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Interactions between co-expressed *Arabidopsis* sucrose transporters in the split-ubiquitin system

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Abstract

Background: The *Arabidopsis* genome contains nine sucrose transporter paralogs falling into three clades: SUT1-like, SUT2 and SUT4. The carriers differ in their kinetic properties. Many transport proteins are known to exist as oligomers. The yeast-based split ubiquitin system can be used to analyze the ability of membrane proteins to interact.

Results: Promoter-GUS fusions were used to analyze the cellular expression of the three transporter genes in transgenic *Arabidopsis* plants. All three fusion genes are co-expressed in companion cells. Protein-protein interactions between *Arabidopsis* sucrose transporters were tested using the split ubiquitin system. Three paralogous sucrose transporters are capable of interacting as either homo- or heteromers. The interactions are specific, since a potassium channel and a glucose transporter did not show interaction with sucrose transporters. Also the biosynthetic and metabolizing enzymes, sucrose phosphate phosphatase and sucrose synthase, which were found to be at least in part bound to the plasma membrane, did not specifically interact with sucrose transporters.

Conclusions: The split-ubiquitin system provides a powerful tool to detect potential interactions between plant membrane proteins by heterologous expression in yeast, and can be used to screen for interactions with membrane proteins as baits. Like other membrane proteins, the *Arabidopsis* sucrose transporters are able to form oligomers. The biochemical approaches are required to confirm the *in planta* interaction.

Background

Sucrose transport activity is essential for distribution of photoassimilates between photosynthetic tissues and tissues of high demand, including growing regions, storage compartments, and non-photosynthetic organs. Trans-

port of sucrose from the apoplast across the plasma membrane into sieve-elements or companion cells is mediated by sucrose transporters belonging to the major facilitator superfamily of membrane proteins. Members of the sucrose transporter family are characterized by twelve

transmembrane spanning domains with cytosolic N- and C-termini [1] and a large central hydrophilic loop.

Arabidopsis contains nine sucrose transporter homologs (SUTs or SUCs) [2], several of which have been functionally characterized. AtSUC2 functions as a high affinity transporter expressed in companion cells [3–5]. The essential role of SUC2 in long distance transport was recently demonstrated by analysis of T-DNA insertional mutants into the *AtSUC2* gene [6]. Export of sucrose from leaves was severely impaired even in heterozygous plants. In contrast, AtSUC1, another high-affinity sucrose transporter closely related to SUC2 [3,7] seems to be involved in pollen tube growth and anther dehiscence [8]. AtSUC5, which is 80% identical (amino acids) to AtSUC1, was isolated as a biotin transporter [9], but also transports sucrose with high affinity. In contrast, AtSUT4 serves as a low affinity transporter and is potentially involved in phloem loading in minor veins [10]. SUT2 was characterized as weak low affinity transporter, and based on circumstantial evidence it has been speculated that SUT2 may be a candidate for a postulated sucrose sensor fulfilling similar functions as the yeast sugar sensors SNF3 and RGT2 [11]. The other *Arabidopsis* genes are highly related to SUC1 and SUC2, indicating recent gene amplification events, but function of these genes is still not defined.

Rapid changes in sucrose transport activity together with coexistence of sucrose transport systems differing in their kinetic properties indicate that uptake and distribution of sucrose according to supply and demand within the plant is highly regulated. One efficient means of regulation of transport activity can also occur through oligomerization, as has been described for the animal glucose transporter GLUT1 [12,13], which constitutes a functional carrier regulated within a tetrameric complex. It would thus be interesting to understand the quaternary structure of sucrose transporters in *Arabidopsis*.

Analysis of mechanisms involved in regulation of transport processes across the plasma membrane is difficult, due to difficult accessibility of plasma membrane proteins to biochemical assays. Moreover, classical two-hybrid studies provide limited means of analysis, since most systems require targeting of the interacting proteins to the nucleus for reporter gene expression. The split ubiquitin system provides a new and powerful two-hybrid tool allowing the analysis of protein-protein interactions between membrane proteins [14–17]. The split-ubiquitin system had so far been used mainly to probe interactions of yeast proteins constituting the Sec62 ER protein translocation complex at the ER [14,18–21]. However, little is known about the use of the split-ubiquitin system for detection of protein-protein interactions between heterologously expressed proteins. Two separately expressed parts

of sucrose transporters were shown to reconstitute a functional sucrose transporter at the yeast plasma membrane [23], indicating that the split-ubiquitin system is suitable to study protein-protein interactions between different sucrose transporters.

The aim of this work was to analyze interactions between sucrose transporters and other integral membrane proteins, and membrane-associated soluble proteins. It could be shown that *Arabidopsis* sucrose transporters are co-expressed in companion cells and interact with themselves and with each other, while no interaction was observed with a variety of other plasma membrane proteins, soluble proteins of sucrose metabolism, or proteins of the endomembrane system, demonstrating that the split-ubiquitin system provides a powerful tool to detect specific interactions among plant membrane proteins by heterologous expression in yeast.

Results

Co-expression of *Arabidopsis* sucrose transporters in companion cells

Promoter-GUS fusions were used to determine the expression pattern of the three sucrose transporters histochemically. The expression pattern of the high affinity *AtSUC2* [4] and the low affinity *AtSUT4* [10], and the putative sucrose sensor *AtSUT2* [11] overlaps in minor veins of leaves of mature plants (Fig. 1A). *AtSUC2::GUS* was expressed in companion cells, consistent with previous immunolocalization studies [5]. This pattern coincides with weak expression of *AtSUT2::GUS* and strong expression of *AtSUT4::GUS* in the same cell type (Fig. 1B). Although differences in expression pattern of the three genes become apparent during the development of the plant, the clear overlap of expression of three transporters in the same cell type may have significant function during specific stages of development. However, it cannot be excluded, that the RNA expression pattern as derived from promoter-reporter studies may not entirely reflect the in-vivo protein localization pattern. The expression of SUT2 in companion cells is in disagreement with immunolocalization data in *Arabidopsis* [24].

Generation of split-ubiquitin fusion constructs

To study the interaction between the co-expressed *Arabidopsis* sucrose transporters, two transporters, i.e. SUT2 and SUC2, were fused with the C-terminal half of ubiquitin and the artificial transcription factor PLV (Fig. 2) as bait constructs. SUT2, SUC2, and SUT4 were fused with a mutated form of the N-terminal half of ubiquitin, NubG (Fig. 2) as prey constructs. A series of NubG-fusion proteins including membrane proteins of different compartments such as a the plasma membrane potassium channel AtKAT1 [25], the plasma membrane monosaccharide transporter AtSTP1 [26], a SEC12 homolog (AtStl2p) as

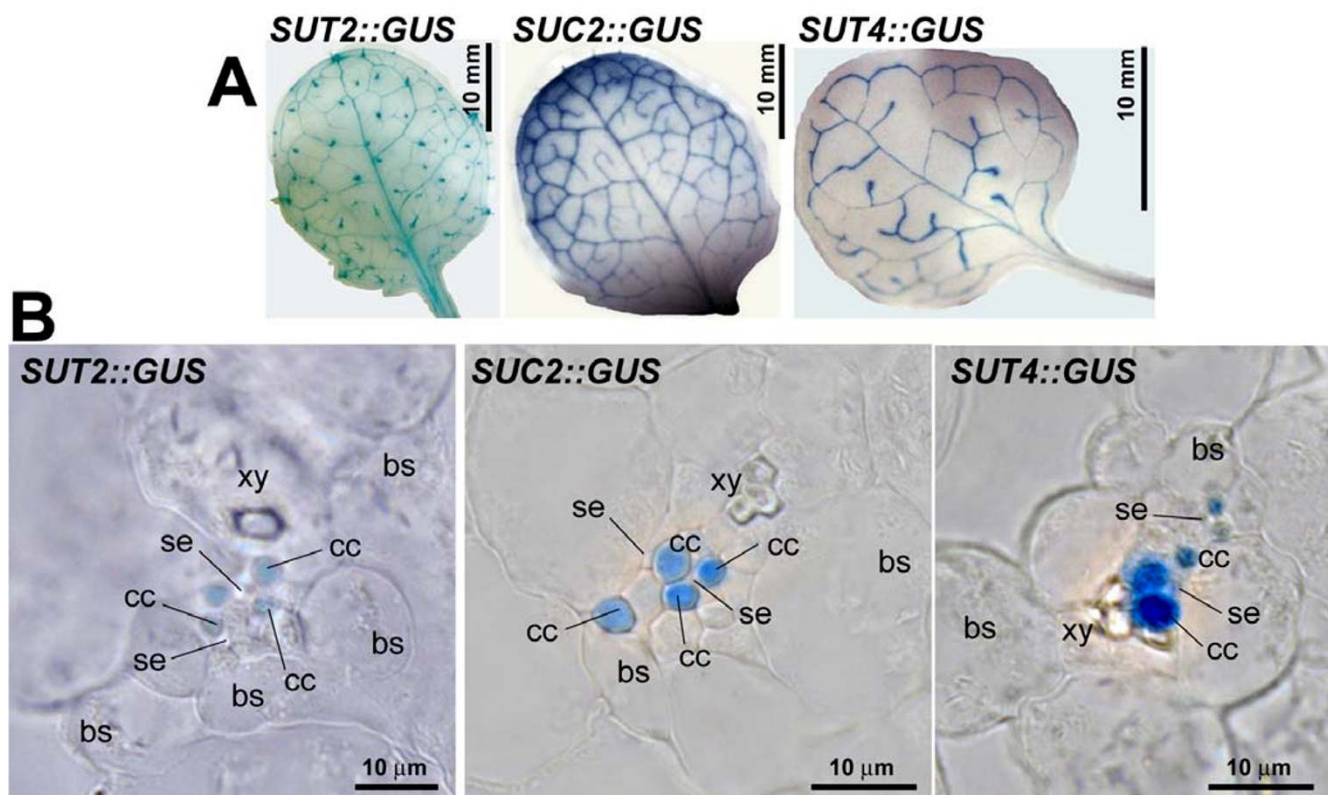


Figure 1
Overlapping expression of sucrose transporters in *Arabidopsis*. **(A)** GUS-expression mediated by promoter fragments of *AtSUC2*, *AtSUT2*, and *AtSUT4* in leaves of *Arabidopsis*. **(B)** Cellular localization of GUS expression in minor veins of leaves embedded in LRwhite on 5 μm sections.

an ER membrane protein [27], and orthologs of the soluble, but also partially membrane-bound sucrose metabolizing enzymes sucrose phosphate phosphatase [28] and sucrose synthase [29], was used to probe the specificity of the interactions and possibly detect interactions between sucrose transporters and enzymes of sucrose metabolism (Fig. 2).

Split-ubiquitin analysis of sucrose transporter interactions

The bait constructs were stably integrated into the genome of the reporter yeast strain. The transformed bait strains were subsequently transformed with the prey constructs. Both *AtSUT2* as well as *AtSUC2* were able to interact with themselves and with NubG-fusions of sucrose transporters *AtSUT2*, *AtSUC2*, and *AtSUT4*, but not with soluble NubG alone (Fig. 3). The results indicate that the three proteins can form oligomers. Interactions between sucrose transporters were confirmed using growth of the auxotrophic strain in absence of histidine. On control plates with histidine, yeast cells bearing the bait constructs *AtSUC2*-CubPLV or *AtSUT2*-CubPLV in combination

with sucrose transporter prey constructs grow equally well as cells bearing bait constructs in combination with NubG only (Fig. 4A). In contrast, on plates without histidine, growth of yeast cells bearing interacting sucrose transporters is significantly stronger compared to cells bearing bait proteins in combination with NubG only (Fig. 4B). Thus, both markers detecting cleavage of the artificial transcription factor from the CubPLV upon protein-protein interaction can be used to study interactions between two membrane proteins.

To confirm the targeting of the NubG-fusion proteins to the plasma membrane and to show their functionality, NubG-*AtSUC2* and NubG-*AtSUT4* were tested for their ability to complement growth of the yeast strain *SUSY7/ura3* on sucrose [11,30]. In both cases, the N-terminal fusion of NubG to the sucrose transporters did not impair their transport function, as demonstrated by the growth of *SUSY7/ura3* containing the respective fusion proteins on sucrose as the sole carbon source (Fig. 5). Kinetic analysis (data not shown) revealed no significant difference

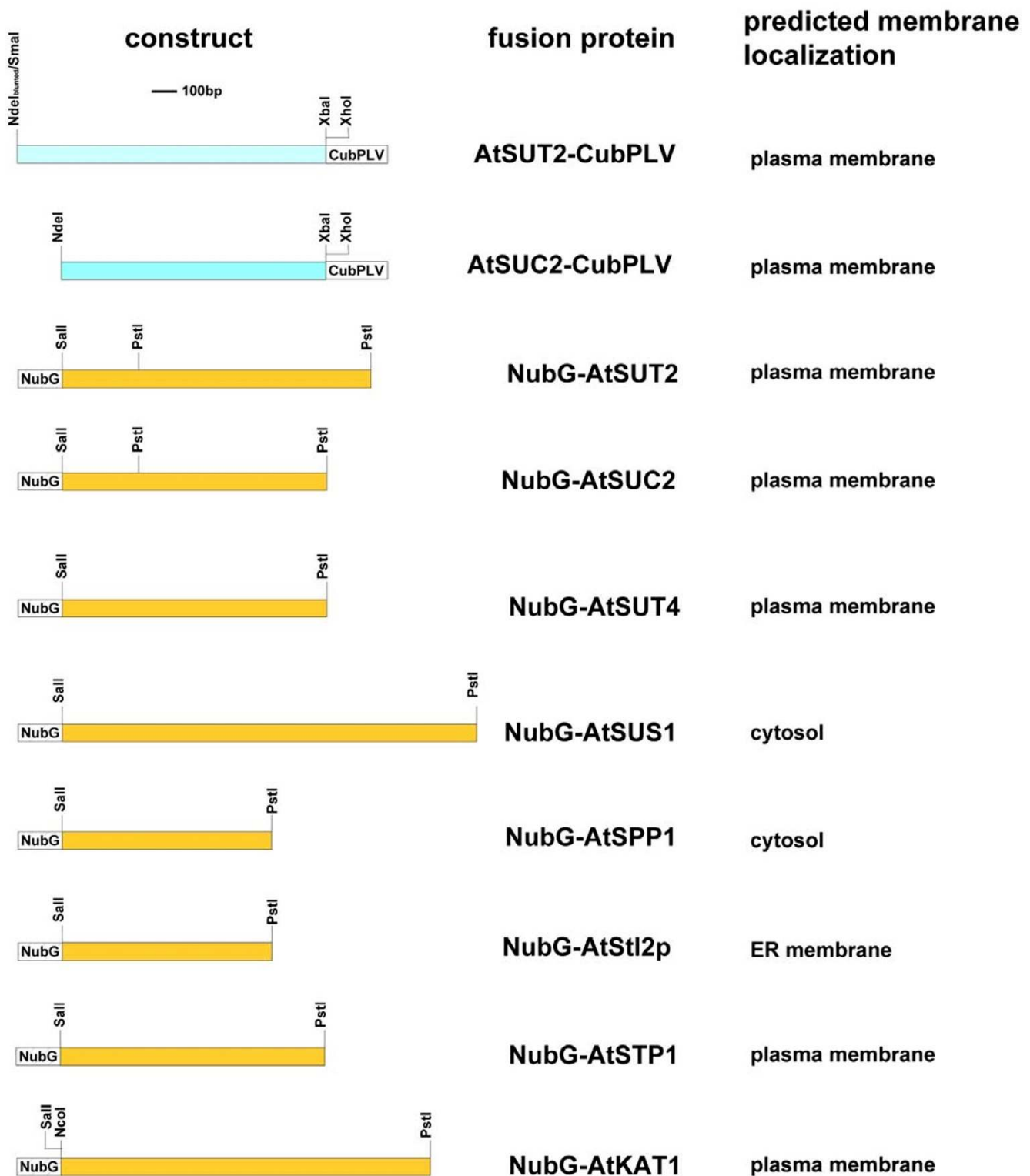


Figure 2
 Constructs for split ubiquitin system. Two sucrose transporters (AtSUT2, AtSUC2) were cloned as CubPLV-fusion proteins, sucrose transporters (AtSUT2, AtSUC2, AtSUT4) and a variety of membrane proteins (AtSTP1, AtKAT1, AtStI2p), as well as cytosolic proteins (AtSUS1, AtSPP1) were cloned as NubG-fusion proteins. The predicted membrane location is indicated for each protein.

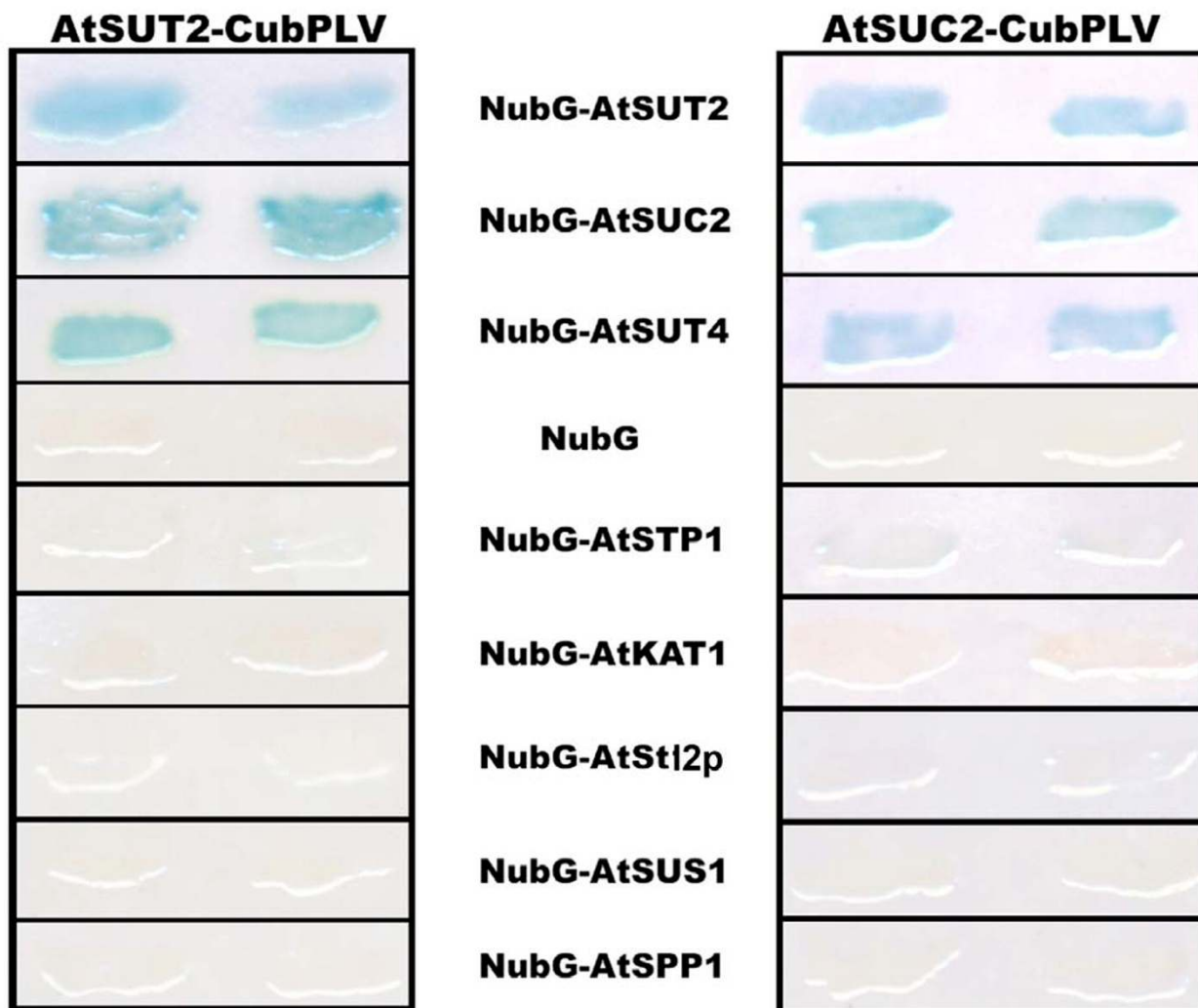


Figure 3
 Interactions of SUT2 and SUC2 as baits with other sucrose transporters detected by the split-ubiquitin system using *LacZ* as a reporter gene. Interaction of SUT2-CubPLV and SUC2-CubPLV as a bait was tested against various NubG-fusions of membrane proteins and soluble proteins as prey. Positive interaction was visualized by β -galactosidase expression in filter assays.

between sucrose affinity of NubG-fusion proteins and published sucrose affinities [3,10].

To exclude non-specific interactions due to random collisions in the plasma membrane, interactions were tested between control baits. No interactions were observed with the glucose transporter AtSTP1 and the potassium channel AtKAT1 [23,31], demonstrating specificity of the interaction between the three different sucrose transporters (Fig. 3,4). Furthermore, no interaction was found between

AtSUT2 and AtSUC2 and the ER-protein AtStl2p excluding non-specific interactions within the endomembrane system during plasma membrane targeting (Fig. 3). The results further support the potential of the *Arabidopsis* sucrose transporters to form homo- or hetero-oligomers.

Since soluble enzymes involved in sucrose metabolism, such as sucrose synthase and sucrose-6-phosphate phosphatase were found to be membrane-associated [32,33], sucrose may also be channeled directly from transport to

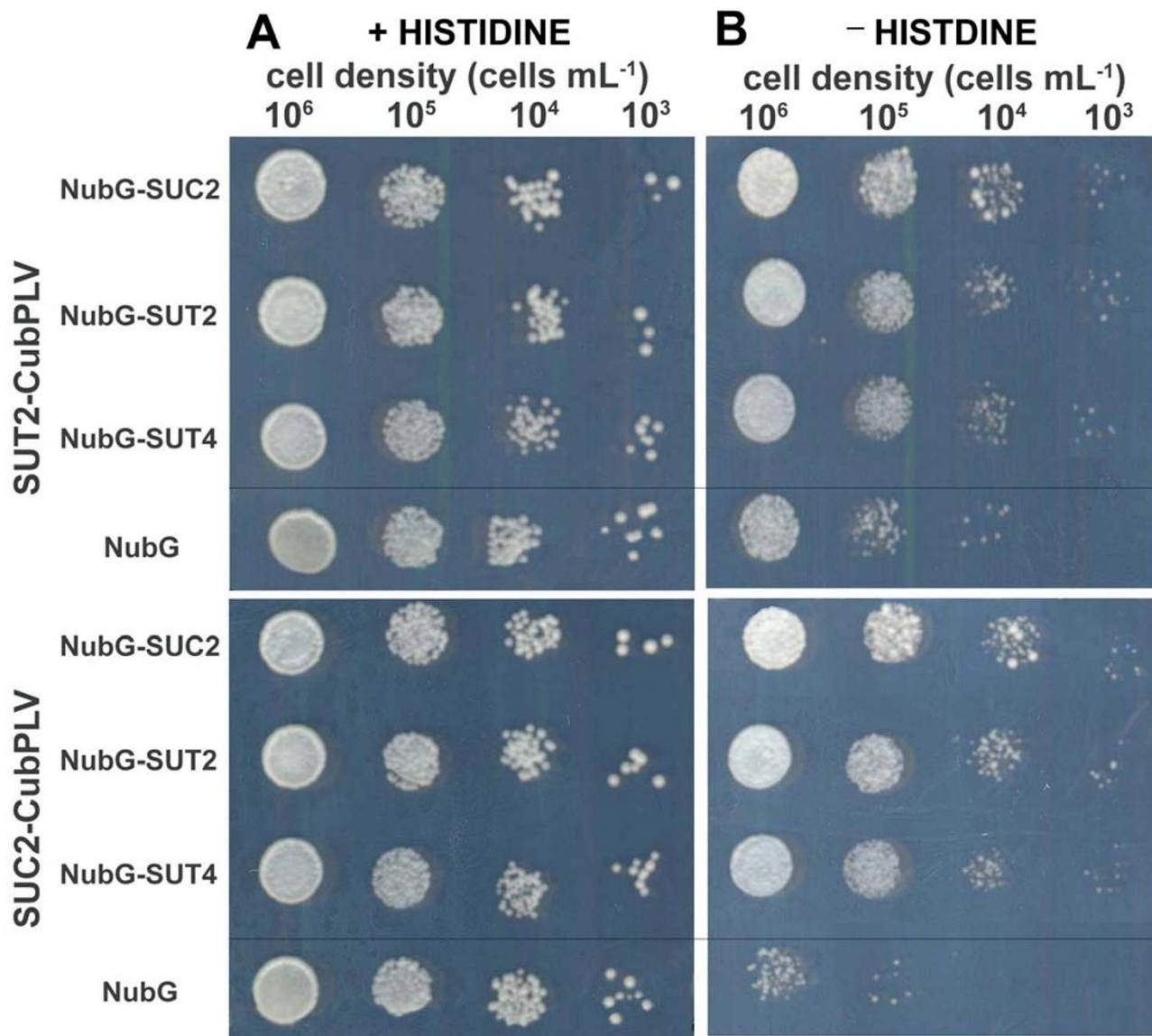
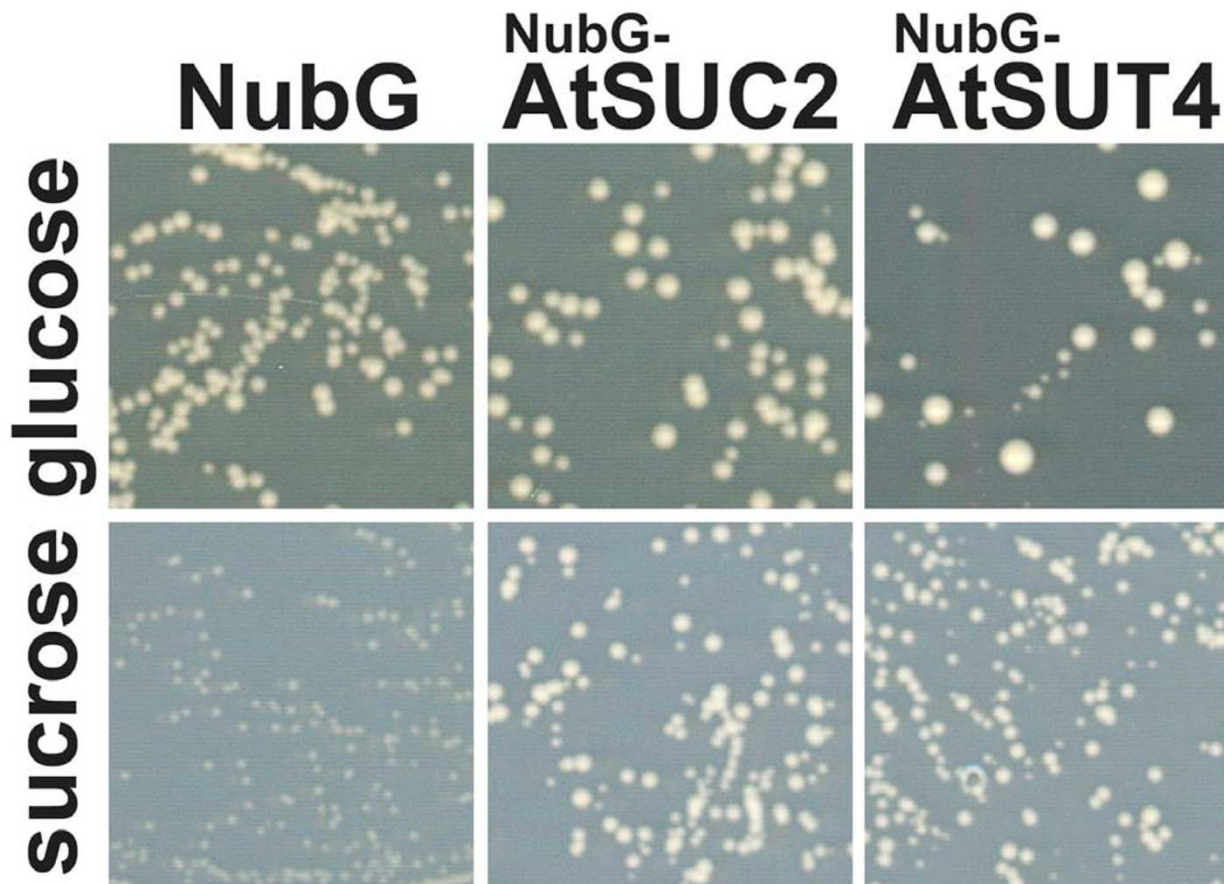


Figure 4

Protein-protein interactions determined by the split ubiquitin system using *HIS3* as reporter gene. **(A)** Growth of yeast cells on control plates with histidine. **(B)** Growth of yeast cells on plates without histidine demonstrating interaction of AtSUT2 and AtSUC2 as baits with other sucrose transporters as prey, and no growth of cells expressing bait constructs and NubG. Cells were spotted on SD medium with adenine and with or without histidine in 10 μ L at cell densities indicated.

metabolism by an interaction of sucrose transporters with key enzymes of sucrose metabolism, similarly to glucose in tight associations of hexokinase with glucose transport [34–36]. Therefore sucrose synthase and sucrose phosphate phosphatase were used as NubG fusions as prey and tested against the different sucrose transporter baits (Fig.

3). No interaction was found between sucrose transporters AtSUT2 or AtSUC2 and sucrose synthase (AtSUS1) or sucrose-6-phosphate phosphatase (AtSPP1).

**Figure 5**

Functionality of NubG-AtSUC2 and NubG-AtSUT4 fusion proteins. Growth of yeast strain SUSY7/ura3 bearing respective fusion proteins or the empty NubG-vector on medium containing 2% glucose or 2% sucrose as the sole carbon source after four days of growth at 28°C.

Discussion

The potential role of homomerization of sucrose transporters

Interactions detected between sucrose transporters provide first indications that sucrose transporters may exist as homodimers and possibly also as heterodimers or even higher order oligomeric structures. Dimerization may not be necessary for function *per se*, as concluded from studies on lactose permease, which is functional also as a monomer [37]. However, dimerization may contribute to protein stability or may have a role in targeting to the plasma membrane. For glucose transporters, it is proposed that native structure is established before translocation of the transporters to the plasma membrane [38]. Dimerization and tetramerization of mammalian glucose transporters was shown to be important for regulation of transport

properties. Binding of substrate to one transport site changes the substrate affinity of the interacting partner in a cooperative manner [13]. This dynamic change in transport capacity is highly relevant for glucose homeostasis in mammalian cells. In addition, regulation of glucose transport is modulated by oligomerization, since tetrameric but not dimeric GLUT1 is subject to direct regulation by cytosolic ATP [39]. In addition, the stabilization of tetrameric states by disulfide bridges between subunits is dependent on the redox-status of the cellular environment [12,38]. Oligomerization is also required for proton co-transport of the bacterial lactose transporter LacS [40], thus the function as homomer may be valid for transporters in general. Sucrose transport is a highly regulated process, which needs to be highly dynamic over a wide range of conditions. Therefore, dynamic regulation of sucrose

transport through formation or stabilization of homo-oligomers may allow fast adaptation of transport to changing conditions.

The potential role of heteromeric sucrose transporter complexes

The co-expressed sucrose transporters AtSUC2, AtSUT2 and AtSUT4 showed the potential of heteromeric interactions. So far, heteromeric associations were not observed for mammalian glucose transporters [41]. However, identification of a larger number of paralogues suggests also the possibility that GLUTs can form hetero-oligomers in certain cell types. The dramatic dominant negative effect observed in a yeast mutant GSF4-1 on overall glucose transport activity, due to a fusion event between HXT1 and HXT4, may be explained by the formation of inactive complexes suppressing activity of many other members of the yeast HXT family. This provides indirect evidence that also in yeast related transporters form hetero-oligomers [42].

The formation of heteromeric structures allows even more dynamic regulation of sucrose transport, especially if heteromers consist of subunits with different substrate affinities, as for example the high affinity AtSUC2 and the low affinity AtSUT4. Considering the hypothesized function of AtSUT2 as a sucrose sensor [11], one can envisage a model in which a putative sucrose sensor can directly modulate the transport activity of an interacting sucrose transporter through cooperative effects. It became obvious from analyses in yeast, that certain micro-environments of the plasma membrane, the so called lipid rafts, can change in composition due to intra- or extracellular stimuli [43], which in turn may favor certain protein-protein interactions resulting in the activation of signaling cascades or transport processes. The typical property of lipid rafts is their stability in the detergent Triton X-100. Thus, a more detailed analysis of the *in planta* biochemistry of interacting sucrose transporters is necessary involving the analysis of Triton X-100 extraction to detect such higher order homo- or heteromeric complexes.

Although co-expression of the three transporters in companion cells of *Arabidopsis* suggests a tight regulatory interaction between these transporters, the interaction between sucrose transporters detected by the split-ubiquitin system has to be validated *in planta*, before a full understanding of sucrose transporter regulation is possible. Given the potential of sucrose transporters to form homo- and heteromers, the observed overlapping but also distinct expression pattern of the three different sucrose transporters may reflect different regions of phloem function.

Conclusions

In conclusion, this work demonstrates that protein-protein interactions among plasma membrane proteins can be detected using the split ubiquitin system. Using a variety of membrane proteins as well as cytosolic proteins as controls, the interactions between plant sucrose transporters were shown to be specific for the family of *Arabidopsis* sucrose transporters, suggesting a general function of MFS transport proteins in higher order complexes similar to mammalian glucose transporters [13]. The split-ubiquitin system is a valuable tool to characterize these interactions in specific and quantitative manner. Moreover, the split-ubiquitin system may serve as tool to screen for interactions with membrane proteins as bait in order to identify interactions with unknown partners.

Methods

Split-ubiquitin constructs

A modified split-ubiquitin system [14] was used to investigate protein-protein interactions between AtSUT2, or AtSUC2 as baits, and other *Arabidopsis* proteins as prey in the yeast strain L40 (*MATa ade3 trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*). Interactions between membrane proteins was monitored by interaction of two membrane bound fusion proteins leading to release of an artificial transcription factor consisting of Protein A, LexA, and VP16 (PLV) and inducing transcription of reporter genes (*LacZ*, *HIS3*). The bait cDNAs were cloned in frame with the C-terminal (Cub) subdomain of ubiquitin followed by PLV. Prey cDNAs were fused to the N-terminal (NubG) subdomain of ubiquitin. Fusion vectors have been described [23] and can be obtained from Igor Stagliar, Institute of Veterinary Biochemistry, University of Zürich. Interaction between "bait" and "prey" fusion proteins leads to cleavage of PLV by ubiquitin-specific proteases, and subsequent activation of reporter genes. The integrative bait vector pY-CubPLV contains the wheat germ agglutinin-binding protein *WBP1*-promoter, allowing constitutive, but low expression of bait fusion protein. *AtSUT2* (At2g02860) and *AtSUC2* (At1g22710) were cloned as fusion proteins with Cub using restriction sites *NdeI* and *XhoI* (Fig. 1). After linearization with *ClaI*, bait-constructs were integrated into *LEU2* of strain L40 using lithium-acetate based transformation [44]. *AtSUT2*, *AtSUC2*, *AtSUT4* (At1g09960), *AtSTP1* (At1g11260), *AtSPP1* (sucrose-phosphate phosphatase; At2g35840), and *AtSUS1* (sucrose synthase; At3g43190), and the ER-protein AtSt12p (At5g50550) were amplified by RT-PCR, sequenced and cloned as NubG-fusion proteins under control of the *ADH1*-promoter using the restriction sites *Sall* and *PstI*, or *NcoI*, *PstI* for NubG-fusion with *AtKAT1* (At5g46240) (Fig. 1).

Yeast complementation

Functionality of the sucrose transporter-NubG-fusions was tested using yeast strain SUSY7/ura3 [11,30] transformed with plasmids bearing respective fusion constructs. To test for growth on sucrose, three colonies from each transformation were resuspended in 5 mL water and 50 µL aliquots were plated both on yeast minimal medium supplemented with tryptophan and either glucose (2%), or sucrose (2%, pH 4). Plates were incubated for 3 (glucose) and 6 days (sucrose) at 28°C. Sucrose was obtained from Carl Roth, Karlsruhe, Germany)

β-galactosidase assay

β-galactosidase activity was determined by filter assays [45]. Cells were streaked on reinforced nitrocellulose membranes, placed on SD agar plates containing adenine and histidine and grown for two days. Filters were removed from the agar, frozen in liquid nitrogen, placed on filter paper saturated with Z-buffer (60 mM Na₂PO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄ pH 7.0) containing 35 mM β-mercaptoethanol and 1.5 mg mL⁻¹ 5-bromo-4-chloro-3-indolyl-3-D-galactoside and incubated at 37°C for two hours.

Promoter GUS analysis

A 2.1 kb fragment of the AtSUC2 promoter was cloned into pGPTV-hpt [46] using *SalI* and *SmaI*. *AtSUT2::GUS* [11], *AtSUT4::GUS* [10], and *AtSUC2::GUS* expression was analyzed in mature plants grown in soil and seedlings grown on 6% sucrose in the dark as described [11] in presence of 3 mM K-ferricyanide and 0.5 mM K-ferrocyanide at pH 7. Leaves of 15-day old plants were fixed in 4% glutaraldehyde, dehydrated in ethanol and embedded in LRWhite and 5 µm sections were prepared [31]. For each gene, several plants and leaves of different age were analyzed, representative photographs are shown.

Authors' contributions

WS and AR carried out the molecular genetic studies, SL participated in the design of the study. JMW and WBF conceived of the study, and participated in its design and coordination. WS and WBF drafted the manuscript. All authors read and approved the final manuscript.

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