Identification of amino acids relevant for activity and regulation of plant sucrose transporters StSUT1 and ZmSUT1

Philipp Burt, Jelena Pustogowa, Undine Krügel, Aleksandra Hackel, Ronja Rote, Christina Kühn*
Humboldt University of Berlin, Institute of Biology, Plant Physiology Department, Philippstr. 13, Building 12, 10115 Berlin
* for correspondence: christina.kuehn@biologie.hu-berlin.de

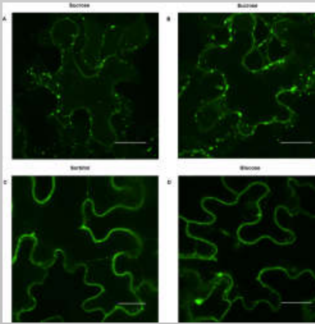
Abstract

Plant sucrose transporters are tightly regulated at the post-translational level by phosphorylation/dephosphorylation, redox-dependent oligomerization, endocytosis and recycling, and potentially also by association to membrane microdomains and protein-protein interactions (Krügel et al., 2008; Krügel et al., 2009; Liesche et al., 2010). Our aim is the identification of stimuli for the internalization of sucrose transporters, the elucidation of the role of dimerization, phosphorylation, and microdomain-association in transport activity. It is also the question whether concentration of sucrose transporters in membrane microdomains affect their lateral mobility. Fluorescence recovery after photobleaching (FRAP) experiments were performed in order to test the impact of sterol depletion or plasmolysis on lateral mobility of these membrane proteins.

We identified a highly-conserved negatively charged amino acid motif by PCR-based site-directed mutagenesis as important for the transport activity at very low pH, of StSUT1 from *Solanum tuberosum* as well as of ZmSUT1 from *Zea mays*. Transport activity was measured electrophysiologically in *Xenopus* oocytes and by yeast complementation.

Western blots revealed that StSUT1 homodimerization and dephosphorylation undergo diurnal oscillation (Krügel et al., 2013). The PhosPhat database helped in predicting potential phosphorylation sites within the StSUT1 amino acid sequence. Two serine residues with the highest prediction score have been replaced by either non-phosphorylatable amino acids (A) or residues mimicking constitutive phosphorylation (D). Yeast complementation strongly argues for an important function of one of these serine residues in phosphorylation-dependent regulation of StSUT1 sucrose transport activity. Further investigations will clarify whether activity-relevant mutations of ZmSUT1 and StSUT1 are related with changes in subcellular localization, oligomerization and/or the protein's concentration within certain membrane compartments.

High amounts of extracellular sucrose induce endocytosis of StSUT1-GFP



Expression of StSUT1-GFP in tobacco leaf epidermis cells under control of the 35S promoter after incubation with 500 mM sucrose **(A, B)** for 1 h resulted in increased vesicle formation compared to the untreated control. The effect can not be observed after treatment with 500 mM sorbitol **(C)** or 500 mM glucose **(D)**. Images are single scans with GFP-specific filter settings. Scale bars: 25 µm. High amounts of extracellular sucrose are able to induce endocytosis of StSUT1 but do not affect lateral mobility.

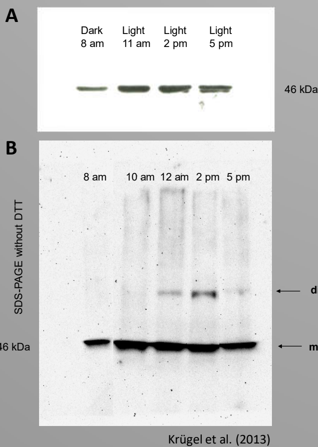
Dephosphorylation of Serin $_{174}$ is important for StSUT1 activation

Sucrose transporters are described to be regulated via phosphorylation/dephosphorylation at the post-translational level and the sucrose transporter BvSUT1 from *Beta vulgaris* is inactivated by phosphatase inhibitors suggesting that the dephosphorylted form of BvSUT1 is the active form (Ransom-Hodgkins et al., 2003).

Yeast complementation of yeast strain EBY-VW4000:
Replacement of Ser_{128} by either Ala (A) or Asp (D) abolished sucrose transport activity completely.
Replacement of Ser_{174} by Ala mimicking the dephosphorylted form of StSUT1 increased sucrose uptake capacity of StSUT1, whereas replacement of Ser_{174} by Asp (D) mimicking permanent phosphorylation abolished sucrose uptake completely.
All mutant proteins of StSUT1 are highly expressed in yeast as confirmed by Western blot analysis (right panel) and immunodetection with StSUT1-specific antibodies.

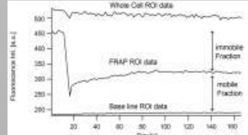
Dimerisation and dephosphorylation of StSUT1 follow diurnal rhythms

(A) Western Blots revealed that StSUT1 protein is highly abundant during the light period and only low protein levels are detectable during the night. At the end of the light period a second band corresponding to the dephophorylated form of the protein is detectable. Inhibitor studies with ocadaic acid and staurosporine confirmed that StSUT1 is dephophorylted during the light period and at the end of the night only the phosphorylated form is detectable.
(B) The dephosphorylation is paralleled by increased dimer formation at the end of the light period as shown by SDS-PAGE in the absence of reducing agents. The homodimer formation of StSUT1 was previously shown to occur in a redox-dependent manner (Krügel et al. 2008).

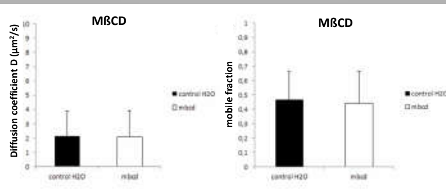


Fluorescence recovery after photobleaching

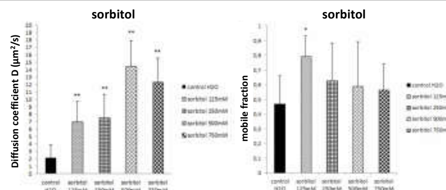
StSUT1 is enriched in the detergent-resistant membrane (DRM) fraction of plant plasma membrane and was detected in membrane microdomains if expressed in yeast (Krügel et al. 2008). It is assumed that membrane proteins in the liquid ordered phase of the plasma membrane are restricted in their lateral mobility.



Fluorescence recover after photobleaching (FRAP) was used to test the impact of various treatments on the diffusion paramters of a StSUT1-GFP fusion protein transiently expressed in tobacco epidermis cells. The diffusion coefficient D was calculated according to the formula $D = 0,224\ \omega^2/\tau_D$ where ω is the radius of the ROI and τ_D is the time of half-maximum recovery. The diffusion coefficient and the percentage of the mobile fraction recovering after photobleaching are given.



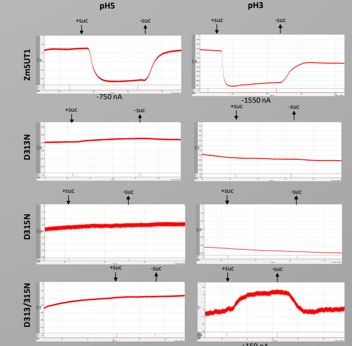
Sterol depletion of the plant plasma membrane using methyl-beta-cyclodextrin (MßCD) has no effect on lateral mobility of StSUT1-GFP. This indicates that association of membrane proteins to membrane rafts or microdomaisn plays inly a minor role for lateral mobility.



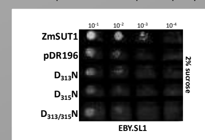
Cell wall constrains lateral mobility of membrane proteins: plasmolysis by sorbitol increases lateral mobility (diffusion coefficient D) and mobile fraction of StSUT1-GFP. This is in agreement with previous findings published by Martinière et al. (2013).

Site directed mutagenesis of ZmSUT1

Site directed mutagenesis of StSUT1 from potato revealed that the highly conserved aspartic acid residues at position D_{308} and D_{310} might play an important role in pH-dependent regulation of sucrose transport capacity since sucrose uptake of StSUT1 D_{308} and StSUT1 D_{310} single mutants show normal sucrose uptake at pH5 but completely abolished sucrose uptake at pH3 (Krügel et al. 2013).
We decided to mutagenize the corresponding aspartic acid residues D_{313} and D_{315} from ZmSUT1 in order to test whether pH-dependent regulation of sucrose uptake is similar in monocotyledons.

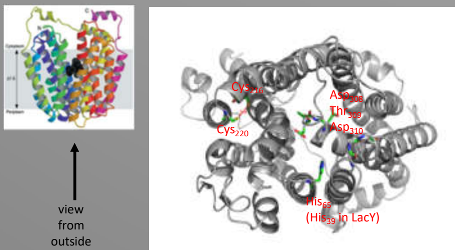


Raw data of electrophysiological two-electrode-voltage clamp measurement at a holding potential of -70 mV are shown. Each buffer was applied for 40 sec. All data are baseline corrected and have been repeated at least three times independently. Note the differences in scale for each single experiment!
Non-injected oocytes or water injected oocytes served as negative control.
ZmSUT1 expressing oocytes show an increasing influx pH7<pH5<pH3 upon sucrose addition, as previously shown (Krügel et al. 2009).
The ZmSUT1 double mutant $D_{313/315}N$ shows no detectable reaction upon sucrose supply at pH7 and pH5, but a slight efflux of positive charge (or influx of negatively charged ions).
Both ZmSUT1 single mutants $D_{313}N$ and $D_{315}N$ behave as the non-injected or water-injected oocytes.
Similar results have been obtained by yeast complementation experiments using ZmSUT1 mutant proteins. None of the mutants were able to complement sucrose uptake capacity of the yeast mutant EBY.SL1.



ZmSUT1 single and double mutants expressed in the yeast expression vector pDR196 are not able to complement sucrose uptake capacity of the yeast mutant EBY.SL1 defective in extracellular sucrose cleavage and sucrose and hexose uptake (Krügel et al. 2013).

Molecular model of StSUT1 based on LacY structure



The 3D-structure was deduced from the known crystal structure from the lactose permease LacY from *E.coli* and plotted using the PHYRE algorithm. According to the 3D-model the two Cysteine residues Cys_{216} and Cys_{220} are engaged in intramolecular disulfid bridge within StSUT1. The 3D-model predict location of the two aspartic acid residues D_{308} and D_{310} (corresponding to D_{313} and D_{315} in ZmSUT1) in the 7th transmembrane spanning domain whereas hydrophobicity analysis according to Kite-Doolittle predict their location in the extracellular loop between the 7th and the 8th transmembrane spanning domain.

Conclusions

- Sucrose at high concentration induces endocytosis of StSUT1.
- MßCD inhibits StSUT1 endocytosis but doesn't affect ist lateral mobility.
- Sorbitol induced plasmolysis of StSUT1-expressing cells increases lateral mobility of StSUT1-GFP suggesting that the cell wall constraints mobility of integral membrane proteins that are concentrated in membrane microdomains.
- The presence or absence of the cell wall seems to have greater impact on lateral diffusion of membrane proteins than their concentration in liquid ordered (l_o) domains.
- The PhosPhat database predicts phosphorylation of $Serin_{128}$, $Serin_{174}$ and $Serin_{500}$ of StSUT1. Replacement of Ser_{174} by Alanin (A) which can not be phosphorylated increases StSUT1 sucrose transport capacity, whereas replacement by Aspartic acid (D) abolished StSUT1-mediated sucrose uptake. This suggests that the dephosphorylated form of StSUT1 is functional.
- Mutagenesis of highly conserved aspartic acid residues D_{313} and D_{315} from maize ZmSUT1 completely abolished ZmSUT1-mediated sucrose transport capacity. D_{313} and D_{315} might represent putative proton binding sites.

References

Krügel, U., Veenhoff, L. M., Langbein, J., Wiederhold, E., Liesche, J., Friedrich, T., Grimm, B., Martinoia, E., Poolman, B. and Kühn, C. (2008) , Plant Cell 20(9): 2497-513. Correction (2009), Plant Cell 21(12): 4059-60.
Krügel, U., Wiederhold, E., Pustogowa, J., Hackel, A., Grimm, B. and Kühn, C. (2013) Biochimie 95(11): 2132-44.
Liesche, J., He, H. X., Grimm, B., Schulz, A. and Kühn, C. (2010) Mol Plant 3(6): 1064-74.
Martiniere, A., Lavagi, I., Nageswaran, G., Rolfe, D. J., Maneta-Peyret, L., Luu, D. T., Botchway, S. W., Webb, S. E., Mongrand, S., Maurel, C. et al. (2012) , *Proc Natl Acad Sci U S A* 109(31): 12805-10.
Ransom-Hodgkins, W. D., Vaughn, M. W. and Bush, D. R. (2003) , *Planta* 217(3): 483-9.
Sun, Y., Lin, Z., Reinders, A. and Ward, J. M. (2012), Biochemistry 51(15): 3284-91.