Function of the cytosolic N-terminus of sucrose transporter AtSUT2 in substrate affinity

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Abstract AtSUT2 was found to be a low-affinity sucrose transporter (K_M = 11.7 mM at pH 4). Chimeric proteins between AtSUT2 and the high-affinity StSUT1 were constructed in which the extended N-terminus and central loop of AtSUT2 were exchanged with those domains of StSUT1 and vice versa. Chimeras containing the N-terminus of AtSUT2 showed significantly lower affinity for sucrose compared to chimeras containing the N-terminus of StSUT1. The results indicate a significant function of the N-terminus but not the central cytoplasmic loop in determining substrate affinity. Expression of *AtSUT2* in major veins of source leaves and in flowers is compatible with a role as a second low-affinity sucrose transporter or as a sucrose sensor.

Key words: Sucrose transporter; Sucrose sensor; Protein chimera

1. Introduction

In plants, sucrose transport activity is essential for distribution of photoassimilates between source and sink tissues. Members of the proton-coupled sucrose uptake transporter (SUT) family within the major facilitator superfamily play essential roles in long-distance transport of sucrose within the vascular tissue of plants. In solanaceous plants, high-affinity sucrose transporter SUT1 is required for phloem loading [1–3]. In *Arabidopsis*, eight SUT genes that encode putative sucrose transport proteins are currently known [4]. Three have been characterized functionally: AtSUC2 is a high-affinity transporter involved in phloem loading in major veins [5,6], AtSUT4 is a low-affinity transporter responsible for phloem loading in minor veins [7], and AtSUC1 is responsible for pollen tube growth and anther dehiscence [5,8,9].

One *Arabidopsis* sucrose transporter homolog, AtSUT2, is structurally different from other identified SUT proteins [4,10]. It has extended domains at the N-terminus (about 30 amino acid residues longer) and central cytoplasmic loop (about 40 amino acid residues longer). These structural differences show analogies to extended domains (C-terminus and central loop) of the yeast glucose sensors Rgt2 and Snf3 compared to other members of the glucose transporter family [11,12]. This had led to the hypothesis that SUT2 homologs may function as sucrose sensors in plants [10]. Rgt2 and Snf3 show no substantial glucose transport activity in a yeast complementation assay [13], but the C-terminal extended domain is capable of complementing null-mutants in the respective genes [11]. Thus, a distinct function in signal transduction is assigned to the characteristic C-terminal domains of Rgt2 and Snf3. In a similar fashion, the extended domains of AtSUT2 may also confer characteristic functions in sucrose transport or signaling.

The aim of this study was to elucidate the functional properties of the distinct sucrose transporter homolog AtSUT2. Sucrose transport activity was analyzed by expression of *AtSUT2* cDNA in yeast, and the expression pattern in plants was analyzed by promoter–GUS fusion. To dissect the function of the extended domains, protein chimeras were constructed in which the distinct domains of AtSUT2 (N-terminus and central cytoplasmic loop) were replaced with the respective domains of the well characterized high-affinity sucrose transporter StSUT1 [14,15], and vice versa. AtSUT2 is shown here to be a low-affinity sucrose transporter, which is expressed in major veins of mature leaves as well as in sink organs. The cytoplasmic N-terminus is involved in determining the affinity of SUTs for their substrate sucrose.

2. Materials and methods

2.1. Construction of protein chimeras between AtSUT2 and StSUT1

The coding region of *AtSUT2* was isolated by reverse transcription-PCR from *Arabidopsis thaliana* (Columbia ecotype) leaves and cloned into the yeast expression vector pDR196 as described [10]. The open reading frame of *StSUT1* was amplified from the *StSUT1* cDNA in pDR195 [14], using primers with restriction sites *Sma*I and *Xho*I, and ligated into the yeast expression vector pDR196.

Chimeric constructs, in which the first 58 amino acids of AtSUT2 were exchanged with the respective N-terminal domain of StSUT1 (28 amino acids), and vice versa, were generated by creating restriction sites by PCR within a conserved region in the first transmembrane domain of AtSUT2 and StSUT1. Subsequently, the PCR fragments of the N-terminal region and the rest of the sequence were cloned into the yeast expression vector pDR196 using the sites *Sma*I and *Pst*I for the N-terminal parts and *Pst*I and *Xho*I for the remaining part of the open reading frames. These chimera are referred to as AtSUT2/StSUT1-N and StSUT1/AtSUT2-N, respectively (Fig. 1A).

Chimeric constructs, in which the central loop of AtSUT2 (89 amino acid residues) was exchanged with the smaller central loop of StSUT1 (42 amino acid residues), and vice versa, were generated by creating restriction sites by PCR within conserved regions of transmembrane spans VI and VII. The N-terminal half, the cytoplasmic loop, and the C-terminal half of the coding regions were amplified by PCR, and cloned into pDR196 by first ligating the three fragments using *Sac*I and *Bcl*I/*Bgl*II for AtSUT2/StSUT1-loop, and *Sac*I and *Bam*HI/*Bgl*II for StSUT1/AtSUT2-loop (Fig. 1A). The chimeric

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DNA was then ligated into the yeast expression vector pDR196 using *Sma*I and *Xho*I.

2.2. Functional analysis of AtSUT2 and chimeric proteins

Sucrose uptake assays using the yeast strain SEY6210 [16] carrying the respective cDNAs in the expression vector pDR196 were performed as described [7]. Non-linear regression was performed using the Michaelis–Menten equation. For analysis of pH dependence, cells were washed in water and then placed in sodium phosphate buffer at the required pH. Sugar competition experiments were performed with a 10-fold excess of the competing sugar added 1 min prior to initiating the assay. The inhibitors antimycin A and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were used at final concentrations of 50 μM and 10 μM, respectively, and were added 1 min prior to beginning the assay.

2.3. SDS–polyacrylamide gel electrophoresis and protein gel-blot analysis

Yeast microsomal membranes were prepared as described [10], followed by the addition of 4× sample buffer and incubation at 37°C for 5 min [10] based on the method of Laemmli [17]. Following electrophoresis, proteins were transferred to PVDF membranes. Immunodetection was performed with NBT and BCIP.

2.4. Isolation of the AtSUT2 promoter and expression analysis

The promoter of *AtSUT2* was isolated by PCR on *A. thaliana* Col-0 genomic DNA using Pfu-polymerase (Stratagene) and transcriptionally fused to the GUS gene within the plant binary vector pGPTV-hpt [18] using *Sma*I and *Sal*I [10]. *Arabidopsis* plants were transformed by vacuum infiltration of transformed *Agrobacterium* GV2260 as described [19]. For analysis, plant material was infiltrated under vacuum with 2 mM X-Gluc in phosphate buffer, pH 7.2 with 0.5% Triton X-100, and incubated at 37°C for 12–16 h [20], and destained in 70% ethanol.

3. Results

In contrast to other sucrose transporters (SoSUT1 [21], StSUT1 [14], LeSUT1, and AtSUT4 [7]), the sucrose transporter homolog AtSUT2 did not complement growth of the yeast strain SUSY7/ura3 [10,21]. Therefore, transport activity was characterized directly by ^{14}C-labeled sucrose uptake using yeast cells expressing sucrose transporters and chimeras. This approach is more sensitive, since the K_M of potato sucrose synthase, which cleaves sucrose internally in the strain SUSY7/ura3, is rather high (65 mM [22]). Therefore high sucrose uptake rates are necessary to enable the SUSY7/ura3 strain to grow on sucrose as the sole carbon source.

3.1. Time course of sucrose uptake by AtSUT2 and kinetics

Sucrose uptake by yeast cells expressing *AtSUT2* was linear for the first 5 min of the assay (Fig. 2A), in which 0.1 nmol sucrose was accumulated by 10^8 cells. Sucrose accumulation by AtSUT2 was significantly ($P < 0.05$) higher compared to yeast cells expressing the empty vector pDR196. The transport rate for *StSUT1* expressing cells was 400-fold higher than for AtSUT2 (insert in Fig. 2A). Kinetic studies of sucrose uptake by AtSUT2 revealed a very low affinity for sucrose. Using the Michaelis–Menten equation and non-linear regression analysis, a K_M value of 11.7 ± 1.2 mM could be determined for AtSUT2 at pH 4 (Fig. 2B). In contrast, StSUT1 showed a 10-fold lower K_M value for sucrose at 1.7 ± 0.2 mM (Fig. 3A).

3.2. pH dependence, substrate specificity, and inhibition

Sucrose uptake by AtSUT2 was pH-dependent (Fig. 2C) with highest uptake rates at pH 4. Sucrose uptake decreased rapidly at less acidic pH values and at pH 6 no sucrose uptake activity could be measured. To determine the substrate specificity of AtSUT2, sucrose uptake was competed with other sugars and sugar-alcohols. Among the variety of substrates, only sucrose, and to a lesser extent maltose, was able to significantly compete with [^{14}C]sucrose for uptake (Fig. 2D). In addition, transport of sucrose by AtSUT2 could be inhibited by the protonophore CCCP and by the inhibitor of mitochondrial ATP generation, antimycin A (Fig. 2D). The substrate specificity and response to inhibitors were similar for StSUT1 [14].

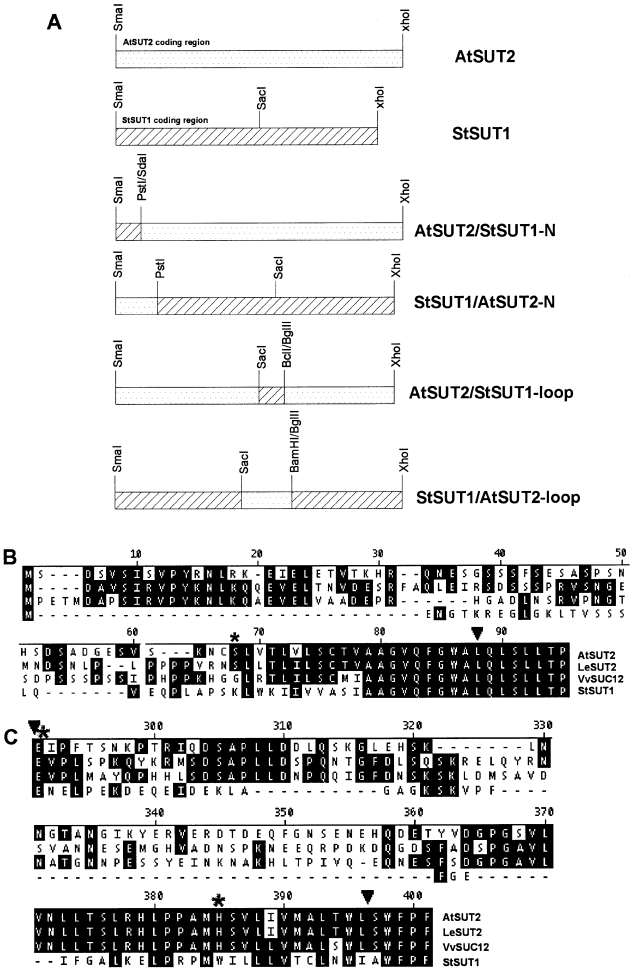

Fig. 1. Description of chimeric constructs. (A) The N-terminal region or central cytoplasmic loop of StSUT1 and AtSUT2 were exchanged. All sequences were cloned into the yeast expression vector pDR196. (B) Alignment of the N-terminal region, and (C) of central loops of members of the SUT2 gene family [10,32] and StSUT1 [14]. Arrows mark the fusion sites for the chimeras, asterisks mark the beginning and end of cytoplasmic domains (N-terminus and loop).

3.3. Sucrose uptake kinetics of sucrose uptake for chimeric proteins

To study the role of the extended domains of AtSUT2, chimeric proteins between AtSUT2 and StSUT1 were analyzed. The chimera AtSUT2/StSUT1-N showed a significantly lower K_M value for sucrose of 3.4 ± 1.6 mM (Fig. 3D) compared to AtSUT2 (Fig. 3B). In contrast, the chimera StSUT1/AtSUT2-N showed a significantly higher K_M value for sucrose of 8.08 ± 1.4 mM (Fig. 3C) compared to StSUT1 (Fig. 3A). Analyses of chimeras in which the central cytoplasmic loops

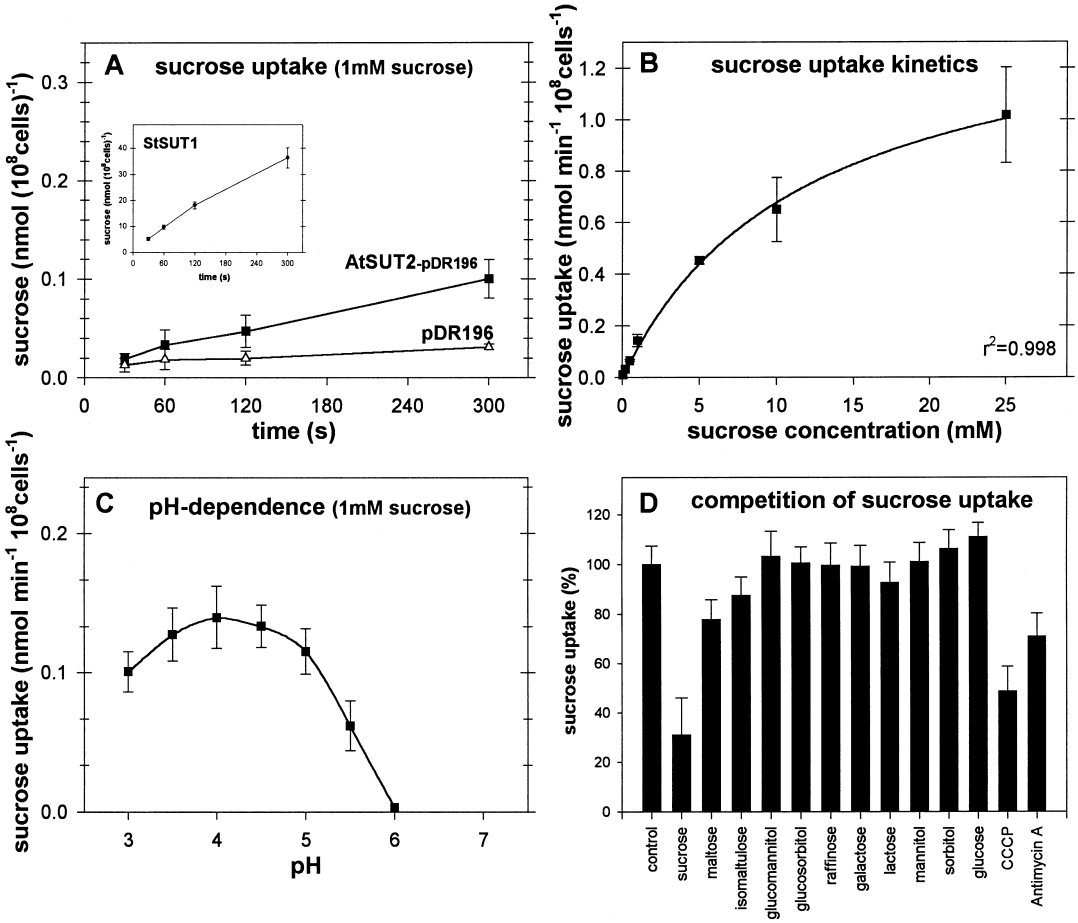

Fig. 2. Kinetics of sucrose uptake by AtSUT2. (A) Time course of [^{14}C]sucrose uptake by yeast cells expressing *AtSUT2*, or the empty vector pDR196. The insert shows sucrose uptake rates for the high-affinity StSUT1, which was used as control. Sucrose uptake was measured at 1 mM sucrose, pH 4. (B) Sucrose uptake rates at different substrate concentrations were measured within the first 5 min of the assay at pH 4. Uptake rates of empty vector pDR196 were subtracted. (C) [^{14}C]Sucrose uptake of AtSUT2 at different pH values, measured at 1 mM sucrose. (D) Inhibition of sucrose uptake by AtSUT2 by different sugars and inhibitors. The competing sugars were added at 10-fold excess. Columns represent the uptake rates in percent of [^{14}C]sucrose uptake without additional sugar. In all figures, values represent the mean ± S.E.M. (N = 3–5 measurements from at least two independent transformants).

were exchanged indicated that the central loop does not contribute to substrate affinity. Thus, AtSUT2/StSUT1-loop (Fig. 3F) had higher K_M values (6.75 mM ± 1.9) for sucrose, whereas the StSUT1/AtSUT2-loop (Fig. 3E) showed a low K_M value (1.4 mM ± 0.3) for sucrose. AtSUT2, as well as the chimeric proteins containing the membrane spanning domains of AtSUT2 (AtSUT2/StSUT1-N and AtSUT2/StSUT1-loop), showed extremely low uptake rates (Fig. 3B,D,E).

The central cytoplasmic loop did not consistently influence sucrose affinity of the chimeric proteins or uptake rates. The presence of the central loop of AtSUT2, when replacing the loop in StSUT1 (Fig. 3E), led to reduced transport rate to 2.5% of the uptake rate for StSUT1 (Fig. 3A). However, the loop of StSUT1 did not, in turn, lead to increased transport rates when exchanged with the loop of AtSUT2 (Fig. 3F).

3.4. Expression levels of chimeric proteins in yeast

The chimeric proteins, as well as AtSUT2 and StSUT1, showed great differences in their K_M values and uptake rates. To relate these differences to potential differences in expression or membrane targeting, protein gel-blots were performed on microsomal fractions of yeast expressing the different proteins. Protein was detected by a primary antibody against the central loop of StSUT1 or against the N-terminus of StSUT1. The chimeric proteins of AtSUT2 and StSUT1 were detected using StSUT1 antibodies. StSUT1 and the chimeric proteins recognized by the antibody against the StSUT1 N-terminus (AtSUT2/StSUT1-N, StSUT1/AtSUT2-loop) were expressed at comparable levels (Fig. 4). However, among the proteins recognized by the antibody against the central loop of StSUT1, expression of AtSUT2/StSUT1-loop was lower compared to the other two proteins. In addition, multiple bands could be detected for those proteins containing the N-terminus of AtSUT2, indicating possible secondary structures or modifications.

3.5. Expression of AtSUT2 in plants

To relate the functional properties of AtSUT2 to possible functions in plants, the expression pattern of *AtSUT2* was analyzed. By RNA blot hybridization, expression of *AtSUT2* was low and present in all tissues tested (data not shown). Promoter–GUS fusions revealed expression in all cells of the shoot of seedlings and young plants (Fig. 5A,B) and roots (Fig. 5C). In older plants, GUS expression in source leaves was highest in major veins and hydathodes of the leaf (Fig. 5D). In the inflorescence stem of flowering plants, no GUS

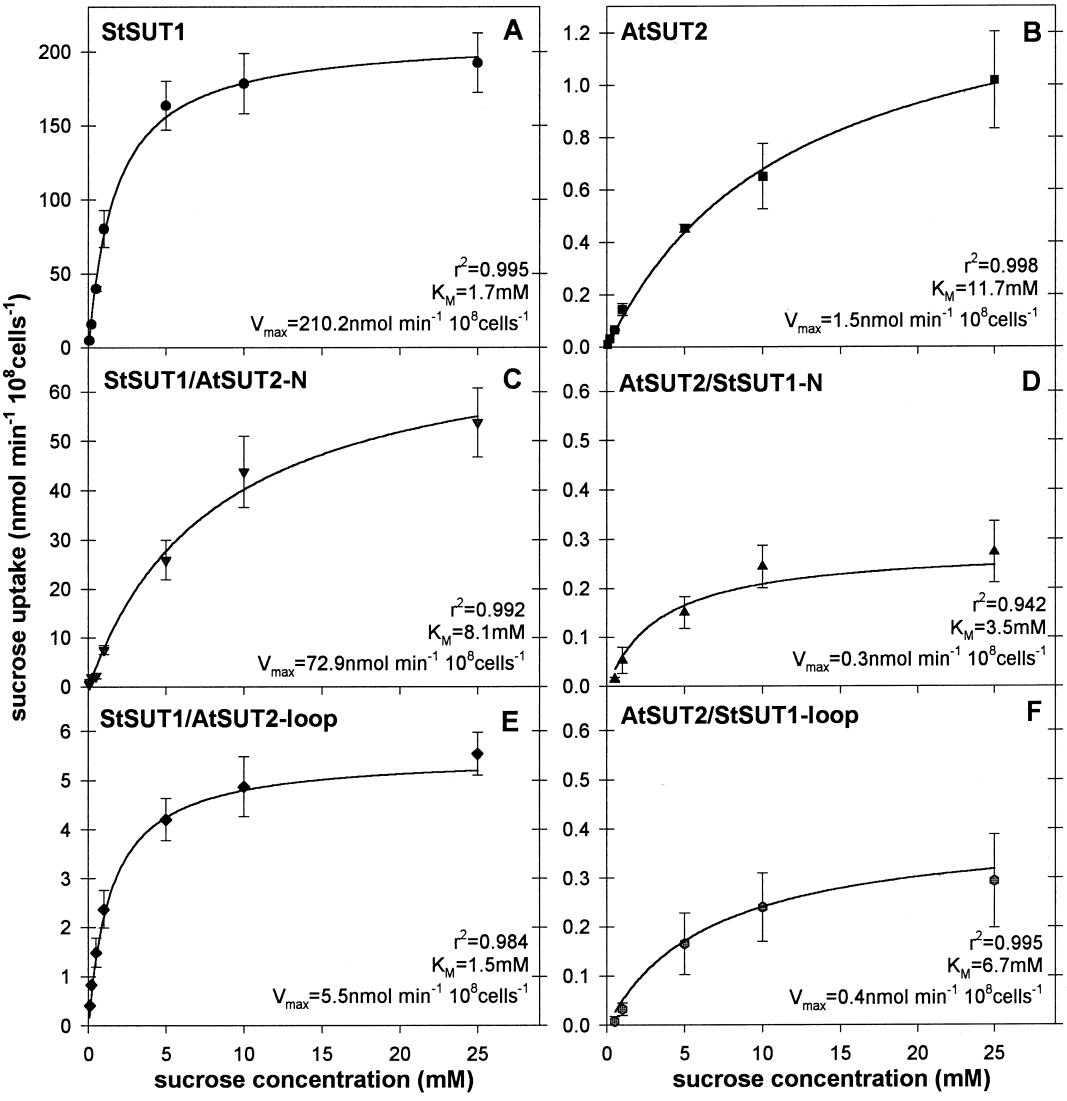

Fig. 3. Sucrose uptake kinetics of the AtSUT2 and StSUT1 chimeric proteins, measured at pH 4. Values are presented as means ± S.E.M. (A) StSUT1, (B) AtSUT2, (C) StSUT1/AtSUT2-N, (D) AtSUT2/StSUT1-N, (E) StSUT1/AtSUT2-loop, (F) AtSUT2/StSUT1-loop.

expression was detected (Fig. 5E). GUS expression was further found in sepals of mature flowers (Fig. 5F), in anthers and in pollen (Fig. 5G). In addition, the *AtSUT2* promoter was active in the stigma of the pistil (Fig. 5F) and later, after pollination, at the peduncle of the developing silique (Fig. 5E).

4. Discussion

Transporters of the SUT family catalyze the H^+-coupled uptake of sucrose and maltose but not other physiological sugars and exhibit a range of affinities for sucrose. This allows plants to differentially express specific SUTs depending on uptake requirements and extracellular sucrose concentrations. Several high-affinity SUTs have been functionally characterized including StSUT1 [14], AtSUC2 [5], and AtSUC1 [6,9]. The SUT4 subfamily represents low-affinity uptake transporters [7]. However, of all sucrose transporters characterized to date, AtSUT2 has the lowest affinity for sucrose (Fig. 2).

The structural basis for the differences in substrate affinity between different SUTs has not been investigated previously. In *Chlorella* glucose transporters HUP1 and HUP2, the first external loop was demonstrated to confer substrate specificity through a single acidic amino acid residue [23]. Replacing a conserved histidine within the first extracellular loop of AtSUC1 with basic amino acids lowers the affinity for sucrose [24]. However, all SUT homologs from plants contain a histidine at this position. Therefore the first extracellular loop is likely to have a role in substrate affinity, but the differences in sucrose affinity of different SUTs must have another structural basis. AtSUT2 contains two domains that are larger compared to other SUTs, which may have functions in controlling affinity and transport rate.

4.1. A role for the N-terminus in substrate affinity

Expression of chimeras in yeast allowed a detailed kinetic analysis of sucrose uptake. Replacing the N-terminus of the high-affinity transporter StSUT1 with that of the low-affinity AtSUT2 resulted in an increase in K_M from 1.7 mM to 8.1 mM ($P < 0.01$, Fig. 3C). Similarly, the StSUT1 N-terminus conferred high affinity on AtSUT2, resulting in a decrease in K_M from 11.7 mM to 3.4 mM ($P < 0.05$, Fig. 3D). There-

fore structural differences in the N-terminus between StSUT1 and AtSUT2 appear to contribute to a high proportion of the differences in substrate affinity.

Based on analysis of PmSUC2 [25], the N-terminus of both StSUT1 and AtSUT2 is predicted to be cytosolic. Therefore, it is unlikely that the N-terminus contributes to substrate affinity by binding sucrose directly. It is more likely that the N-terminus influences affinity through intramolecular interactions with other cytosolic domains. For potassium channels, the N-terminus also controls the flux of ions through the pore [26]. In this case, only transport rate and not affinity is influenced by the N-terminus. Structural analysis [27,28] of members of the SUT2 subfamily has revealed helical regions in the first 15 amino acid residues of the N-terminus, which were not present in StSUT1 or other SUTs. These regions may be important for determining substrate affinity.

4.2. Analysis of central loop chimeras

Exchange of the central loops between StSUT1 and AtSUT2 indicated that the central loop has no significant function in controlling substrate affinity. In lac permease from *Escherichia coli*, another member of the MFS, expression of the protein in two separate halves, devoid of a central loop, led to transport activities similar to wild-type [29]. This indicates that the central loop is not required for transport activity. However, in the intact lac permease, the central loop must be of sufficient length and hydrophilicity for efficient insertion of the protein into the membrane and transport activity [29]. The central loop of StSUT1 is shorter than that of AtSUT2 and both are highly hydrophilic (Fig. 1C). Replacement of the StSUT1-loop with that of AtSUT2 led to a dramatic decrease in V_{max} from 210.2 to 1.5 nmol sucrose/min/10^8 cells. By Western blot it appears that the expression level of StSUT1 is similar to that of StSUT1/AtSUT2-loop. Therefore, the decrease in V_{max} could be due to inhibition of the transporter by the AtSUT2-loop, or failure of the chimera to be targeted to the plasma membrane efficiently.

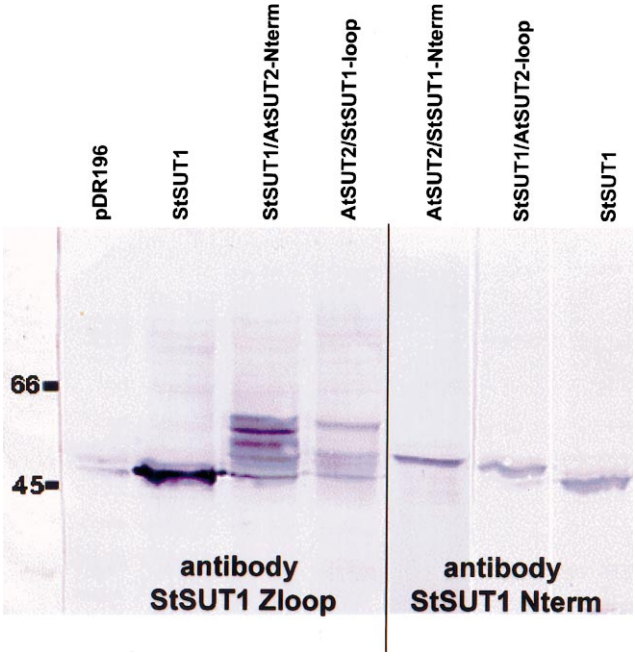

Fig. 4. Protein gel-blots of microsome fractions of yeast expressing the different protein chimeras. Proteins were detected using a primary antibody against the StSUT1 central loop or against the StSUT1 N-terminus and an alkaline phosphatase-coupled secondary antibody.

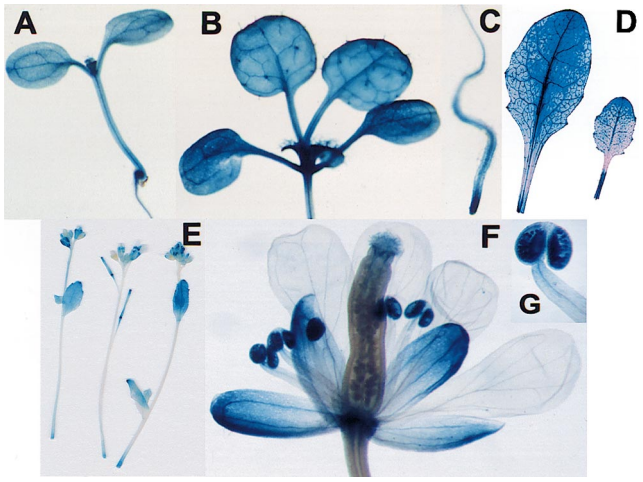

Fig. 5. Histochemical detection of 1.2 kb AtSUT2P–GUS expression in *Arabidopsis*. (A) Seedling, 8 days old. (B) Young plant, 18 days old. (C) Root of young plant. (D) Expanded source leaf of older plant. (E) Inflorescence with developing siliques. (F) Single mature flower. (G) Magnification of an anther from image (F).

AtSUT2 transport activity when expressed in yeast has a very low V_{max} compared to StSUT1. One hypothesis is that this low activity is due to the central loop. However, the reciprocal experiment, replacement of the AtSUT2-loop with that of StSUT1 (Fig. 3F), does not support an auto-inhibitory role for AtSUT2 loop. The AtSUT2/StSUT1-loop construct resulted in a V_{max} even lower than that of wild-type AtSUT2, which may be explained by a lower number of transporters in the plasma membrane as a result of incorrect targeting.

4.3. Is AtSUT2 a flux sensor?

AtSUT2 has been hypothesized to function as a sucrose sensor. This hypothesis is based on its distinct extended domains which show analogies to the extended domains of yeast glucose sensors Snf3 and Rgt2 in yeast [10]. In addition, *AtSUT2* shows poor similarity to the consensus for high rates of translational initiation [30]. Also the non-functionality of SUT2 in yeast complementation supported the 'sensor' hypothesis, since the glucose sensors Snf3 and Rgt2 also cannot complement growth of a deletion mutant in all members of the hexose transporter family [13].

AtSUT2 in this study was shown to transport sucrose, even though transport rates are extremely low. This does not contradict the 'sensor' hypothesis. SUT2 may function as a flux sensor, which measures sucrose through conformational changes during transport instead of substrate binding. The non-functionality in transport of Rgt2 and Snf3 does not contradict this theory, since the yeast sensors may have low transport activity not detected by the complementation assay, whereas low transport rates of AtSUT2 could only be detected by direct measurement of sucrose uptake. In analogy to Snf3, the low affinity of AtSUT2 would then make it a low-affinity sensor. The advantage of a flux sensor is that by measuring the transport rate, information concerning the transmembrane gradients, as well as concentration of sucrose and H^+ are detected.

4.4. Is AtSUT2 a second low-affinity transporter?

AtSUT2 functions as a sucrose transporter with low affinity and low transport rates. A proton-coupled mechanism of AtSUT2 may be concluded from the inhibitor studies (Fig. 2C). In that respect, AtSUT2 is not different from the other members of the plant sucrose transporter family [5,7,14]. However, in contrast to the previously characterized SUTs, the transport rates and affinities measured with AtSUT2 were the lowest reported so far for a plant sucrose transporter. The K_M of 11.7 mM for AtSUT2 at pH 4.0 was about 2-fold higher than reported for the low-affinity sucrose transporter AtSUT4 [7]. The expression pattern of *AtSUT2*, however, is very different from that of *AtSUT4*. Thus a role as a second low-affinity transporter in major veins of source leaves is suggested by the complementary expression pattern of *AtSUT2* compared to *AtSUT4*. Moreover, a role of AtSUT2 in sucrose partitioning to sink organs becomes obvious from its high expression in sepals, anthers, the pistil of flowers, and young siliques.

In conclusion, concerning the function of the extended domains of AtSUT2, the N-terminal region may contribute to substrate affinity. However, no clear function in transport kinetics could yet be assigned to the central cytoplasmic loop. Potentially the conserved regions CCB1 and CCB2 [10] could function in a signal transduction pathway or interactions with other proteins. In yeast, the conserved regions in the C-terminus were shown to interact with proteins from the signal transduction cascade [31]. Further experiments involving the analysis of transgenic plants are necessary to clarify the function of the distinct low-affinity sucrose transporter AtSUT2 in plants.

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