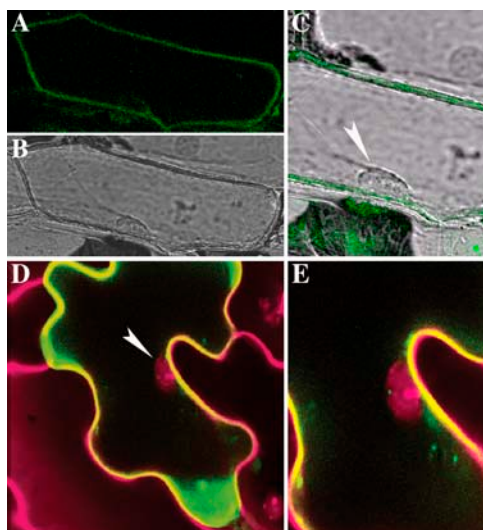
To confirm the tonoplast localization of HvSUT2 and AtSUT4, GFP fusion proteins were transiently expressed in Arabidopsis leaves and onion (*Allium cepa*) epidermal cells. For both transporters, fluorescence was detected at the vacuolar membrane of Arabidopsis and onion epidermal cells (Fig. 3, A–J). As a control, we cloned AtSUC2, a well-known plasma membrane Suc transporter of the companion cells (Stadler and Sauer, 1996), as a GFP fusion protein. Based



**Figure 2.** Comparison between the barley and Arabidopsis vacuolar membrane proteome. Barley tonoplast proteins were BLAST-searched for homologous proteins that were reported in the Arabidopsis tonoplast proteome. Proteins with a similarity of at least 30% were accepted as significant homologs. The number in brackets indicates the total number of identified proteins in each study and the bold numbers represent the number of overlapping proteins. Forty of the detected proteins were only identified in barley, suggesting a significant function at the vacuolar membrane for monocotyledon cereals.



**Figure 3.** Subcellular localization of HvSUT2, AtSUT4, and AtSUC2 by transient expression of GFP fusion proteins in onion epidermal cells and Arabidopsis epidermal cells. Cell walls and nuclei of Arabidopsis were stained in red with propidium iodide. Nuclei are indicated by arrowheads. A to E, Tonoplast localization of HvSUT2 in onion epidermal cells (A–C) and Arabidopsis epidermal cells (D and E). Fluorescence of the HvSUT2-GFP fusion protein in onion epidermal cells (A) and transmission picture (C) of the same cell. The tonoplast is located in the internal side of the cytoplasm including the nucleus as shown in the overlay of the detail pictures (B and E). F to J, Localization of the AtSUT4-GFP fusion protein at the tonoplast, transiently expressed in onion epidermal cells (F–H) and Arabidopsis epidermal cells (I and J). As shown in detail pictures (G and J), the green fluorescent tonoplast surrounds the nucleus. K and L, Plasma membrane localization of AtSUC2-GFP in Arabidopsis epidermal cells.



**Figure 4.** Subcellular localization of AtSUC1 by transient expression of GFP fusion proteins in onion epidermal and Arabidopsis leaf cells. AtSUC1-GFP localizes at the plasma membrane of onion epidermal cells (A–C) and Arabidopsis epidermal cells (D and E). Nuclei are indicated by arrows in overlay pictures (C and D). Cell walls and nuclei of Arabidopsis were stained in red with propidium iodide.

on fluorescence intensity, expression of AtSUC2-GFP was similar to AtSUT4-GFP and HvSUT2-GFP. However, unlike AtSUT4 and HvSUT2, AtSUC2 localizes to the plasma membrane (Fig. 3, K and L).

In contrast to this localization study, LeSUT4 and StSUT4, the closest homologs of HvSUT2 and AtSUT4, were immunolocalized in the plasma membrane of enucleate sieve elements, a cell type that does not contain vacuoles. Thus far, SUT4 of Arabidopsis has not been localized at the subcellular level. However, promoter- $\beta$ -glucuronidase studies showed high expression of AtSUT4 in companion cells (Schulze et al., 2003). High expression in phloem companion cells does not exclude vacuolar localization since these cells contain many small vacuoles. These vacuoles may play a role in temporary storage of Suc that can be used at night as an energy reserve in these energy-demanding cells. The results of our localization study pose the question of whether orthologs might be differently localized or whether the unusual cell structure of sieve elements, cells without nuclei, vacuoles, ribosomes, and Golgi, might entail another subcellular localization of the same protein.

Shimaoka et al. (2004) detected in their Arabidopsis tonoplast proteomic analysis the Suc transporter AtSUC1, which is strongly expressed in Arabidopsis flowers (Stadler et al., 1999). AtSUC1 was also identified in the plasma membrane proteomic approach of Alexandersson et al. (2004). To investigate if AtSUC1 is localized like AtSUT4 at the vacuolar membrane, we expressed AtSUC1-GFP in Arabidopsis leaves and onion epidermal cells. As shown in Figure 4, AtSUC1 is targeted to the plasma membrane as AtSUC2. This observation stresses on the necessity to confirm the

localization of novel candidates obtained in proteomic approaches by GFP-fusion proteins.

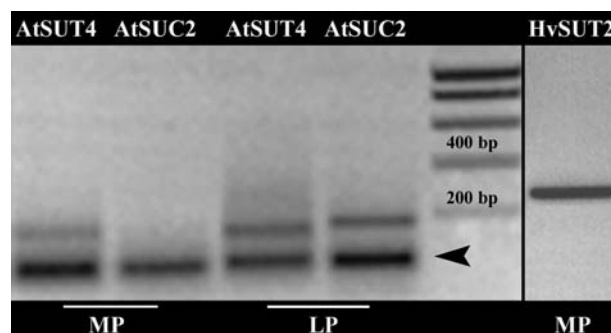
#### Expression of AtSUT4 and HvSUT2 in Mesophyll Cells

AtSUT4 promoter- $\beta$ -glucuronidase fusion plants exhibited a strong expression in companion cells (Schulze et al., 2003). To investigate whether AtSUT4 is expressed exclusively in companion cells or if transcripts are also present in mesophyll cells, where photosynthetic Suc is stored inside the vacuoles, we performed a reverse transcription (RT)-PCR analysis with isolated leaf mesophyll protoplasts.

Thirty to 40 mesophyll protoplasts of source leaves were discriminated from smaller companion cell protoplasts, chloroplast-free sieve element protoplasts, and large epidermal protoplasts by bright-field microscopy and collected in a microcapillary. RT-PCR was conducted with primers specific for the amplification of HvSUT2 and AtSUT4. AtSUC2 was used as a marker gene for companion cell contaminations. As illustrated in Figure 5, only AtSUT4 transcripts were detected in the mesophyll protoplast preparation. While in a typical leaf protoplast mixture, both AtSUC2 and AtSUT4 transcripts were present. HvSUT2 transcripts were detected in mesophyll cells (Fig. 5). These results prove that HvSUT2 and AtSUT4 are vacuolar Suc transporters of leaf mesophyll cells.

#### CONCLUSION

Our results demonstrate that the combination of a high throughput technique, the tonoplast proteomic approach, and subsequent localization by GFP is a powerful approach to identify transporters awaiting identification for a long time. We localized HvSUT2 and the Arabidopsis homolog AtSUT4 as the first Suc transporter at the tonoplast and showed expression of these Suc transporters in leaf mesophyll cells. The results



**Figure 5.** Expression analysis of AtSUT4 and HvSUT2 in mesophyll protoplasts. RT-PCR products of AtSUT4 (180 bp) and HvSUT2 (270 bp) are visible in mesophyll protoplast preparations (MP). AtSUC2 RT-PCR products (190 bp), a marker gene for companion cell contaminations, are only detected in the typical leaf protoplast mixture (LP). Actin RT-PCR products (100 bp) are indicated by an arrow.

suggest that AtSUT4 and HvSUT2 are involved in the exchange of Suc between vacuole and cytoplasm.

## MATERIALS AND METHODS

### Plant Material

Barley (*Hordeum vulgare*) var. Baraka was grown in soil in a controlled environment chamber (16 h light/8 h dark, 300  $\mu\text{E m}^{-2} \text{s}^{-1}$ , 22°C, 60% relative humidity), and *Arabidopsis thaliana* ecotype Columbia was cultivated in soil in a controlled environment chamber (8 h light/16 h dark, 150  $\mu\text{E m}^{-2} \text{s}^{-1}$ , 18°C, 60% relative humidity).

### Tonoplast Isolation

Barley mesophyll vacuoles were isolated from 8-d-old plants according to Rentsch and Martinoia (1991), omitting bovine serum albumin in the isolation medium. Tonoplast vesicles were isolated by sonication of vacuoles and subsequent ultracentrifugation for 1 h at 100,000g. The supernatant was removed and the tonoplast fraction was resuspended in 20 mM HEPES-KOH, pH 7.2, at a concentration of approximately 200  $\mu\text{g protein/mL}$ . The vacuolar membranes were either analyzed directly or treated by washing with 0.3 M KI or 0.5 M NaOH. Proteins were quantified according to Bradford (1976) using the Bio-Rad protein assay (Reinach).

### Tonoplast Proteome Analysis and Interpretation of MS Data

Tonoplast proteins were separated using SDS-PAGE (Laemmli, 1970) with 10% acrylamide gels. The SDS gels were cut into 35 sections, and gel slices were immediately subjected to in-gel tryptic digestion (Shevchenko et al., 1996). Tryptic peptides were further fractionated by reverse-phase chromatography coupled online to an LCQ Deca XP ion trap mass spectrometer (Thermo Finnigan). The peptides were analyzed by MS full scan and MS/MS scans of the three most intense parent ions.

MS/MS data sets were interpreted according to the standards of Carr et al. (2004). The SEQUEST software (Thermo Finnigan) was used to search the National Center for Biotechnology Information (NCBI) protein database of the Liliopsida (GI4447) including 139,384 proteins. dta files were created by the SEQUEST software for every MS/MS scan with a total ion count of at least  $5 \times 10^4$ , minimal peak count of 35, and a precursor ion mass in the range of 300 to 2,000 mass-to-charge ratio. Data were searched against the database restricted to tryptic peptides without modifications (except for carboxyamidomethylated Cys 57.0513 and oxidized Met 15.9994), allowing a parent mass error tolerance of 2 D and daughter ion error tolerance of 0.8 D. All SEQUEST data were analyzed by Peptide and Protein-Prophet (Nesvizhskii et al., 2003). Protein-Prophet is based on a statistical model that allows one to assess the reliability of protein identifications on the basis of MS/MS data. The Protein-Prophet score gives a probability estimate for the protein identification. We accepted scores above 0.9; at this cutoff, the rate of false positive protein identifications is less than 10% (Table II). Mostly, we identified the same protein from different biochemical fractions, further compounding the reliability of the identification (Table II).

For the identification of *Arabidopsis* homologs, we used the BLAST search of the Munich Information Center for Protein Sequences (MIPS) database ([http://mips.gsf.de/proj/thal/db/search/search\\_frame.html](http://mips.gsf.de/proj/thal/db/search/search_frame.html)). In Tables II, III, and IV, the closest *Arabidopsis* homologs are listed as well as the second and third homologs if the similarity differed not more than 10% from the closest homolog.

### Western Blotting

Western blotting was carried out using antibodies for  $\gamma$ -TIP (Conceicao et al., 1997), ATP- $\alpha$ , luminal BiP (member of the heat shock family), AOX (Elthon et al., 1989), and PIP (Frangne et al., 2001). Secondary antibodies (anti-rabbit or anti-chicken, coupled to alkaline phosphatase [AP] or horse radish peroxidase [HRP]; Promega) were diluted 1:3,000 (AP) or 1:25,000 (HRP) in TBST (0.2 M Tris-HCl, pH 7.5, 0.5 M NaCl, 0.05% Tween 20). AP detection mixture was prepared by adding 6.6  $\mu\text{L}$  of nitroblue tetrazolium (Promega) and 3.3  $\mu\text{L}$  of 5-bromo-4-chloro-3-indolyl phosphate (Promega) to 1 mL of AP buffer

(0.1 M Tris-HCl, pH 9.5, 0.5 M  $\text{MgCl}_2$ ). The activity of HRP was detected using a Chemiluminescence Blotting Substrate kit (SuperSignal Chemiluminescent Working Solution; Pierce) according to manufacturer's instructions.

### Localization Study

To localize HvSUT2 (gi|7024412), AtSUC1 (At1g71880), and AtSUC2 (At1g22710), the respective cDNAs were cloned in frame to the N terminus of GFP into the vector pGFP2 (Haseloff and Amos, 1995). The AtSUT4-GFP fusion construct was cloned into the expression vector pART7 (Gleave, 1992). GFP fusion constructs were transiently expressed in onion (*Allium cepa*) and *Arabidopsis* epidermal cells using a Helium Biolistic Particle Delivery system (Bio-Rad). Cell walls and nuclei of *Arabidopsis* epidermal cells were stained with 1 mM propidium iodide. Fluorescent cells were imaged by confocal microscopy (Leica).

### Collection of Mesophyll Protoplasts

The epidermis of *Arabidopsis* rosette leaves was rubbed off with glass paper P 80. Leaves were transferred into digestion buffer, pH 5.6 (0.5 M sorbitol; 1 mM  $\text{CaCl}_2$ ; 10 mM MES) containing 0.75% cellulase YC (w/v) and 0.03% pectolyase Y23 (w/v) (both from Kyowa Chemical Products) and incubated for 1.5 h at 30°C. After digestion, protoplasts were recovered by centrifugation (2,000g for 5 min) and purified by a Percoll gradient. The protoplasts were mixed 3:1 (v/v) with 100% Percoll, pH 6 (0.5 M sorbitol; 1 mM  $\text{CaCl}_2$ ; 20 mM MES), overlaid with 25% Percoll, pH 6, and betaine buffer, pH 6 (0.4 M betaine; 30 mM KCl; 20 mM HEPES) followed by centrifugation at 1,500g for 5 min. Protoplasts from the interface between 25% Percoll, pH 6, and betaine buffer were recovered. The leaf protoplast suspension was visualized by bright-field microscopy to distinguish between mesophyll protoplasts and other protoplasts. Thirty to 40 mesophyll protoplasts were drawn up into a microcapillary with a tip opening of approximately 100  $\mu\text{m}$ . Total RNA was extracted from the protoplasts using the PicoPure RNA isolation kit (Arcturus).

### RT-PCR

First-strand cDNA was prepared with the DNA-free total RNA using the First-Strand cDNA Synthesis kit (Amersham Biosciences). The following primers were used for RT-PCR: AtSUT4(for) 5' gtc atc cca cag gta att gtg tct gtt ggc 3', AtSUT4(rev) 5' gcg gcc gct cat ggg aga ggg atg gg 3', AtSUC2(for) 5' cat tgt cgt ccc tca gat ggt aat atc tg 3', AtSUC2(rev) 5' ctc gag atg aaa tcc cat agt agc ttg gag g 3', Actin(for) 5' gga aca gtg tga ctc aca cca tc 3', Actin(rev) 5' aag ctg ttc ttt ccc tct acg c 3', HvSUT2(for) 5' cac aat ctt agg agc acc tct gtc gat cac g 3', and HvSUT2(rev) 5' cat ggg tac ctc gtt ggg tgg ttt tct tct tc 3'.

### ACKNOWLEDGMENTS

We thank Prof. Masayoshi Maeshima (University of Nagoya) for his kind supply of antibodies to BiP and Prof. Francis Marty (University of Bourgogne) for providing us with antibodies to  $\gamma$ -TIP and AOX.

Received February 22, 2006; revised February 22, 2006; accepted March 16, 2006; published March 31, 2006.

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