Phloem-localized, Proton-coupled Sucrose Carrier ZmSUT1 Mediates Sucrose Efflux under the Control of the Sucrose Gradient and the Proton Motive Force*

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Armando Carpaneto‡§, Dietmar Geiger‡, Ernst Bamberg**, Norbert Sauer‡‡, Jörg Fromm§§, and Rainer Hedrich†††

From the §Julius-von-Sachs-Institute for Biosciences, Molecular Plant Physiology and Biophysics, Julius-von-Sachs-Platz 2, D-97082 Würzburg, Germany, the ‡Istituto di Biofisica, Consiglio Nazionale delle Ricerche, Via De Marini 6, I-16149 Genova, Italy, the **Max-Planck-Institute for Biophysics, Marie Curie Strasse 15, D-60439 Frankfurt am Main, Germany, the †††University Erlangen-Nürnberg, Molecular Plant Physiology, Staudtstrasse 5, D-91058 Erlangen, Germany, and the §§Technical University München, Holzforschung, Winzererstrasse 45, D-80797 München, Germany

The phloem network is as essential for plants as the vascular system is for humans. This network, assembled by nucleus- and vacuole-free interconnected living cells, represents a long distance transport pathway for nutrients and information. According to the Münch hypothesis, osmolytes such as sucrose generate the hydrostatic pressure that drives nutrient and water flow between the source and the sink phloem (Münch, E. (1930) Die Stoffbewegungen in der Pflanze, Gustav Fischer, Jena, Germany). Although proton-coupled sucrose carriers have been localized to the sieve tube and the companion cell plasma membrane of both source and sink tissues, knowledge of the molecular representatives and the mechanism of the sucrose phloem efflux is still scant. We expressed ZmSUT1, a maize sucrose/proton symporter, in Xenopus oocytes and studied the transport characteristics of the carrier by electrophysiological methods. Using the patch clamp techniques in the giant inside-out patch mode, we altered the chemical and electrochemical gradient across the sucrose carrier and analyzed the currents generated by the proton flux. Thereby we could show that ZmSUT1 is capable of mediating both the sucrose uptake into the phloem in mature leaves (source) as well as the desorption of sugar from the phloem vessels into heterotrophic tissues (sink). As predicted from a perfect molecular machine, the ZmSUT1-mediated sucrose-coupled proton current was reversible and depended on the direction of the sucrose and pH gradient as well as the membrane potential across the transporter.

To ensure adequate partitioning of sucrose throughout the plant body, sucrose has to be translocated from the mesophyll cells to the sieve element-companion cell complex. Because of the energy-dependent sucrose/H+ symporter in apoplasmic loading plant species, the transport sugar accumulates at concentrations of several hundred mM to 1 molar in the conduct-
MATERIALS AND METHODS

Aphid Breeding—Aphids of the species *Rhopalosiphum padi* were bred on barley and maize grown in a climate chamber under a 14-h photoperiod.

Experimental Setup—Plant aphid cages were applied to the mature leaves of a 4-week-old potted maize. Aphids feeding on a leaf were dissected from their styles using a laser as described previously (10). The recording electrodes were brought in contact with the phloem exudate appearing at the cut end of the style. The leaf was cut 15 cm proximal to the tip, and the cut end was incubated with artificial pond water containing the reference electrode (silver/silver chloride) and 1 mM NaCl, 0.1 mM KCl, 0.1 mM CaCl2, 100 mM sorbitol, and 1 mM MES, adjusted to pH 6.0 with Tris. Sucrose pulses were applied by perfusion of artificial pond water solution. Phloem potential measurements were recorded according to (10).

Two-electrode Voltage Clamp (TEVC) Analysis in Xenopus Oocytes—ZmSUT1 cRNA was prepared using the mMESSAGE mMACHINE™ RNA transcription kit (Ambion Inc., Austin, TX). Oocyte preparation and cRNA injection have been described elsewhere (11). In TEVC studies, oocytes were prepared at 22 °C in a standard solution containing 30 mM KC1, 1 mM CaCl2, and 1.5 mM MgCl2 based on Tris/MES buffers for pH values from 5.6 to 8.0 or based on citrate/Tris buffers for the pH values 4.5 and 5.0. The sucrose concentrations and pH values are indicated in Figs. 2–4 and 6 (and the corresponding legends) and throughout the text where noted. All solutions were adjusted to 220 mosmol kg

\[ I = I_{\text{max}}\frac{[S]}{[S]+K_{\text{m}}} + I_{\text{n}} \]

(1) where the substrate (S) is either [sucrose] or [H]. These fits yielded in the maximal currents \( I_{\text{max}} \) for sucrose and \( I_{\text{n}} \) for H and the half-maximal ligand concentrations \( K_{\text{m}} \) for H⁻ and \( K_{\text{n}} \) for sucrose.

**Intracellular pH Measurements**—PH-sensitive microelectrodes were pulled from borosilicate capillary (TW100F-3; WPI, Sarasota, FL) using a laser puller (P2000; Sutter Instruments, Novato, CA) and silanized with dimethyldichlorosilane (Fulka, Steinheim, Germany) at 200 °C for 15 min. The tips of the pH microelectrodes were brought in contact with the phloem exudate containing 40 mM KH₂PO₄, 2 mM NaOH, and 150 mM NaCl (pH 6.8). Only electrodes with a linear slope of 55–60 mV/pH unit over the calibration range from 4.5 to 5.0 were used. Currents were calculated by subtracting the currents in the absence of sucrose from the currents in its presence. The sucrose-induced steady state currents were measured in respect to ligand concentrations and membrane potential. At each test potential the currents were measured in respect to sucrose affinity gradients and proton motive force. We be shown that the addition of sucrose reversibly depolarized the phloem potential (Fig. 1). To elucidate the transport characteristics of the underlying sucrose/H⁻ transporter activity with respect to sucrose affinity gradients and proton motive force, we heterologously expressed ZmSUT1 in *Xenopus laevis* oocytes.

Functional analysis was performed using both the TEVC technique and the patch clamp technique. Oocytes expressing ZmSUT1 efficiently import radio-labeled sucrose with uptake rates of 6 nmol per hour and oocyte, whereas non-injected oocytes did not accumulate sucrose in detectable amounts (Fig. 2A). To monitor the movement of protons accompanying the sucrose transport, we simultaneously recorded sucrose-induced ionic currents and changes in cytoplasmic pH by TEVC and proton-selective microelectrodes (18). Upon the addition of sucrose to the external solution, large inward currents were elicited (Fig. 2B, **upper trace**). Inward currents were accompanied by a decrease in pH by up to 0.5 units within 10 min (Fig. 2B, **lower trace**). After the removal of sucrose from the bath medium, the inward currents returned to the pre-sucrose level again, whereas the recovery of pH was delayed. Control oocytes showed neither sucrose-induced currents nor sucrose-dependent changes in pH. Stepwise increases in sucrose concentrations resulted in a gradual rise in ZmSUT1-mediated currents (Fig. 2C). In the current clamp mode, membrane depolarization in response to different sucrose concentrations could be recorded as well (Fig. 2D). Like the current response in Fig. 2C, the degree of membrane depolarization dependent on the sucrose concentration applied (up to 50 mM with 10 mM sucrose). When the steady-state currents recorded in presence of extracellular sucrose concentrations between 0.5 and 50 mM were plotted against the membrane potential, ZmSUT1 currents increased upon hyperpolarization and

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1 The abbreviations used are: MES, 4-morpholinethanesulfonic acid; pH, internal pH; TEVC, two-electrode voltage clamp.
FIG. 1. Phloem potential measurements. **Top**, side-view of *R. padi* in feeding position on the upper side of a maize leaf (32×). **Bottom left**, front view of *R. padi* sucking on maize with its stylet inserted into a sieve element of a vascular bundle. **Bottom right**, after the aphid is separated from its stylet by a laser pulse, the stylet stump exuded sieve tube sap to which the tip of a microelectrode was attached (400×). Application of sucrose via the apoplast depolarizes phloem potential, pointing to a proton-coupled cotransporter. Upon removal of sucrose, the membrane potential repolarized.

FIG. 2. ZmSUT1 is a sucrose/H+ symporter. **A**, uptake of 14C sucrose (5 mM final concentration) into ZmSUT1-injected and non-injected *Xenopus* oocytes over a time scale of 60 min at pH 5.6. **B**, parallel measurements of sucrose-dependent inward currents (upper trace) and the cytosolic pH (lower trace) of a ZmSUT1-injected oocyte in response to 5 mM sucrose at an external pH of 5.6 and a holding potential (V_h) of -60 mV. Sucrose-induced currents are accompanied by a decrease in cytosolic pH. **C**, sucrose-dependent inward currents were monitored in response to a stepwise increase in sucrose concentrations. V_h = -60 mV. **D**, sucrose concentration-dependent membrane depolarization caused by a series of different sucrose concentrations at pH 5.6.
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Figure 3. Voltage-, sucrose-, and pH-dependence of ZmSUT1. A. steady-state, sugar-dependent, inward currents (mean ± S.D.; n = 4) at different potentials at pH 5.6 were plotted as a function of the external sucrose concentration. Steady-state currents (currents in the absence of sucrose were subtracted) were normalized to the current induced by 10 mM sucrose and a membrane potential of -100 mV. Currents were fitted with a Michaelis-Menten function. B, apparent affinity constants of ZmSUT1 $K_m^S$ (deduced from panel A) as a function of the membrane potential. $K_m^S$ decreases exponentially upon hyperpolarization. Data were fitted with a single exponential function ($I/I_{max} = I_{0} \cdot \exp (V/\tau_0)$, where $I$ is substrate) and extrapolated to more positive and more negative voltages. The fitting parameters at pH 5.6 were $S_0 = 16.1$ mM ± 0.7 mM and $\tau_0$ = 122 mV ± 8 mV, and at pH 6.5 $S_0 = 67$ mM ± 3 mM and $\tau_0$ = 185 mV ± 10 mV. The half-maximal proton concentration $K_m^H$, was determined from the Michaelis-Menten fit (not shown) and plotted against the membrane potential. Like $K_m^S$, $K_m^H$ is voltage-dependent and could be fitted with a single exponential function in panel B, with $S_0 = 15.4$ $\mu$M ± 0.3 $\mu$M and $\tau_0$ = 98 mV ± 5 mV. The currents at selected voltages were plotted against pH values with increasing proton concentration and hyperpolarization. At pH values > 7.0 no significant inward currents could be detected. The currents at selected voltages were plotted against the H$^+$ concentration (not shown) and fitted by a single Michaelis-Menten equation to calculate $K_m^H$ and $I_{max}^H$ (not shown). The proton affinity $K_m^H$ of ZmSUT1 exponentially increased with hyperpolarizing membrane potentials (Fig. 3C). This behavior is in line with the results for the sucrose affinities $K_m^S$ (compare Fig. 3B). Thus, both the apparent affinity constants and the $I_{max}$ values for sucrose as well as for protons decrease upon hyperpolarization.

To study the inverse transport mode of ZmSUT1 and its affinity toward cytosolic sucrose, we applied the giant patch clamp technique to ZmSUT1-expressing oocytes. In the inside-out configuration we varied the “cytosolic” sucrose concentration in the presence of either 0.5, 5, or 50 mM extracellular (pipette) sucrose. Upon a stepwise increase in cytosolic sucrose from 0 to 50, 100, 200, and 500 mM in the presence of 50 mM in the pipette, a progressive decrease in inward current was measured (Fig. 4A). This effect was completely reversible; inward currents reached their pre-stimulus levels after the removal of cytosolic sucrose. When plotting the average currents shown in Fig. 4A as a function of the cytosolic sucrose concentration, data could be fitted by a Michaelis-Menten equation (Fig. 4A, continuous line) characterized by an apparent $K_m^S$ of 160 mM (Fig. 4D). The inset of Fig. 4D depicts the extrapolation of the sucrose-induced currents from 2 to 3 mM, a concentration range in which ZmSUT1 currents would reverse direction ($I = 0$ at 2.38 mM sucrose). When the extracellular sucrose concentration was decreased to 5 mM or even 0.5 mM, the ZmSUT1-mediated currents reversed direction at physiological cytosolic sucrose levels (Fig. 4, B and C). In the presence of 5 mM external sucrose, a $K_m$ of 278 mM was calculated (Fig. 4E). A rise in cytosolic sucrose concentration above 314 mM even inverted the current direction. Upon a further decrease in extracellular sucrose concentration to 0.5 mM and the absence of cytosolic sucrose, only very small inward currents remained (Fig. 4C). Under these conditions, however, a rise in cytosolic sucrose concentration to just 50 mM inverted the ZmSUT1 current already. From the Michaelis-Menten fit a $K_m$ of 362 mM and a zero current value at 31 mM was obtained (Fig. 4F). When plotting the $K_m$ values versus the extracellular sucrose concentration, a decrease in $K_m$ with the rise in extracellular sucrose concentration became evident (not shown).

Likewise, the cytosolic sucrose concentration causing the ZmSUT1 current to change direction was plotted as a function of external sucrose (Fig. 5). Under the equilibrium conditions depicted in Equation 2,

$$n_{suc} \Delta \mu_{suc} + n_{H} \Delta \mu_{H} = 0 \quad (\text{Eq. } 2)$$

where $n_{suc}$ and $n_{H}$ are the number of moles of sucrose and protons transported through the membrane, $\Delta \mu = \mu_{suc}^{cyt} - \mu_{suc}^{ext}$ is the difference between the cytosolic and external chemical poten-
tial (or molar free energy) of sucrose, and $\Delta \mu_{\text{fit}} = \mu_{\text{fit}}^{\text{cyt}} - \mu_{\text{fit}}^{\text{ext}}$ is the difference between the cytosolic and external electro-chemical potential of protons. Therefore, Equation 3, shown here,

$$[\text{Suc}]_{\text{ext}} = \frac{[\text{Suc}]_{\text{cyt}}^0 \cdot 10^{n_{\text{fit}}} \cdot \text{pH}_{\text{cyt}} \cdot \text{pH}_{\text{ext}}}{V_m \cdot 2.303 \cdot R \cdot T}$$  \quad (\text{Eq. 3})$$

is another way in which Equation 2 can be written. The continuous lines in Fig. 5 are obtained by Equation 2 with $V_m = 0$ mV and using different values for $n_{\text{Suc}}$ and $n_{\text{H}}$. This analysis revealed that the ZmSUT1 transporter has a 1 Suc/1 H$^+$ stoichiometry (compare Refs. 19 and 21).

In agreement with a perfectly coupled thermodynamic machine, the positive current in Fig. 4 represents the sucrose gradient-driven efflux of protons against the proton gradient. To study the two transport modes of ZmSUT1 in the absence of the proton motive force, in Fig. 6A we stepped the cytosolic sucrose concentration from 0 to 500 mM (5 mM sucrose in the

**Fig. 4.** Changes in cytosolic sucrose feedback on the magnitude and direction of ZmSUT1 currents. A–C, ZmSUT1 currents recorded in inside-out giant patches in the presence of 50 mM (A), 5 mM (B), and 0.5 mM external sucrose (C). Schematic representations above each graph depict the proton and sucrose concentrations; cytosolic and external pH was 7.5 and 5.6, respectively, and cytosolic sucrose concentrations were elevated from 0 to 50, 100, 200, and 500 mM as indicated. The membrane potential was clamped to 0 mV. D–F, averaged currents gained from experiments shown in panels A–C were plotted versus the corresponding cytosolic sucrose concentrations. Data were fitted by the Michaelis-Menten equation $I = I_0 + \frac{[\text{Suc}]_{\text{cyt}}}{K_m + [\text{Suc}]_{\text{cyt}}}$, where $I_0 = 11.0$ pA, $K_m = 161$ mM, and $I_0 = 10.3$ pA (E); $I_1 = 4.83$ pA, $K_m = 278$ mM, and $I_0 = 2.56$ pA (F); and $I_1 = 536$ fA, $K_m = 362$ mM, and $I_0 = 42.7$ fA (G). The inset of panel D shows the current extrapolation for cytosolic sucrose concentrations ranging from 2 to 3 M.

**Fig. 5.** Stoichiometry between H$^+$ and sucrose of ZmSUT1. The cytosolic sucrose concentration that induces zero current, obtained by experiments as shown in Fig. 3, is plotted against the external sucrose concentration. The continuous lines were obtained by the equilibrium equation (Equation 3) with $V_m = 0$, $\text{pH}_{\text{cyt}} - \text{pH}_{\text{ext}} = 1.9$, and different values for $n_{\text{Suc}}$ and $n_{\text{H}}$ (the thicker line corresponds to a 1:1 stoichiometry of the ZmSUT1 transporter). The same experimental conditions as those in Fig. 3 were used.
pipette) in the absence of a pH gradient. With $[\text{Suc}]_{\text{cyt}} = 0 \text{ mM}$ and the absence of a membrane potential, we recorded an inward current as expected from the steep inward-directed sucrose gradient. Inverting the sucrose gradient by increasing $[\text{Suc}]_{\text{cyt}}$ to 500 mM, the carrier current reversed direction. In the presence of an inward-directed pH gradient, however, the magnitude of outward currents was smaller (compare Fig. 4). Inward currents could be re-established again upon removal of the disaccharide. Following a rise in the extracellular sucrose concentration from 5 to 50 mM and the absence of cytosolic sucrose, carrier currents remained inward (Fig. 6B). During bath perfusion to $[\text{Suc}]_{\text{cyt}} = 500 \text{ mM}$, currents changed direction. These experiments indicate that the sucrose gradient can drive the proton flux and vice versa. In the experiment depicted in Fig. 4B, the ZmSUT1 currents were subject of a fast “run-down,” most likely due to the loss of regulatory cytosolic factors. Interestingly, in Fig. 6B the decay of both inward and outward currents could be fitted by single exponential functions (dashed lines) with the same time constant. This indicates that both transport modes of ZmSUT1 are perfectly coupled via the sucrose gradient and proton motive force.

Under the conditions of the sink phloem, the sucrose gradient drives the efflux of protons and sucrose. To mimic this situation in the oocyte system in Fig. 6C, ZmSUT1-expressing oocytes were injected with $[14\text{C}]\text{sucrose}$ (final concentration of 50 mM), and the release of the radioactively labeled sucrose was measured. In ZmSUT1-oocytes, but not in water-injected control-oocytes, pronounced sucrose release was measured. As expected from our thermodynamic assumptions, the sucrose-release was enhanced when the cytosol was acidified by acetate treatment.
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Because of the localization of a sucrose/H+ transporter in sink tissues, it has previously been speculated that phloem unloading may be mediated by the same sucrose-H+ symporters that are responsible for phloem loading (for example, Ref. 22). The direct demonstration that ZmSUT1, a member of the phloem sucrose carrier family, acts either in the source mode or sink mode for the life-maintaining uptake, and the adsorption of sucrose is underpinned by genetic evidence. Arabidopsis mutants, which lack the ZmSUT1 homologue AtSUC2, are strongly impaired in phloem loading and unloading of sucrose, which results in stunted growth, retarded development, and sterility (23). Phloem unloading of sucrose is required for starch formation in storage tissues, such as the grains of cereals or potato tubers. When the copy number of StSUT1, a ZmSUT1 orthologue expressed in the phloem of developing tubers, is reduced by antisense repression, reduced fresh weight accumulation during tuber development was observed (4, 5).

Furthermore, indirect measurements with the proton-coupled monosaccharide transporter ChHUP1 from the green alga Chlorella and the SGLT1 Na+/glucose transporter from human and rabbit suggest that these sugar carriers from single-celled organisms can act in the inverse transport mode to release their substrates (24–27). To study the inverse mode of ZmSUT1, we performed patch clamp experiments in the giant inside-out configuration. Varying the cytosolic sucrose concentration, we were for the first time able to determine the cytosolic affinity constant for sucrose. Upon variation of the sucrose gradient we could reverse the direction of the proton current, e.g. by increasing the cytosolic sucrose concentration. The direction of the transport of the ZmSUT1 symporter is therefore dependent on the sum of the free energies of both the sucrose and the proton gradient across the membrane. In agreement with the above considerations, we could demonstrate that sucrose could drive protons through ZmSUT1. Recently, the reversibility of the human and rabbit Na+/glucose co-transporters has been documented by measuring the reversion of the glucose-coupled Na+ current. Like the proton-coupled dicarboxylic acid carrier ZmSUT1, the sodium-coupled SGLT1 shows more than one order of magnitude difference between the sugar affinities of the two transport modes, indicating a functional asymmetry of both carrier types. Under physiological conditions the inverse transport mode of SGLT1 is highly improbable because of the low affinity of the sugar carrier. In the plant phloem, however, both transport modes of ZmSUT1 are probable (see model in Fig. 7). In maize source leaves, extracellular sucrose concentrations of 2.6 mM were measured (28). Assuming a pH gradient of ~1.5 units and a phloem membrane potential of ~150 mV (29), a perfect proton-coupled ZmSUT1 would allow a theoretical phloem sucrose accumulation of up to 26 M (according to Equation 3, with $n_S/n_{Suc} = 1$). Direct measurements in these sink phloem cells revealed membrane potentials of only ~60 mV.5 At an apoplastic sucrose concentration of 1 mM, a phloem sap sucrose concentration of 0.85 m, and a pH gradient of 1 unit of sucrose release would occur at membrane potentials positive from ~115 mV (according to Equation 3 with $n_S/n_{Suc} = 1$). This regime directs ZmSUT1 into the inverse transport mode, and sucrose is released.

The present work revealed the functional asymmetry of the phloem sucrose carrier ZmSUT1. Our data, for the first time, demonstrate the “sink mode” of this pivotal carrier type, provide for the molecular mechanism of phloem sucrose release, and explain the severe phenotype of phloem H+/sucrose carrier loss-of-function mutants and antisense-repression plants. In contrast to symplasmic unloading, this sucrose/H+ symporter-mediated mechanism drives unloading of sucrose under the control of both the sucrose and the pH gradients as well as the membrane potential of the phloem and the surrounding tissues.

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