Macromolecular Trafficking Indicated by Localization and Turnover of Sucrose Transporters in Enucleate Sieve Elements

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The leaf sucrose transporter SUT1 is essential for phloem loading and long-distance transport of assimilates. Both SUT1 messenger RNA (mRNA) and protein were shown to be diurnally regulated and to have high turnover rates. SUT1 protein was detected by immunolocalization in plasma membranes of enucleate sieve elements (SEs) in tobacco, potato, and tomato. Analysis by in situ hybridization showed that SUT1 mRNA localizes mainly to the SE and is preferentially associated with plasmodesmata. Antisense inhibition of *SUT1* expression under control of a companion cell (CC)–specific promoter indicated synthesis of *SUT1* mRNA in the CC. These results provide evidence for targeting of plant endogenous mRNA and potentially SUT1 protein through phloem plasmodesmata and for sucrose loading at the plasma membrane of SE.

In plants, most cells are separated from each other by cell walls. Despite this separation, the majority of cells are interconnected by plasmodesmata, analogous to gap junctions. The function of these structures in intercellular trafficking remained obscure for many years. The finding that plasmodesmata allow the trafficking of both endogenous and viral mRNAs and proteins has led to a new understanding of their functions (*1*). However, little is known about plasmodesmal structure at the molecular level.

Plants contain cell types that, because they lack nuclei, require import of RNAs or proteins. The phloem—the long-distance transport system for assimilate transport—consists of various cell types, the main conduits being sieve elements (SEs). They are derived from unequal cell divisions that result in the formation of closely associated cell types: SEs and companion cells (CCs). In many plant species, including potato, the SE-CC complex is symplastically isolated from surrounding cells but the two cell types are closely linked with one another by specific plasmodesmata (*2*). During maturation, SEs lose their nuclei and many organelles, retaining only a modified endomembrane system, plastids, and mitochondria (*3*). Sieve elements remain active over an entire growth period, that is, for several months in annual plants or up to decades in plants such as palms (*4*). Because they lack nuclei, active SEs must either retain proteins stable enough to function for the lifetime of the cell or mRNAs and proteins must be imported from neighboring CCs. The presence of proteins and enzymatic activities (*5–7*), rapid turnover of certain phloem proteins (*8*), and constitutive efflux of an identical spectrum of polypeptides for days (*9*) indicate that SEs are constantly supplied with new proteins or mRNAs from neighboring CCs. This conclusion is supported by the finding that mRNA of typical SE P-proteins is localized in CCs, indicating protein movement into mature SEs through plasmodesmata (*10*).

Complementation of an artificial yeast mutant deficient in sucrose uptake was used to isolate a H^+/sucrose symporter gene *SUT1* (*11*). Several lines of evidence underscore the essential role of SUT1 in phloem loading and long distance transport (*12*). When expressed in yeast, SUT1 has biochemical properties similar to those of sucrose uptake into plasma membrane vesicles from leaves (*13*). SUT1 is expressed in the phloem, and its induction correlates with the development of export capacity in leaves (*14*). Most importantly, antisense repression of SUT1 in transgenic plants inhibits sucrose export from leaves (*15*, *16*). Furthermore, antisense repression of SUT1 with a CC-specific promoter indicates that *SUT1* is transcribed in CCs (*13*).

For direct localization of SUT1, four different affinity-purified peptide antisera specific for distinct hydrophilic domains of SUT1 (the NH_2-terminus, central loop region 1, central loop region 2, and seventh loop) were generated [here and (*13*)]. Each antiserum recognized a single polypeptide of 47 kD in microsomes and plasma membranes from yeast and potato leaves (Fig. 1A). By immunofluorescence and silver-enhanced immunogold staining, SUT1 was detected exclusively in SEs of minor veins (Fig. 2A). The presence of sieve plates (Fig. 2B) and the absence of nuclei allowed unambiguous identification of SEs (Fig. 2C). SUT1 was found both in adaxial (inner) and abaxial (external) phloem in leaves and stems, respectively. The protein was found not only in minor veins of source leaves (Fig. 2, A and E) but also in stems (Fig. 2, B and C), petioles (Fig. 2F), and roots (Fig. 2G). Analysis of immunogold-stained sections by electron microscopy revealed that SUT1 is localized at the plasma membrane of SE (Fig. 2H). SUT1 could thus contribute to the high density of particles on the SE plasma membrane (*17*). Identical results were obtained in

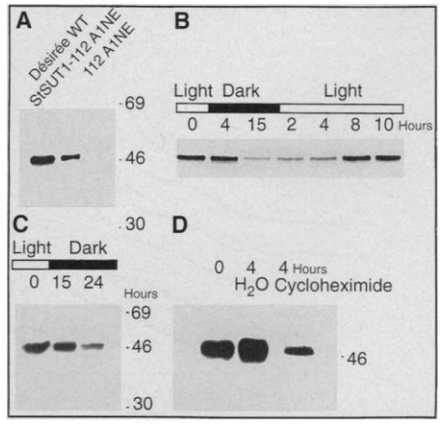

Fig. 1. Turnover of SUT1 mRNA and protein. (**A**) Immunoblot analysis of *Solanum tuberosum* SUT1 (StSUT1) (P2 antiserum, 1:1000 dilution) in microsomal fractions of potato leaves and yeast cells expressing StSUT1 (StSUT1-112A1NE); control, vector alone (112A1NE). Molecular sizes are indicated on the right (in kilodaltons). (**B**) Northern (RNA) blot analysis of light-dependent expression of *Lycopersicon esculentum* SUT1 (LeSUT1) mRNA in tomato leaves. (**C**) Light-dependent turnover of StSUT1 protein as analyzed by immunoblot of microsomal fractions from leaves of potato plants exposed to darkness for extended periods. (**D**) Effect of 4-hour treatment with 30 μM cycloheximide supplied to detached potato leaves on StSUT1 protein amounts in microsomal fractions. For antisera to StSUT1, rabbits were immunized with synthetic peptides (central loop: P1, RENELPEKDEQEIDE; P2, EIDEKLAGAGKSK; NH_2-terminus: P3, MENGTKREGLGKL; putative loop VII: P4, KEVFGGQVGDARLYD). For immunoblotting, yeast cells were homogenized with glass beads, and membranes were centrifuged at 100,000*g*. Proteins were solubilized in dodecylmaltosid. Plasma membranes were purified as described (*13*, *14*). Immunodetection was performed with P2 antiserum (1:1000 dilution). Abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; and Y, Tyr.

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tobacco (Fig. 2I) and tomato (*18*). In agreement with the homology in the respective region, P1 antiserum detected SUT1 in protein immunoblots and immunogold studies in potato and tobacco, but not in tomato, whereas P2 antiserum localized SUT1 in tomato but not in tobacco. Furthermore, positive reactions could be competed by addition of fusion proteins containing the respective peptide, but not by control proteins (*18*).

To resolve the seemingly contradictory results of *SUT1* transcription in CCs (*13*) and the presence of SUT1 protein only in SEs, we performed in situ localization of *SUT1* mRNA. Similarly, *SUT1* transcripts were found at high levels in SEs and at lower levels in CCs (Fig. 3A). Analysis by electron microscopy revealed the presence of transcripts at the orifices of the plasmodesmata between CCs and SEs (Fig. 3, B and C). Thus, the simplest explanation is that *SUT1* mRNA is synthesized in the CC, the mRNA is translocated through plasmodesmata by specific targeting mechanisms to the SE, and subsequent translation occurs in the SE. Alternatively, SUT1 mRNA or protein (or both) are already present in SE-CC mother cells before division, and both RNA and protein are stable for several months (that is, the life-span of SEs). Young SEs still containing a nucleus already express SUT1 protein (Fig. 2D). However, *SUT1* mRNA levels are highest in mature leaves where SE development is complete (*11*). Furthermore, SUT1 mRNA and protein levels decrease after 15 hours of exposure to darkness or after 4-hour incubation in cycloheximide (Fig.1, B to D). The turnover correlates with diurnal regulation of sugar export from leaves (*19*). This high turnover points to a specific mechanism that controls the number of active carriers in the plasma membrane and suggests the involvement of endocytosis as in mammalian glucose transporters or yeast amino acid permeases (*20*). Moreover, it demonstrates that SUT1 mRNA must be imported from the neighboring CC.

Asymmetric RNA transport within cells has been observed during *Drosophila* oogenesis and in neurons (*21*, *22*). Trafficking of RNA is also involved in viral transport facilitated by movement proteins (*23*, *24*) and in transport of RNA for the transcription factor Kn1 (*1*). In several instances, both protein and RNA are transported. It remains to be shown whether, in addition to trafficking of *SUT1* mRNA, trafficking of SUT1 protein also occurs. If *SUT1* mRNA is transported from the CC to the SE, it must be translated in the SE. However, most ribosomes are lost during SE maturation (*25*). Nevertheless, SEs are able to synthesize proteins (*26*). This observation is reminiscent of mammalian erythrocytes that lose their nuclei but have a half-life of about 120 days. Hemoglobin mRNA is stored in the mature cells that contain sufficient ribosomes to translate hemoglobin mRNA (*27*). It will therefore be essential to reevaluate the paradigm for the absence of ribosomes in the SE by using immunolabeling techniques because direct assessment of SUT1 synthesis in SE is currently not possible.

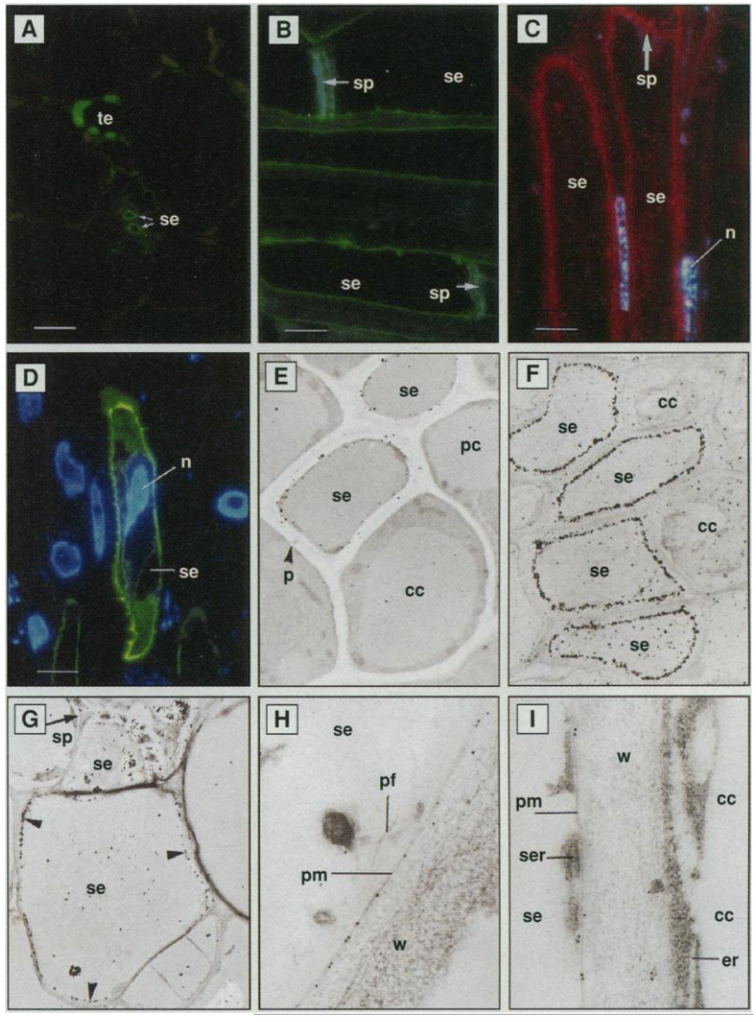

Fig. 2. Immunocytochemical localization of StSUT1 protein in SE by fluorescent detection (**A** to **D**) (1-μm-thick sections; bar, 40 μm) or immunogold labeling (**E** to **I**) [100-nm ultrathin sections; silver-enhanced 1-nm gold-coupled anti-rabbit immunoglobulin G (IgG) (E to G) or 10-nm gold-coupled anti-rabbit IgG (H to I)]. Fluorescent immunodetection of StSUT1 was performed as described (*31*) with modifications. For immunogold labeling, material was fixed, dehydrated, and embedded in methacrylate (Unicryl, British Biocell). Ultrathin sections (100 nm) were mounted on gold grids, blocked, and incubated with affinity-purified anti-StSUT1 diluted 1:50 in phosphate-buffered saline, 0.1% bovine serum albumin (BSA) and then with 10-nm gold-coupled goat anti–rabbit IgG (Amersham). (A) Transverse section of seventh-order vein of potato source leaf. Antibody detection was by anti–rabbit IgG–fluorescein isothiocyanate (FITC) conjugate. (B) Longitudinal stem section of potato. Double staining of sieve plate callose with aniline blue (arrows) and the anti-StSUT1–FITC conjugate. (C) Histochemical staining of nuclei with 4′,6′-diamidino-2-phenylindole (DAPI) on a longitudinal stem section treated with anti-StSUT1–cy3 conjugate. (D) SUT1 protein in young nucleated SE and CC in longitudinal section from not fully extended internodes of potato, counterstained with DAPI for nuclear localization. (E) SE of a minor vein showing peripheral labeling with P2 antiserum. Branched plasmodesma (arrowhead) pore-contacts identify neighboring companion cells (arrowhead) (magnification, ×5900). (F) Petiole cross section of potato with four SEs with silver-enhanced immunogold label at the plasma membrane (×4500). (G) Cross section of potato root showing metaphloem SE with silver-enhanced gold particles at the plasma membrane (arrowheads). Starch grains of SE plastids show nonspecific labeling (×3800). (H) Petiole cross section of potato SE showing specific labeling of the plasma membrane (×50,000) (I) Petiole cross section of tobacco (×50,000); cc, companion cell; er, endoplasmic reticulum; n, nucleus; se, sieve element; ser, sieve element reticulum; sp, sieve plate; te, tracheary element; p, plasmodesmos; pc, phloem parenchyma cell; pf, P-protein filaments; pm, plasma membrane; w, cell wall.

These data raise the question of where the driving force for active sucrose uptake into the SE is generated. Support comes from the finding that a steep gradient for sucrose can exist between the SE and CC (*14*). Electric coupling between the two cell types would allow active loading of sucrose into the SE on the basis of the electromotive force (*28*). In this instance, the H^+/adenosine triphosphatase (ATPase) in *Vicia faba* (*29*) and the *AHA3* H^+/ATPase in *Arabidopsis* (*30*), which are localized in the CC, would energize sucrose uptake into the SE. SUT1 mRNA and protein were detected not only in minor veins of mature exporting leaves but also in petioles, stems, and roots (Fig. 2). Thus, in addition to its role in phloem loading, SUT1 may also be involved in retrieval of sucrose leaking out of the SE along the translocation path (*31*).

Our results appear to contradict data obtained for SUC2 sucrose transporters in *Arabidopsis* and *Plantago* (*32*). However, since localization of SUC2 by electron microscopy was not performed, the possibility remains that protein detected is not localized at the plasma membrane. Otherwise, we have to assume that species-specific differences exist and may be related to different loading mechanisms (*33*). Alternatively, sucrose might be loaded in a stepwise manner involving different carriers in the CC (SUC2) and SE (SUT), as suggested by physiological analyses (*34*).

The localization of *SUT1* mRNA and protein in the SE together with the high turnover rates indicates that *SUT1* mRNA is transported through plasmodesmata. In three investigated *Solanaceae*, these data are consistent with direct uptake of sucrose by the SE. Identification of signal sequences in conjunction with biochemical approaches should provide an excellent tool to study the role of plasmodesmal proteins in macromolecular transport between plant cells.

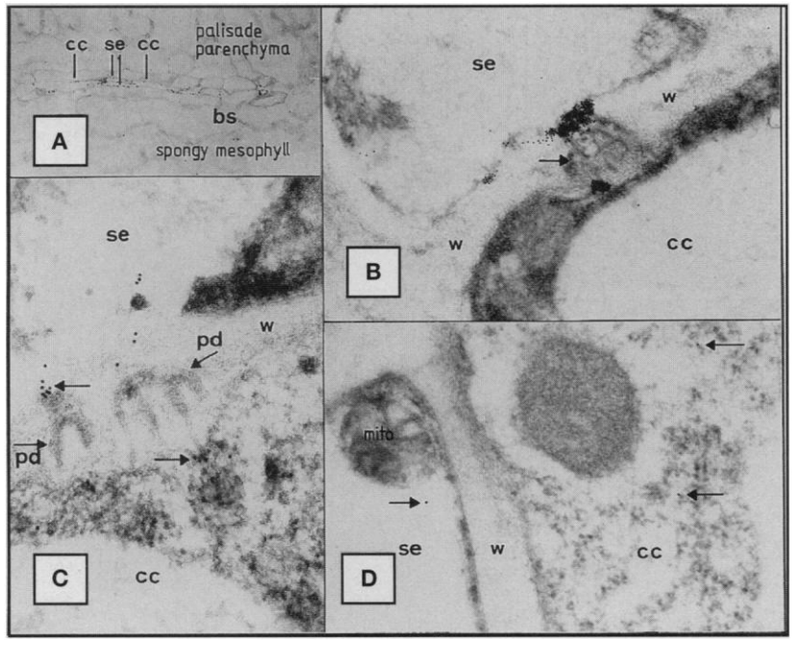

Fig. 3. Localization of *StSUT1* mRNA by in situ hybridization. Sections were stained for 6 hours with 1.25% glutaraldehyde, 2% paraformaldehyde in 50 mM Pipes (pH 7.2) at 4°C and then were dehydrated with ethanol and embedded in LR white. Sections (1 μm for light microscopy) were placed in H_2O on Digene slides and dried. For transmission electron microscopy (TEM), sections were cut to gold interference color and picked onto uncoated nickel grids. Materials were blocked in 0.1% poly(vinylpyrrolidone) (PVP). Sense and antisense probes were made with the Boehringer Genius system. Hybridization was performed in standard buffer with 50% dextrane under sealed cover slips or as grids in microvials in a moist chamber overnight at 56°C. After several washes in 2× saline sodium citrate (SSC), samples were treated with ribonuclease A and washed in 2× SSC–50% formamide at 50°C for 5 min and then in 0.1× SSC at room temperature. Slides were blocked with tris-buffered saline (TBS)–BSA–0.1% PVP–0.5% whole sheep serum for 1 hour at room temperature and then incubated with anti-digoxigenin–gold (1:300 in TBS-BSA for 2 hours at room temperature). After several washes in TBS, silver enhancement was performed only for slides, according to the manufacturer's directions (Amersham). Slides were poststained for 30 s with 0.1% Safranin O. Grids were poststained with 1% potassium permanganate–2% uranyl acetate (1:4) and examined with Jeol 1200EX TEM or Leitz Aristophot light microscopy (see legend to Fig. 2). (**A**) Localization of mRNA in potato source leaves by light microscopy (×290). (**B**) Potato leaf cross section after incubation with labeled *StSUT1* antisense transcripts. Labeling is detecable in both the SE and CC preferentially near plasmodesmal structures (×45,000). (**C**) High-magnification image of StSUT1 mRNA localized in potato source leaf. StSUT1 antisense transcripts localize StSUT1 mRNA to orifices of plasmodesmata (×90,000). (**D**) As a control, leaf cross sections were incubated with labeled StSUT1 sense transcripts where only weak background labeling was detectable (×60,000).

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