TITLE

Comparative analysis of STP6 and STP10 rationalizes molecular selectivity in Sugar Transport Proteins

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ABSTRACT

Sugar distribution plays essential roles in plant energy, signaling, and defense mechanisms. Sugar Transport Proteins (STPs) are responsible for proton-driven cellular uptake of glucose, derived from sucrose in the apoplast. Few STPs also facilitate uptake of fructose, the other constituent of sucrose but the molecular features that define differences in specificity are unknown. Here we present a 3.2 Å crystal structure of *Arabidopsis thaliana* STP6 in an inward occluded conformational state with glucose bound and show that it is both a glucose and fructose transporter. We perform a comparative analysis with the glucose-selective STP10 using *in-vivo* and *in-vitro* based systems and disclose how the kinetic transport properties are influenced by different experimental setup. Our work provides detailed insights into the fine-tuned dynamics of affinity-induced specificity for hexose uptake, ultimately showing how the position of a single methyl group in the binding site is sufficient to direct substrate specificity. These findings advance the understanding of hexose trafficking by STPs and extend the framework for engineering STP, and more broadly sugar transport, in the future.

INTRODUCTION

In plants, sugar serves both as an energy source and essential signaling molecules controlling tissue development. Cellular uptake of monosaccharides is facilitated by Sugar Transport Proteins (STPs) that import monosaccharides by proton driven symport, utilizing the proton motive force (PMF) (Sauer *et al*, 1994; Truernit *et al*, 1996; Schneidereit *et al*, 2005; Büttner *et al*, 2000). STPs form a distinct subgroup in the Sugar Porter (SP) family, which in turn constitutes the largest family within the Major Facilitator Superfamily (MFS) (Büttner, 2007; Niño-González *et al*, 2019). 14 STPs are found in *Arabidopsis thaliana* (STP1 to STP14) where they are primarily expressed in symplastically isolated cells in tissues such as seeds, flowers, pollen, and roots (Büttner, 2010). They undergo extensive transcriptional regulation during lateral root formation and pollen maturation, essential to ensure correct tissue development (Schneidereit *et al*, 2003; Scholz-Starke *et al*, 2003). In addition, they aid in the plant's defense machinery against invading pathogens by sequestering monosaccharides from the apoplast and are directly linked to environmental stress responses triggered by challenges such as drought or extreme temperatures (Huai *et al*, 2022; Huai *et al*, 2020; Milne *et al*, 2019; Nørholm *et al*, 2006; Lemoine *et al*, 2013; Yamada *et al*, 2016; Reyer *et al*, 2021).

A. thaliana STP10 have provided the foundation for the current understanding of the STP transport mechanism (Paulsen *et al*, 2019; Bavnhøj *et al*, 2021). STP10 has been characterized in multiple experimental setups, where it has been shown to transport glucose with high affinity (K_M^{apps} = 2-20 μM) in a proton dependent manner (Paulsen *et al*, 2019; Rottmann *et al*, 2016). Two glucose epimers, galactose and mannose, inhibit STP10's uptake of glucose in competition assays (Paulsen *et al*, 2019). The structures of STP10 solved in complex with glucose, in outward occluded and inward open conformational states, provided detailed insights into the alternating access mechanism of STPs used for transport (Jardetzky, 1966; Drew & Boudker, 2016; Drew *et al*, 2021; Andersen *et al*, 2023; Paulsen *et al*, 2019; Bavnhøj *et al*, 2021). STPs adopt the canonical MFS fold with 12 transmembrane helices (M1-M12) present in two membrane-spanning domains (N and C) (Parker & Newstead, 2014; Sun *et al*, 2014; Ri *et al*, 2024). The two domains are related by rotational pseudo symmetry, with the sugar binding site sandwiched in-between. A distinct protonation site in M1 (Asp42 in STP10) is linked to the substrate binding site, ensuring tight proton-sugar coupling (Paulsen *et al*, 2019; Bavnhøj *et al*, 2021). As in all other SP family proteins, the N and C domains are connected by an intracellular cytosolic helix bundle (ICH) domain (Sun *et al*, 2012; Nomura *et al*, 2015; Wisedchaisri *et al*, 2014; Deng *et al*, 2014). The ICH domain also holds the SP signature motif, which is often seen to interact with the MFS specific A motif, localized between M2 and M3, M8 and M9 (Custódio *et al*, 2021). The A motif is involved in forming interdomain salt-bridge networks, known to stabilize the transporter in outward facing conformations (Bavnhøj *et al*, 2021; Nomura *et al*, 2015, 5; Quistgaard *et al*, 2013). Both the A and the SP motifs have been suggested to play a key role in regulating transport kinetics by several studies conducted on SP family members (Custódio *et al*, 2021; Wisedchaisri *et al*, 2014; Jia *et al*, 2020). A unique feature of the STPs is the presence of a disulfide bridge present on a small extracellular Lid domain located between M1 and M2, tethering the N domain to the C domain. A previous study has shown that the disulfide bridge is needed for high glucose affinity, possibly helping the Lid domain shield the protonation site from the extracellular environment (Paulsen *et al*, 2019).

Transport activity has been characterized for several STPs across species in different experimental setups. While glucose is the preferred substrate, with few exceptions, most STPs are promiscuous and transport a range of monosaccharides (Rottmann *et al*, 2018; Büttner *et al*, 2000; Büttner, 2010). Notably, the hydrolysis of photosynthesis-derived sucrose in the apoplast yields equimolar amounts of

glucose and fructose (Tauzin & Giardina, 2014; Bavnhøj *et al*, 2023), only STP6, STP8, and STP13, have been proposed to facilitate significant uptake of fructose (Scholz-Starke *et al*, 2003; Liu *et al*, 2021; Nørholm *et al*, 2006). With a 52% sequence identity between STP6 and STP10 and their shared involvement in pollen tube formation, the underlying explanation for their divergent substrate profiles remains unknown.

Here we present a structural and biophysical characterization of *A. thaliana* STP6, including a direct comparative analysis with STP10 addressing differences in substrate specificities. With a 3.2 Å crystal structure of STP6 in complex with glucose, we detail a previously unresolved inward occluded state of the STP transport cycle. Our biophysical characterization shows that STP6 transports a range of hexoses with varying preference and demonstrates dependency on the PMF. We identify affinity-determining sites and show how the position of a single methyl group can be used to rationally modify the transport affinities of STP6 and STP10 towards glucose and fructose. Finally, our work represents the use of diverse experimental setups in parallel to demonstrate how transporter kinetics are highly dependent on the context of transporter environment, relevant for comparative analysis of membrane transporter biophysics between related targets.

RESULTS

STP6 adopts an inward occluded conformation distinct from previous structures of STP10

To enable a strict comparative analysis of STP6 and STP10, we established a shared protocol for expression and purification, to ensure identical baseline working conditions and samples (Supplementary Figure 1).

Since prior structural information was only available for STP10, we initiated our analysis with crystallizing STP6 in complex with glucose and solved the structure to a resolution of 3.2 Å. STP6 crystallize in a $P3_221$ space group with a single monomer in the asymmetric unit. The final model includes residues 17 to 492 (of 507 residues total), glucose, cholesteryl hemisuccinate (CHS), and was refined to an R-free of 25.2% (Figure 1A, Supplementary Table 1 and Supplementary Figure 2). STP6 displays the MFS fold, with 12 transmembrane helices (M1-M12) divided between an N domain and a C domain (M1-M6 and M7-M12, respectively). Four of the five-bundle intracellular helix domain (ICH1-5) connects the N and C domains. The extracellular helical Lid domain (L1-3) is found between M1 and M2, where it is covalently linked to the C domain by a disulfide bridge formed between Cys72 (L3) and Cys442 (M11) (Figure 1A and B). The positioning of three CHS molecules mimics the inner leaflet of the plasma membrane (Supplementary Figure 2). One of the CHS head groups interacts with the A motif through Arg340 (M8) (Supplementary Figure 3).

STP6 adopts a substrate-bound inward occluded conformational state with glucose bound in the central cavity (Figure 1A, C and D). The majority of the interactions between glucose and binding pocket are to the C domain, by a distinct network of polar interactions to Gln290, Gln291, Asn296, Asn327, Gly399, Trp403, Asn426 and Thr430 (Figure 1C). The N domain binds the glucose molecule predominantly through hydrophobic interactions mediated by Ile39 and Ile179 and T-shaped π-stacking between Phe35 and the glucosyl ring (Figure 1C). In STP6, the proton donor/acceptor site, Asp38, is positioned on the M1 helix in close proximity to the bound glucose. Arg137 is found in close vicinity to Asp38, on M4, wherefrom it controls the pKa of Asp38. Together, they form a classical proton donor-acceptor pair (Figure 1A and C) (Buch-Pedersen *et al*, 2009). Collectively, these observations reflect the structural arrangement of the key residues needed to ensure tight coupling between glucose-binding and protonation of Asp38, previously described for STP10 and

characteristic for proton-coupled symporters in the SP family (Bavnhøj *et al*, 2021; Paulsen *et al*, 2019; Wisedchaisri *et al*, 2014).

The inward occluded state reveals new insights of structural dynamic during sugar translocation

Compared to the known structures of STP10 determined in the outward occluded and inward open states, STP6 adopts a distinct inward occluded conformational state (Figure 1D). The inward occluded state shares many of the characteristics observed in the inward open state of STP10, as demonstrated by plotting the domain-paired root mean square deviation (RMSD) between the corresponding C-alpha backbone positions (N domain RMSD(Cα) 1.141 Å, C domain RMSD(Cα) 0.734 Å) (Figure 2A and Supplementary Figure 4). The low RMSD of the C domain reflects that both of the inward states exhibit the same helical rearrangement when progressing from the outward state. In this process the C domain inverts into a "substrate release conformation", which includes outwards kinking of M11b that in turn pushes the core of M7 down towards the substrate binding site. This joint movement forces the Lid domain to bend away from the body of the transporter and adopt a more open conformation. Simultaneously, a mimetic residue swap between Tyr71 (L3) and Tyr301 (M7b) takes place as part of the dynamic hydrophobic gate controlling access to the binding site (Figure 2B and Supplementary Figure 4), as previously also described for STP10 (Bavnhøj *et al*, 2021). As indicated by the RMSD plot the transporter is seen to undergo subtle rearrangements when transitioning from the inward occluded to the inward open state, with structural deviations being most pronounced in the flexible regions of the C domain, including the loop between M9 and M10 and the C terminal (Figure 2A). Changes are also noticeable in helical regions, including movement of ICH3 and the joint movement of M10b and M11 (Figure 2A and B). When looking at the other half of the transporter the most pronounced deviation results from rearrangement of the Lid domain, which curls away from the central cavity during the transition from the inward occluded to the inward open state (Figure 2A, B and C). The positioning of Tyr71 and Tyr306 is unaffected in this process (Figure 2B and C), suggesting a continuous restriction of the binding site towards the extracellular side. Further movement is found in the ICH1-connected TM region spanning M4-M6, which includes the noticeable outwards kinking of M5a and rearrangement of the polar interactions between the SP and A motifs (Figure 2A, B and Supplementary Figure 3). Accompanied by the large conformational changes in the Lid domain, movement of the TM helices lining the central cavity determines access to the binding site; M1, M2 and M7 come together to secure the binding site towards the extracellular side, going from outward occluded to inward occluded, and kept in the inward open state (Figure 2C and 3A). Towards the intracellular side the inward transition extends the distance between M4, M5, M10 and M11, successfully creating a path for substrate release (Figure 2B and 3B). This expansion is initially minor, as the transporter transitions from the outward occluded to the inward occluded state, but dramatically increases to ~8 Å as it progresses to the inward open state (Figure 2B and 3B). In order to visualize how the global rearrangements affect the local environment of the substrate binding pocket, we compared the glucose binding of STP6 to both STP10 structures by superposition of the C domains. Comparing STP6 to the outward occluded state of STP10 reveals a clear displacement of Phe35 and Gln172, directly linked to the rearrangement of M1 and M5 (Figure 3C, Panel 1). Interaction with Phe35 and Gln172 is known to be crucial for high-affinity glucose transport (Bavnhøj *et al*, 2021), and the displacement thus results in a looser binding pose, effectively lowering the affinity when transitioning to the inward state (Figure 3B). The glucose molecule remains bound as escape is not possible in the inward occluded state. Progression from the inward occluded to the

inward open state confers further displacement of Phe35 (M1) and Gln172 (M5) now fully disengaged from the glucose, completing the exit path and allowing substrate release (Figure 2B and 3C Panel 2). In contrast to the observed changes in the N domain, substrate interactions with the C domain remain unchanged throughout the transitioning (Figure 3D). Thus, the majority of the defining structural rearrangements happening upon transporter transitioning take place in the N domain. The 5 Å distance between Asp38 and Arg137 observed in STP6 (Figure 3C) supports protonation of Asp38 and suggests that STP6 represents a proton-loaded state (Bavnhøj *et al*, 2021). Based on the comparison of the STP6 and STP10 structures, it is clear that STP6 and STP10 have virtually identical binding pockets, differing only by the position of a methyl group at a single position: Ile39 in STP6 corresponding to Leu43 in STP10 (Figure 2C, D and Supplementary Figure 5).

Transport characteristics of STP6 are comparable to STP10

STP6 transport activity was initially characterized by measuring uptake of radioactively labelled substrates in *Saccharomyces cerevisiae.* This allows a comparison of the transport profile of STP6 with previous characterizations of STP10 performed under identical experimental conditions (Bavnhøj *et al*, 2021; Paulsen *et al*, 2019).

Broadly, the transport characteristics of STP6 resemble those of STP10. STP6 transports glucose with high affinity (K_M^{app} = 25 µM) in a pH dependent manner, with uptake rates peaking at pH 5, appropriate for functioning in the resting apoplastic pH (Figure 4A and B) (Paulsen *et al*, 2019). These observations correspond well with affinity measurements previously reported from yeast uptake assays of STP6 (20 µM) and STP10 (19 µM), as well as the pH preference shown for STP10 (pH 4 – 6.5) (Scholz-Starke *et al*, 2003; Rottmann *et al*, 2016; Paulsen *et al*, 2019). STP6-mediated transport in this setup is highly dependent on the PMF demonstrated by a significantly reduced transport (~76 % decrease) in the presence of the proton gradient decoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP), as seen for other STPs including STP10 (Rottmann *et al*, 2016; Sauer *et al*, 1994; Truernit *et al*, 1996; Schneidereit *et al*, 2005). The activity of STP6 is compromised by a range of inhibitors known to affect SP proteins, such as curcumin and phloretin (Supplementary Figure 6) (Paulsen *et al*, 2019; Gunnink *et al*, 2016; Opekarová *et al*, 1999). Competition with glucose uptake by STP6 shows that both galactose and mannose can compete for binding (Figure 4C). Using radiolabeled galactose and mannose show that both are in fact transported with intermediate affinity by STP6 (galactose K_M^{app} = 129 µM, Mannose K_M^{app} = 530 µM) (Figure 3D and E). Xylose and sucrose do not compete with glucose, supporting the expected preference for monosaccharides, predominantly hexoses (Figure 3C). This competition pattern correlates well with the pattern previously described for STP10, with fructose as the exception, as it only competes with glucose in STP6 (Figure 3C). This observation agrees with previous reports of possible fructose transport by STP6, but the lack of competition in STP10 is surprising given the virtually identical binding sites of STP6 and STP10. Collectively, these data confirm STP6 as a proton/hexose symporter with high affinity for glucose and transport dynamics broadly comparable to STP10, apart from noticeable differences in transport of fructose.

***In vitro* and *in vivo* assessment of STP6 and STP10 reveals different perceptions of fructose**

To compare the transport activities of STP6 and STP10 side-by-side *in vitro*, we reconstituted purified protein in proteoliposomes, again using identical experimental setups, and conducted activity measurements by solid supported membrane (SSM) electrophysiology. STP6 and STP10 display similar affinities towards glucose, with STP6 having a K_M^{app} of 452 µM and STP10 a K_M^{app} of 637 µM

(Figure 5A and Supplementary Figure 7). Notably, the K_M^{apps} in this setup represent ~20-30-fold weaker affinity than determined in the yeast uptake assays described above. We next tested the SSM-signal towards the substrates described above, including L-arabinose. Here, both STP6 and STP10 showed activity in the presence of glucose, galactose, and mannose but negligible signal in the presence of sucrose and arabinose. In addition, STP6 showed a significant response to fructose, while almost no response was observed for STP10 (Figure 5B). Except for the transport signal observed for xylose with STP10 (Figure 5B) , these results match the competition profiles from the yeast uptake assays (Paulsen *et al*, 2019; Rottmann *et al*, 2016).

To address the affinity differences between the yeast uptake assay and the proteoliposome-based SSM assays, we performed an additional *in-vivo* comparison using *Xenopus laevis* oocytes. In oocytes, STP6 and STP10 display affinities for glucose transport that are 2-3-fold stronger than those observed in yeast uptake assays, with respected K_M^{apps} of 12.6 µM and 6.8 µM (Figure 5C). Next, we used radiolabeled fructose to quantify transport in STP6 and STP10. Here, fructose transport was observed for both transporters, however, while STP6 display intermediate affinity, (198 µM, ~15-fold weaker than for glucose) a considerably lower fructose affinity is observed for STP10 (K_M^{app} = 3000 µM, ~440-fold weaker than for glucose) (Figure 5D). The drastically lower fructose affinity of STP10 compared to STP6 correlates well with the differences observed in competition assays and the transport currents measured in proteoliposomes.

Probing selectivity differences between STP6 and STP10

Based on our analysis we created point mutations to probe the activity and selectivity of STP6 and STP10. We continued our analysis in *X. laevis* oocytes since the signal/noise is superior compared to the other experimental setups tested. Alanine mutation of the suggested proton binding site in STP6 (Asp38) and the corresponding well-known site in STP10 (Asp42) abolish uptake of glucose, further underlining the fundamental role of this residue in coupling sugar transport to the PMF (Supplementary Figure 8).

To explore which features enhance fructose affinity of STP6 compared to STP10, we recreated the binding site of STP10 in STP6, by introducing the mutation I39L, shifting a methyl group by one carbon atom. STP6 I39L displays a 3-fold reduction in fructose affinity ($K_M^{app}{}_{STP6\ I39L}$ = 631 µM vs. $K_M^{app}{}_{STP6\ (WT)}$ =198 µM), and a 2-fold increase in glucose affinity ($K_M^{app}{}_{STP6\ I39L}$ = 5.1 µM vs. $K_M^{app}{}_{STP6\ (WT)}$ = 12.6 µM) (Figure 5E and F), reflecting the glucose affinity of WT STP10. The inverse trend was found in the reverse experiment, recreating the binding site of STP6 in STP10, by introducing the mutation L43I. STP10 L43I shows a 3-fold improved affinity for fructose ($K_M^{app}{}_{STP10\ L43I}$ = 894 µM vs. $K_M^{app}{}_{STP10\ (WT)}$ = 3000 µM) while glucose affinity is reduced 2-3-fold ($K_M^{app}{}_{STP10\ L43I}$ = 16 µM vs. $K_M^{app}{}_{STP10\ (WT)}$ = 6.8 µM) (Figure 5E and F), reflecting the glucose affinity of WT STP6.

DISCUSSION

In this study, we characterize and compare *A. thaliana* STP6 and STP10, and present a 3.2 Å crystal structure of STP6 with glucose bound. The STP6 structure captures a previously unresolved "inward occluded state" of the STP transport cycle. This state has previously been resolved only for the bacterial homolog XylE (Mitrovic *et al*, 2023; Wisedchaisri *et al*, 2014). By expanding our structural knowledge with the inward occluded state of STP6, we have gained new insight into the dynamics governing substrate release in the STP transport cycle. The STP structures collectively point to a model where substrate release takes place in two consecutive steps: First, global rearrangement of the Lid domain and helical regions brings the transporter into the inward facing orientation. Next, the

central cavity of the transporter is expanded towards the extracellular side, shaping the substrate exit path. In this process Phe35 (M1) and Gln172 (M5) are retracted from the binding pocket, enforcing substrate release by lowering the local affinity. The interaction observed between CHS and Arg340 (M8) in the C domain A-motif of STP6 likely stabilizes the transporter in the inward conformation, suggesting that the immediate environment may be involved in regulation of the conformational equilibrium. This idea aligns with findings from other studies on SP family members and ties into a more general discussion of how transporters are affected by membrane composition (Custódio *et al*, 2021; Neumann *et al*, 2022; Martens *et al*, 2018, 2016; Opekarová *et al*, 1999).

Considering the effects of external factors is important when assessing transport properties in biophysical assays. Challenges with quantifying protein levels lead to a lack of meaningful Vmax values for membrane protein transporters, limiting affinity assessments to apparent K_M-values (Hammes & Pedersen, 2024). In this paper we have determined apparent K_M-values using three different experimental setups and shown a significant variation between them. While the difference is most pronounced between *in vitro* and *in vivo* based systems (∼20-100 fold), consistent differences are also noticeable between *in vivo* systems (2-10 fold). These differences ultimately arise from a number of factors; membrane composition including the presence/absence of sterols, buffer composition, and differences in contribution from the PMF, all directly affecting the observed transport kinetics. This is clearly observed in the proteoliposome-based SSM electrophysiology assays. These experiments are conducted in absence of a pH gradient across the membrane and transport is solely driven by the difference in substrate concentration, here giving rise to significantly weaker apparent affinities. Our work demonstrates the necessity of using identical experimental setups, when possible, for comparing transport properties of different proteins.

In our side-by-side comparison of STP6 and STP10, we investigate how STPs discriminate between substrates. We find a 15-fold difference in fructose affinity between WT STP10 and WT STP6 in oocyte uptake assays. Next, we show that we can modulate fructose accommodation by changing the position of a single methyl group in the binding pocket, resulting in a gain of affinity for fructose, as seen for STP10 L43I. Enhancing fructose affinity comes with the price of a reduction in glucose affinity, implying the general ligand interaction concept of high affinity imposing high specificity (Eaton *et al*, 1995). Changing the equivalent position in STP6 similarly led to a decreased fructose affinity and increased glucose affinity.

These results correspond well with our previous specificity-affinity concept suggested for the STPs, where the polar network of the C domain confers hexose specificity, showing here that affinity differences between different hexoses arises from hydrophobic interactions with the N domain (Bavnhøj *et al*, 2021; Paulsen *et al*, 2019). Furthermore, a previous study on a homolog from green algae revealed that the binding site position equivalent to Ile39/Leu43, could be used to modulate affinities of glucose and galactose in a similar manner to here (Will *et al*, 1998). This suggest a conserved mechanism for controlling affinity induced specificity by transporters belonging to the SP family, which we can now expand on. Substrate interactions mediated by residues localized in the N domain control affinity by direct interactions, as seen for Phe35 (M1) and Gln172 (M5) (Bavnhøj *et al*, 2021), or indirectly by shaping and tightening of the binding pocket, as seen for Ile39 (M1). As demonstrated here, indirect interactions are highly sensitive and minute changes can be used to modulate the affinity-induced specificity. For fructose binding, we hypothesize a binding scheme closely mimicking the one displayed by glucose. Fructose is found in a pyranose form and furanose form in a ~2:1 equilibrium in solution, as opposed to glucose which is principally only found on the

pyranose form (99%) (Cocinero *et al*, 2013; Ferraris *et al*, 2018). Therefore, we speculate that for fructose, the pyranose form is selectively transported over the furanose form by STPs.

In summary, we have shown that changing the position of a single methyl group is sufficient to manipulate substrate affinities of STPs, showing that subtle variation in the hydrophobic binding site is a key regulator of selectivity. The less-than-perfect fructose affinity complementation of STP6 I39L and STP10 L43I to their respective WT counterparts implies the presence of other yet undiscovered regulatory elements and/or kinetic influence by other regions of the protein (Qureshi *et al*, 2020; Jiang *et al*, 2020). Another interesting feature of the STPs is the Lid domain. Given its exofacial localization and great sequence variability (Supplementary Figure 9), it is tempting to speculate on a potential role as a regulatory element, which may give rise to unique transport properties or substrate promiscuity. For instance, recent studies have shown disaccharide transport by some STPs found in crops (Li *et al*, 2020; Reyer *et al*, 2021; Huai *et al*, 2020, 3). This shows that while we have established a strong understanding of how monosaccharides interactions are mediated by STPs, there is still much to uncover about potential regulatory elements and substrate promiscuity. Uncovering how this is achieved and how it relates to specific stages of plant development will be valuable and can provide a foundation for plant engineering in the future.

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Sample preparation: CA, LB
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Competing interests:
The authors declare no competing interests.

Data and materials Availability:
Atomic model and structure factors for STP6 have been deposited in the Protein Data Bank (PDB) with the accession code 9G11.

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Materials and Methods

Protein expression and purification

The gene encoding *A. thaliana* STP6 (UniProt entry number Q9SFG0) was cloned into an expression vector containing a C-terminal purification tag with a thrombin cleavage site followed by a 10x histidine tag. Protein expression was performed using *S. cerevisiae* (strain DSY-5) grown in a culture vessel. High cell density was obtained by exponential fed-batch, induced with galactose, and harvested after ∼18 hours. Harvested cells were washed in cold water, spun down and resuspended in lysis buffer (600 mM NaCl, 100 mM Tris-HCl pH 7.5 and 1.2 mM PMSF), followed by opening of cells using beating in cooled bead canisters filled with 0.5 mm glass beads. Opened cells were centrifuged for 20 min, 17.000 rcf (check this) at 4 °C, followed by isolation of membranes by ultracentrifugation of the supernatant at 200.000 rcf for 2 h. Pelleted membranes were homogenized in membrane buffer (500 mM NaCl, 50 mM Tris-HCl pH 7.5, 20% (w/v) glycerol) and flash frozen in liquid nitrogen. Protein purification was carried out with 6 g of thawed membranes, solubilized for 30 minutes in solubilization buffer (150 mM NaCl, 50 mM Tris pH 7.5, 5% glycerol, 1% n-dodecyl-β-D-maltoside (DDM) and 0.1% cholesterol hemisuccinate (CHS)) in a total volume of 100 mL. Unsolubilized material was removed by centrifugation followed by filtration of the supernatant using a 1.2 µM filter. 20 mM imidazole pH 7.5 was added prior to loading solubilized membranes on a pre-equilibrated 1 mL Ni-NTA column (GE Healthcare) at 1 mL min-1. The column was washed with 50 column volumes of W60 buffer (150 mM NaCl, 50 mM Tris pH 7.5, 0.1% DDM, 0.01% CHS, and 60 mM imidazole pH 7.5), followed by washing 30 column volumes in G20-buffer (20 mM Tris-HCl, 200 mM NaCl, 20 mM imidazole pH 7.5, 0.12% OGNG, 0.012% CHS or 0.02% DDM, 0.002% CHS). Bound protein was cleaved from the column by circulating in 5 mL G-buffer supplemented with 200 U of bovine thrombin for ∼14 hours at 19 oC. A final wash was performed using 15 mL of G-buffer supplemented with 40 mM imidazole pH 7.5. The sample was concentrated using 50 kDa MWCO spin column (Merck) to a final volume of ∼450 µL prior to size-exclusion chromatography performed on Bio-Rad system equipped with a Superdex200inc. column (GE Healthcare), equilibrated in G-buffer (20 mM Tris-HCl, 200 mM NaCl, 0.12% OGNG, 0.012% CHS or 0.02% DDM, 0.002% CHS). The choice of detergent depended on downstream protein application. OGNG was used in crystallization assays whereas DDM was used for proteoliposome reconstitution.

Yeast uptake assay

Functional characterization in yeast was performed by introducing the A. thaliana STP6 gene into the p426Met25 vector, allowing constitutive expression under a methionine-inhibited promoter. Transformation was carried out into S. cerevisiae lacking endogenous hexose transporters (strain EBY.WV4000) (Wieczorke *et al*, 1999) and plated on SDA plates without uracil supplemented with 2% maltose. After three days a streak of cells was inoculated into 50 mL of complementary media a grown to an OD600 of ∼1.5. Cells were washed with 25 mM NaPO4 buffer pH 5.0, followed by centrifugation and resuspension, achieving a final OD600 of 10. Experiments were carried out using 20 µL of cells diluted to a final volume of 200 µL with NaPO4 buffer (pH directed by the experiment). Reactions were started upon addition of substrate containing 1 µCi of radioactively [3H]-labelling. 100 µM of the tested sugars were used for time dependent assays whereas 10 µM was used in pH dependent and competition assays. Competing sugars were added in 25x excess and for inhibitors 500 µM was used, except for CCCP where 100 µM was used. All inhibitors were dissolved in DMSO. Reactions were stopped in noted intervals by collecting cells on a cellulose filter (0.8 µm) under vacuum. Three wash cycles were then performed before retracting each filter into individual

scintillation tubes and addition of 3 mL of scintillation liquid. Quantification of [3H]-signal was done using Tri-Carb 5110TR Liquid Scintillation Counter (Perkin Elmer). Data analysis was performed in GraphPad Prism 9 where linear regression (time dependent uptake) or Michaelis-Menten fit (concentration dependent uptake) was utilized and normalized-

Solid-supported membrane (SSM)-based electrophysiology assays

SSM-based electrophysiology experiments were performed by reconstituting purified STP6 and STP10 into proteoliposomes analyzed utilizing a SURFE2R N1 instrument (Nanion Technologies). For all experiments sensors preparation was performed as described in (Bazzone & Barthmes, 2020). Proteoliposomes were prepared using desiccated yeast polar lipid extract (Avanti), resuspended in surfer buffer (30 mM HEPES pH 7.4, 140 mM NaCl and 5 mM MgCl2). Liposomes were homogenized in size by extrusion through a 0.4 µM membrane followed by pulse sonication in water batch for 1 min. Liposomes were destabilized by addition of 1% (v/v) n-octyl-β-D-glucoside prior to mixing with purified protein, achieving a final LPR=5, or with addition of buffer in equal volume (control). Detergent was extracted by stirring suspensions overnight with 400 mg mL-1 BioBeads (Bio-Rad) at 4 ºC. Proteoliposomes were diluted 1:5 in surfer buffer prior to application onto 3 mM sensors. Sensors were centrifuged at 2100 g for 30 min. Activating and non-activating buffers were prepared as the surfer buffer, however, the activating buffer was supplemented with 0-40 mM of D-glucose (concentration dependent assays) or 10 mM of the analyzed sugar (substrate specificity assays). A single solution exchange workflow was used for all experiments. Michaelis-Menten curves represent the mean of three individual experiments and the SD represents deviation across all data points. Data handling was performed in GraphPad prism where data was either fitted to Michaelis-Menten kinetics or grouped into bar plots.

***Xenopus* Oocyte assays**

Uptake assays in *Xenopus* oocytes were performed as described in (Bentley *et al*, 2012), with few modifications. The gene encoding A. thaliana STP6, STP10 and mutagenic versions, created with Q5 site-directed mutagenesis (NEB), were encoded into the pNB1-U vector. Template for in vitro cRNA transcription was amplified using primers annealing to the 5' and 3' UTR implementing a poly(A)-tail (FW-HNN49: AATTAACCCTCACTAAAGGGTTGTAATACGACTCACTATAGGG and RV-HNN50: TTTTTTTTTTTTTTTTTTTTTTTTTTTTATACTCAAGCTAGCCTCGAG). In vitro transcription was carried out with the mMESSAGE mMACHINE T7 Transcription Kit (Thermo Fisher) using 200 ng of template. *X. laevis* oocytes (EcoCyte Bioscience) were injected with 25 ng of cRNA or RNAse free water (control) using the Nanoject III (Drummond Scientific) equipped with capillary needles. Oocytes were incubated at 16 oC for 3 days in MBS buffer pH 7 supplemented with 10 IU/mL gentamycin. Uptake assays were performed with 2.5-40 µM D-glucose supplemented with 1 µCi/mL [3H]-D-Glucose or 0.16-5 mM of D-fructose supplemented with 5 µCi/mL [14C]-D-fructose in ND-96 buffer (96 mM NaCl, 1 mM MgCl2, 2 mM KCl, 1.8 mM CaCl2, 5 mM MES pH 5). Oocytes were pH-equilibrated for 5 min in ND-96 pH 5 buffer addition of substrate. Oocytes were incubated with substrate for 30 min (unless stated otherwise), then washed three times in excess ND-96 buffer pH 5, before transferring into scintillation vials. Oocytes were lysed by addition of 200 µL 10% SDS, followed by sonication in sonicator bath. 3 mL of scintillation liquid was added before quantification using Tri-Carb 5110TR Liquid Scintillation Counter (Perkin Elmer). Each oocyte was handled as a unique experiment and each datapoint represents 3-5 oocytes. Data analysis was performed in GraphPad Prism 9 where data was normalized to the relative Vmax before fitting of

Michaelis-Menten kinetics (concentration dependent uptake) or linear regression (time dependent uptake).

Crystallization

Peak fractions from size-exclusion chromatography were concentrated to 4-6 mg mL-1 using a 50 kDa MWCO spin column (Merck), followed by addition of 30 mM D-glucose. Crystallization was carried out as vapor diffusion with sitting drops in MRC 2-well plates and MRC MAXI (SWISSCI) set up with the Mosquito lipid handler (TTP Labtech). A protein:reservoir ratio of 1:1 was used with the initial drop size of 400 nL later scaled up to 1.2 µL carried out at 19 oC. Crystals appeared after 2-3 days in 28-32% PEG smear (PEG2000, PEG3000, PEG3350, PEG4000 and PEG5000), 0.1 M Hepes, pH 8.0 and 0.2-0.5 M NaCl. The final dataset was collected at Diamond Light Source I24 with a wavelength of 0.9686 Å.

Data processing

Datasets were processed in XDS followed by quality assessment in phenix.xtriage suggesting crystal twinning. Space group validation was carried out in Zanuda (Lebedev & Isupov, 2014) and P3221 was given as the most likely space group, with merohedral twin operators (-h,-h,l) and one copy in the asymmetric unit (∼77% solvent content). Final data-scaling was carried out in AIMLESS (Evans & Murshudov, 2013) giving a resolution range of 84-3.2 Å. The phase problem was solved using Molecular Replacement in phenix.phaser (McCoy *et al*, 2007) with the inward open structure of STP10 (PDB 7AAR), prepared with Sculptor, as search model. Manual model building was done in Coot (Emsley *et al*, 2010) and subsequent model refinement in phenix.refine (Adams *et al*, 2010) with final refinement parameters embedding individual ADP weighting and grouped TLS (2 groups). The final model has an R-work of 22.65 and an R-free of 25.16 (Supplementary Table1). MolProbity (Chen *et al*, 2010) evaluation of Ramachandran plot showed 96.62 favored regions and 0.0% outliers.

MAIN TEXT FIGURES, TABLE AND LEGENDS

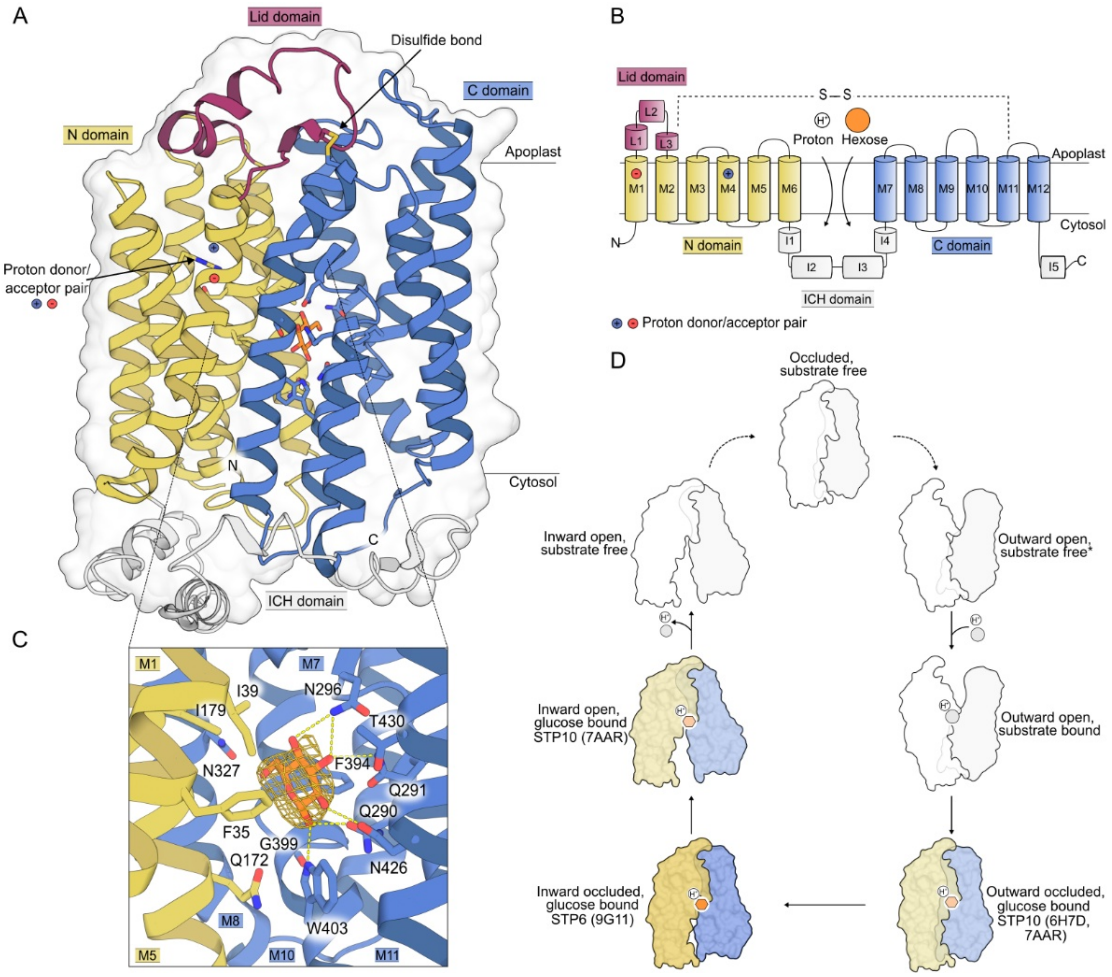


Figure 1. Structure of STP6 in the inward occluded conformation with glucose bound.
A) Cartoon and surface representation of the STP6 crystal structure in an inward occluded conformational state with glucose bound in-between the N domain (yellow) and the C domain (blue). Residues involved in substrate interactions are shown as sticks. The proton donor/acceptor pair and the disulfide bond connecting the Lid domain and the C domain are highlighted with arrows.
B) Schematic representation of STP6 topology. The proton donor/acceptor pair is located in M4 and M1, respectively and the disulfide bond is formed between L3 and M11, as indicated.
C) Close-up view of the glucose binding site. Interacting residues are shown as sticks and yellow dashes represent hydrogen bonding (2.6-3.6 Å distance). The 2Fo-Fc map for glucose is shown as gold mesh (contoured 2σ).
D) Illustration of key conformational states in the STP transport cycle. The * marks the start of the transport cycle. Binding of substrate takes place in the outward open state, substrate transitioning via occluded states and lastly release is permitted in the inward open state. Following substrate release the transport is reset through substrate empty occluded states before the cycle can be repeated. Experimentally determined states are highlighted with colors.

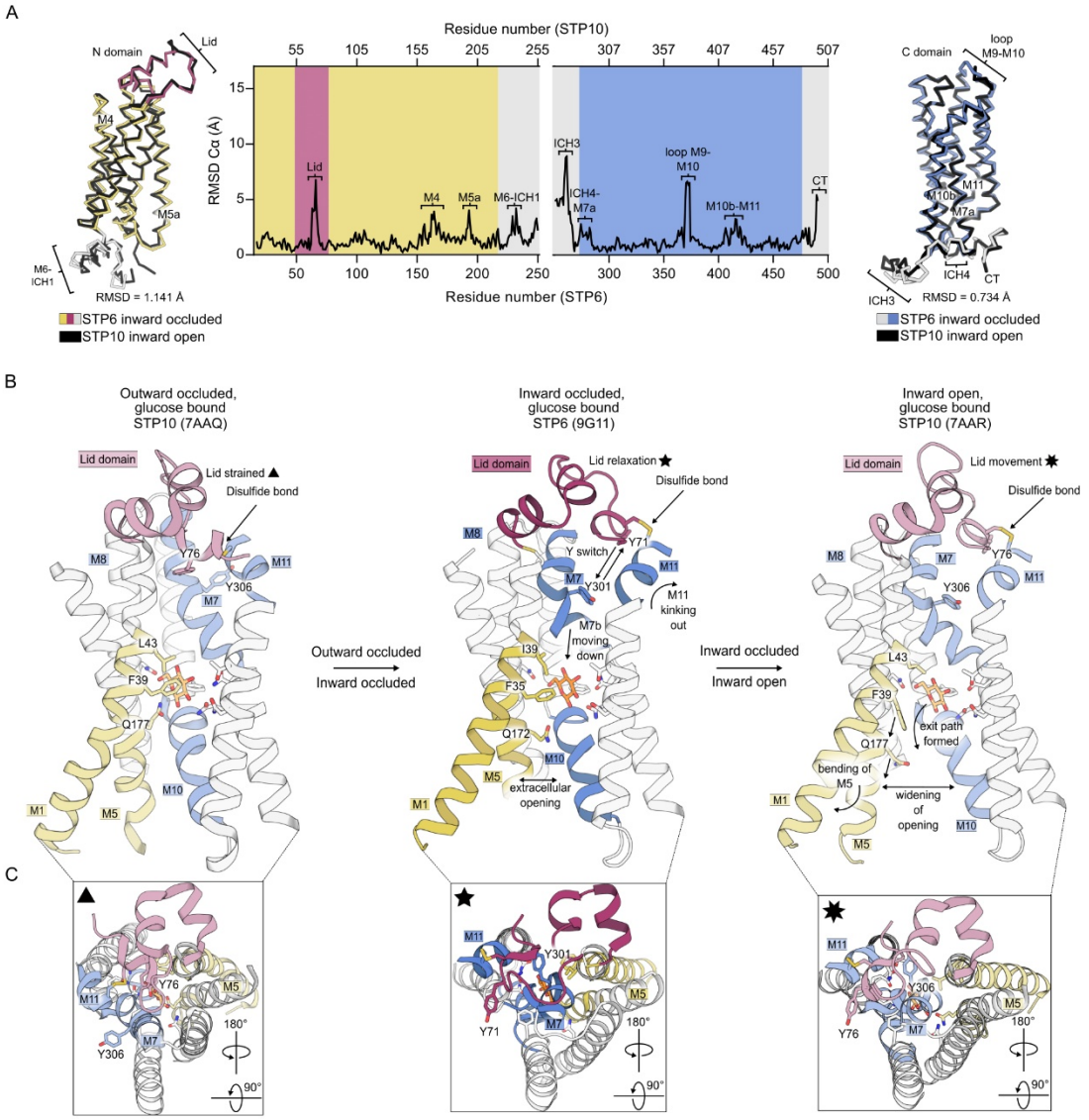


Figure 2. Transporter dynamics during the transitioning from the inward occluded to the inward open conformation

A) Cα backbone superposition and the RMSD plotted as a function of backbone position between the inward occluded (STP6, colored) and inward open (STP10, black) states of the N domain (STP6: 17-250 to STP10: 22-255) and C domain (STP6: 251-491 to STP10: 258-498). Regions of interest are highlighted on the ribbon representations of each domain.

B) Structural changes in the Lid domain (shown in close-up in panel C) and central helical bundle happening upon progression from the outward occluded to the inward open state. Regions of interest are highlighted with colors, pale for STP10 and bright for STP6. Arrows are used to indicate movement and relevant residues are shown as sticks.

C) Close-up view of rearrangements happening in the Lid domain during progression in the transport cycle from outward occluded to inward open as annotated by the symbols. Regions of interest are annotated.

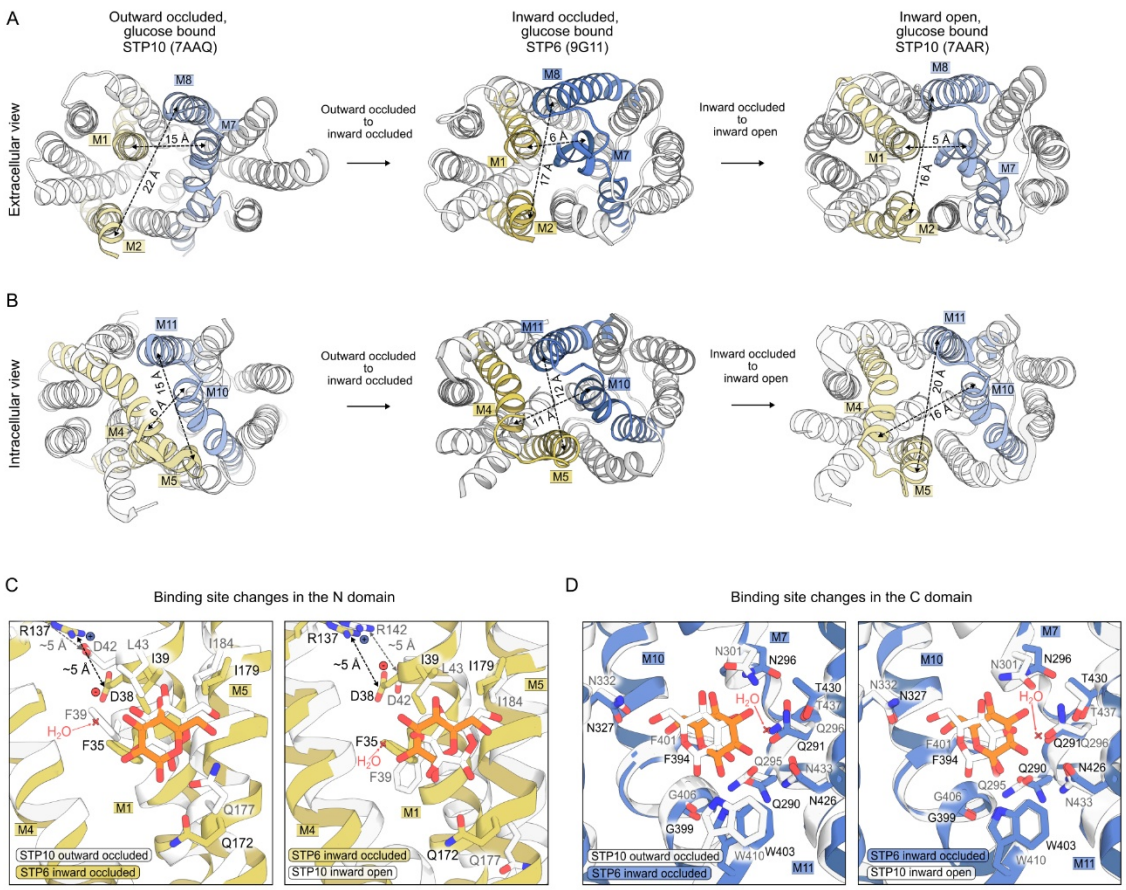


Figure 3. Extended view of transporter dynamics happening throughout substrate translocation
A) Top views showing transporter occlusion towards the extracellular side of the three conformational states, outward occluded, inward occluded and inward open. Cavity lining helices; M1, M2, M7 and M8 are highlighted with colors and the distances between them are shown.
B) Bottom views showing transport opening towards the intracellular side during progression from outward occluded to inward open conformational state. Distances between cavity lining helices; M4, M5, M10, M11 (colored) are shown.
C) Comparison of binding pocket interactions found in the N between both of the outward occluded and inward open (STP10) states and the inward occluded (STP6) state.
D) Comparison of binding pocket interactions found in the C domain. For both C and D structural superposition has been performed on the C domain. STP10 structures are shown in white, while STP6 is colored (N domain in yellow and C domain in blue). The depicted water molecules belong to the STP10 structures.

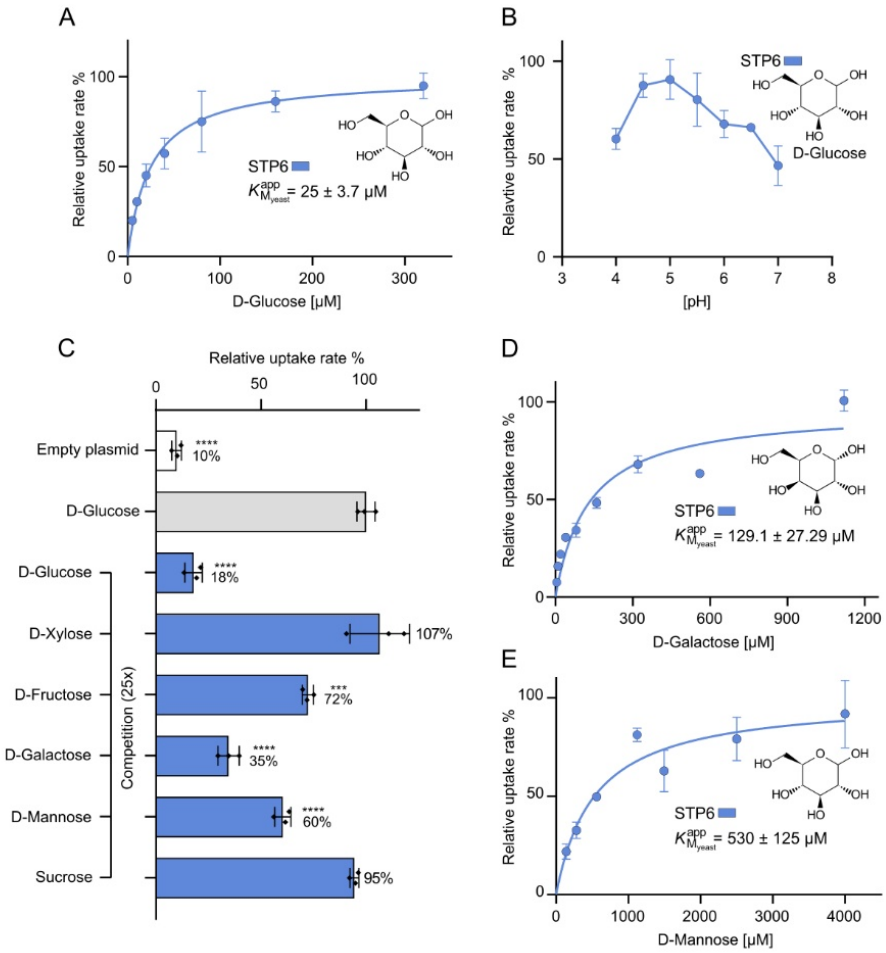


Figure 4. Transport characteristics of STP6 analyzed in yeast uptake assays

A) Michaelis-Menten analysis of D-glucose uptake by STP6 in yeast cells.

B) pH dependency of glucose uptake by STP6. Assays were performed using 10 µM D-glucose in yeast cells at different pH values.

C) Substrate specificity of STP6 assed by competition assay performed in yeast cells with 10 µM glucose and 250 µM of the competing sugar. *P* values have been obtained by one-way ANOVA analysis *** stars $p = 0.0002$ and **** = $p < 0.0001$.

D) Michaelis-Menten analysis of D-galactose uptake by STP6 in yeast cells. E Michaelis-Menten analysis of D-mannose by STP6 in yeast cells. Datapoints represent the mean ± SD of n = 3 experiments. Assays were performed at pH 5 unless stated otherwise.

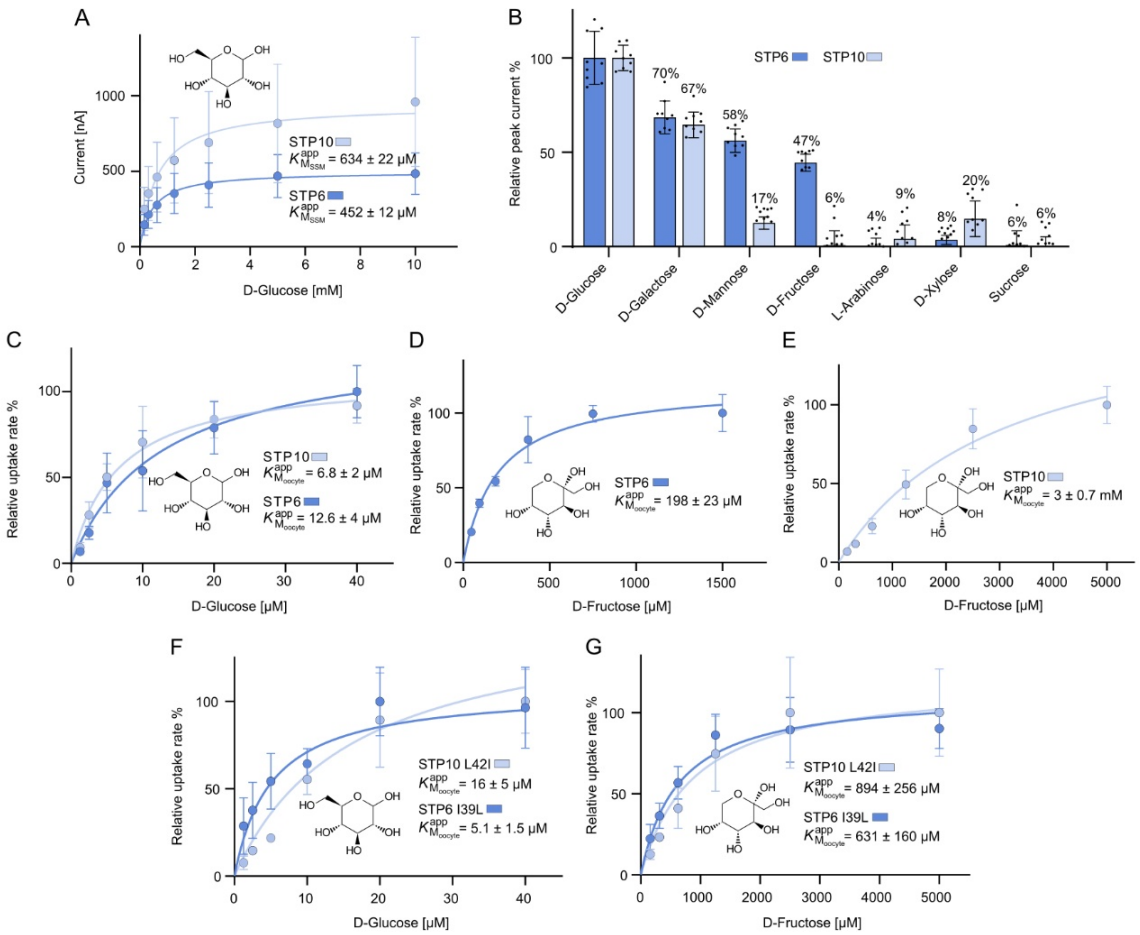


Figure 5. *In vivo* and *in vitro* side-by-side comparison of STP6 and STP10 transport characteristics

A) Michaelis-Menten analysis of peak currents measured by SSM-based electrophysiology on STP6 and STP10 reconstituted in proteoliposomes when titrated with D-glucose.

B) Substrate specificity comparison of STP6 and STP10 measured by peak currents conducted from SSM-based electrophysiology when subjected to 10 mM of a range of sugars. Datapoints represent peak currents measured for each substrate at a concentration of 10 mM. Datapoints represent the mean ± SD of n = 3 experiments. *P* values shown were obtained by one-way ANOVA analysis. SSM experiments were conducted at a symmetrical pH of 7.4

C) Michaelis-Menten analysis of D-glucose uptake by STP6 and STP10 determined in *Xenopus* oocytes.

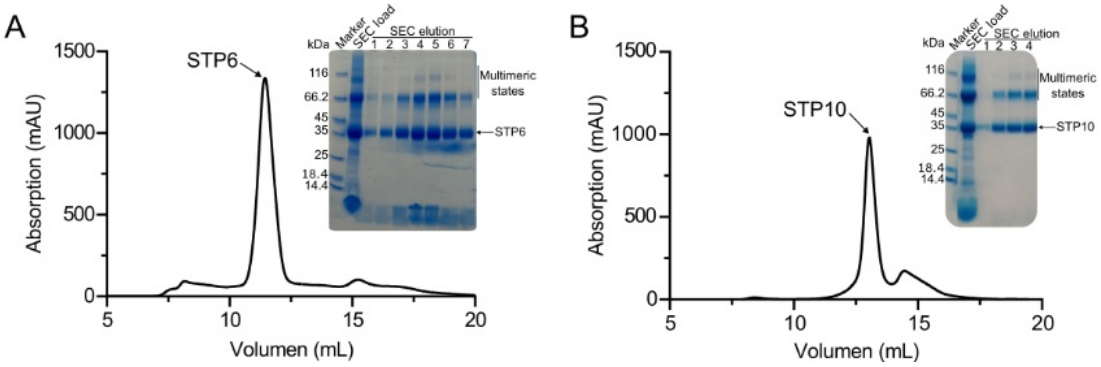
D) Michaelis-Menten analysis of D-fructose uptake by STP6 in *Xenopus* oocytes.

E) Michaelis-Menten analysis of D-fructose uptake by STP10 in *Xenopus* oocytes.

F) Michaelis-Menten analysis on D-glucose uptake by binding site switch mutants, STP6 I39L and STP10 L43I in *Xenopus* oocytes.

G) Michaelis-Menten analysis on D-fructose uptake by binding site switch mutants, STP6 I39L and STP10 L43I in *Xenopus* oocytes. Datapoints represent the mean ± SD of n = 4-6 biologically independent experiments. *Xenopus* oocyte experiments were performed at pH 5.

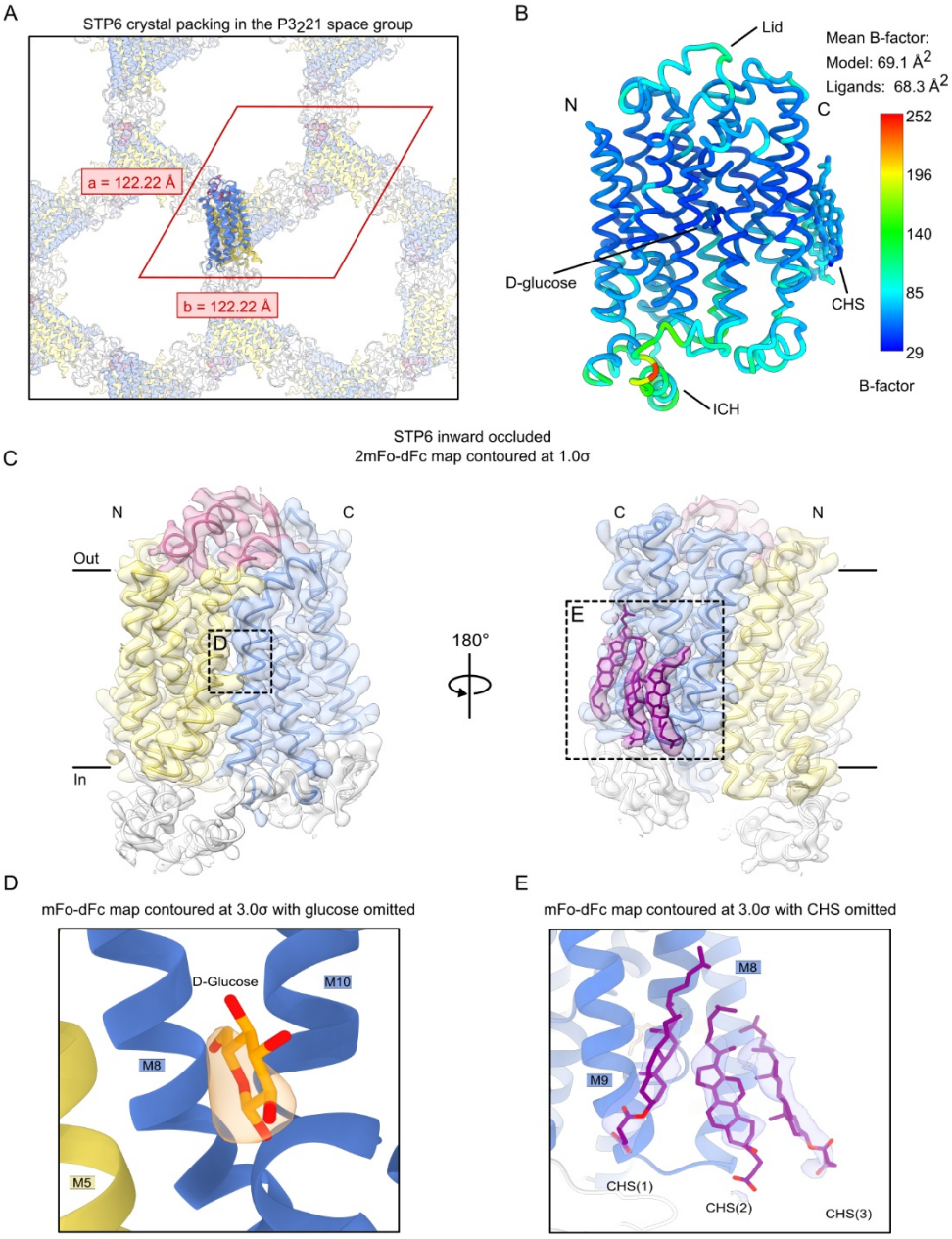
SUPPLEMENTARY FIGURES, TABLE AND LEGENDS



Supplementary Figure 1. Expression and purification profiles of STP6 and STP10

A) Size-exclusion chromatography profile of STP6 with representative SDS-PAGE depicting protein purity.

B) Size-exclusion chromatography profile of STP10 with representative SDS-PAGE depicting protein purity. Expression and purification of both targets have been carried out multiple times with reproducible results.



Supplementary Figure 2. STP6 crystal packing and validation of ligands present in the asymmetric unit

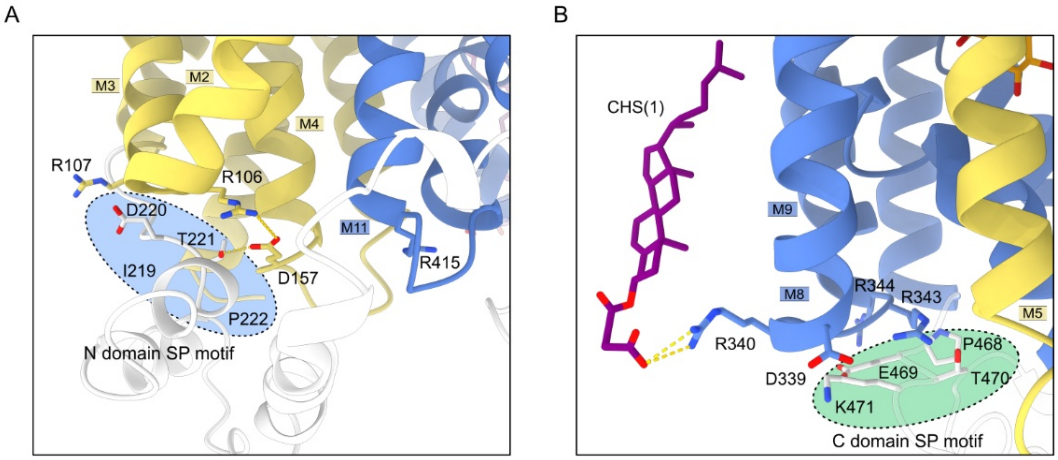
A) The crystal packing of STP6 with the asymmetric unit highlighted by the red rhombus. The a and b axis are shown in red.

B) Ribbon representation of the STP6 backbone colored according to the atomic displacement factor (B- factor) with a gradient from blue (29 $Å^2$) to red (252 $Å^2$).

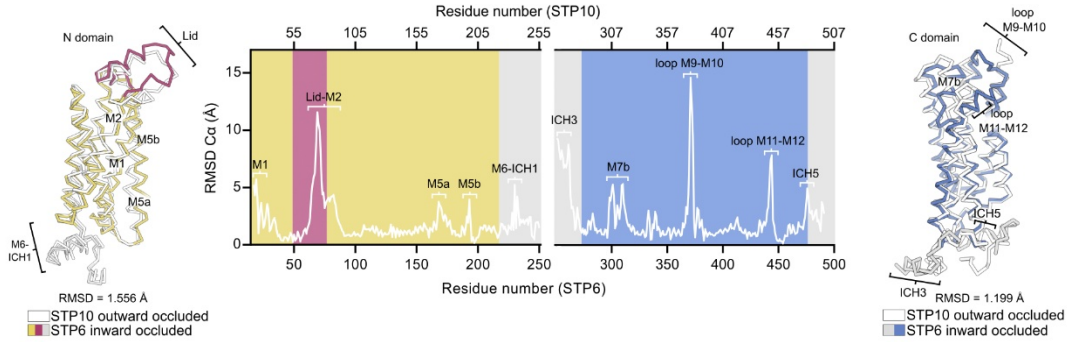
C) The 2mFo-dFc electron density after the final refinement (contoured at 1σ) and shown as transparent surface, with the final model overlaid. Ligand bound regions are highlighted in panel D and E.

D) The mFo-dFC map (contoured at 3σ) calculated after omitting the glucose in the final model.

E) The mFo-dFC map (contoured at 3σ) calculated after omitting the CHS molecule in the final model.

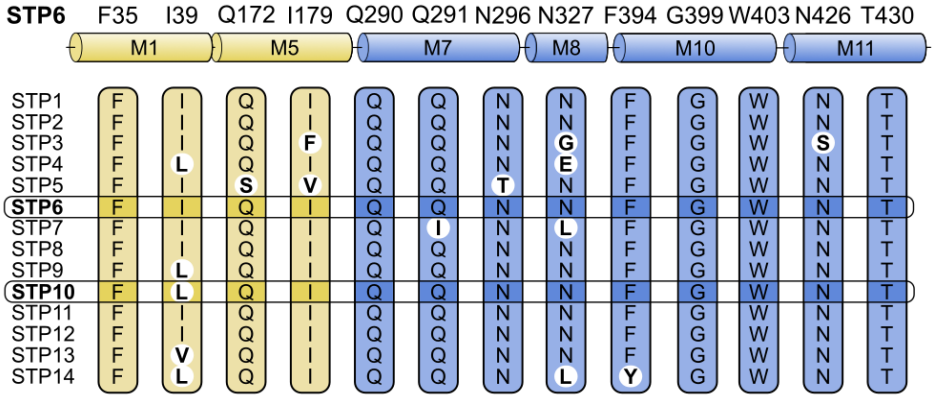


Supplementary Figure 3. Close-up view of the SP and A motifs in the STP6 crystal structure
A) Interactions present between the SP and A motifs in the N domain. Residues from both motifs are shown as sticks and the SP motif is highlighted by the blue oval.
720 **B)** The SP and A motif of the C domain including a close-up view of the salt-bridge formed between R340 and the CHS head group. Residues belonging to the SP motif are highlighted by the green oval. Yellow dashes indicate interactions between residues.



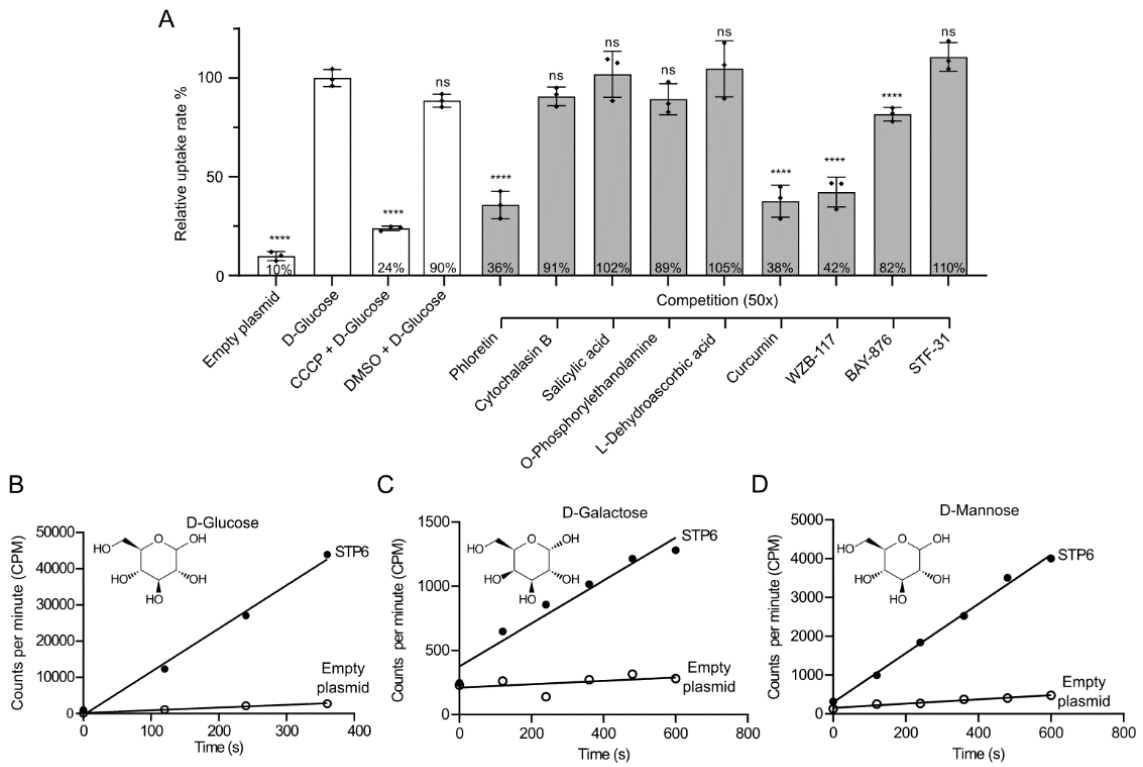
Supplementary Figure 4. Transporter dynamics during progression from the outward occluded to the inward occluded conformational state

Cα backbone superposition and the RMSD plotted as a function of backbone positioning between the inward occluded (STP6, colored) and outward occluded (STP10, white) states of the N domain (STP6: 17-250 to STP10: 22-255) and C domain (STP6: 251-491 to STP10: 258-498). Regions of interest are highlighted on the ribbon representations of each domain. The STP6 domain coloring follows the schematic shown in the main figures. N domain yellow, Lid domain magenta, ICH domain white and C domain blue.



Supplementary Figure 5. Multiple sequence alignment of residues found in the binding site of *A. thaliana* STPs

Binding site alignment of: STP1 (accession number P23586), STP2 (accession number Q9LNV3), STP3 (accession number Q8L7R8), STP4 (accession number Q39228), STP5 (accession number Q93Y91), STP6 (accession number Q9SFG0), STP7 (accession number O04249), STP8 (accession number Q9SBA7), STP9 (accession number Q9SX48), STP10 (accession number Q9LT15), STP11 (accession number Q9FMX3), STP12 (accession number O65413), STP13 (accession number Q94AZ2) and STP14 (accession number Q8GW61). Residues located in the N domain are shown in yellow bars and residues located in the C domain are shown in blue bars. The corresponding helix topology is shown on the top with numbers referring to STP6. Non-conserved residues are highlighted with a white circle and bold font. The STPs concerning this study, STP6 and STP10, are outlined. The only difference in the binding site between STP6 and STP10 is seen on position 39 (STP6) in M5, where an isoleucine instead is a leucine in STP10. STP5 and STP7, which both contain unique sequence deviations have been reported to be non-functional (Büttner, 2010).



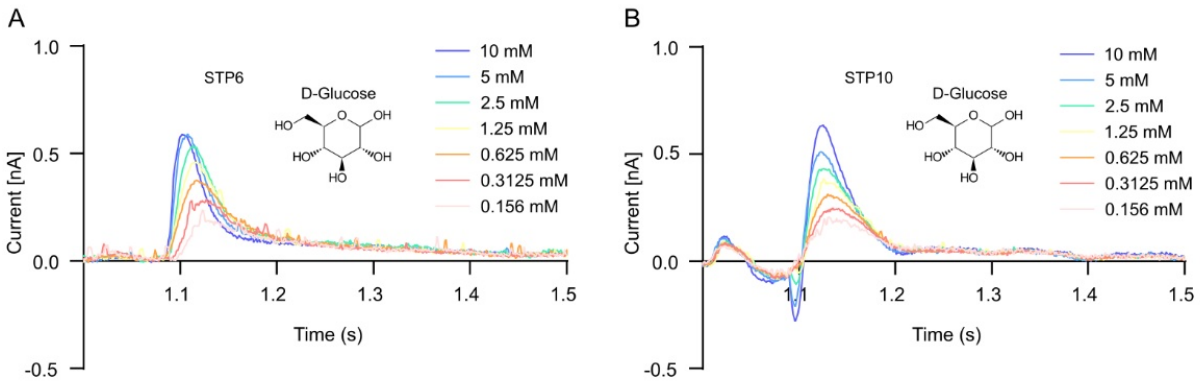
Supplementary Figure 6. Functional characterization of STP6 in yeast uptake assays

A) Glucose competition assay on STP6 with a range of potential inhibitors performed in yeast cells using 10 µM of D-glucose and 500 µM of each of the tested inhibitors, expect for CCCP where 100 µM was used. Datapoints represent mean ± SD of n = 3 replicate experiments. P-values for the competition assay were calculated by one-way ANOVA followed by Dunnett's multiple comparisons test. **** = $p < 0.0001$.

B) Time dependent uptake of D-glucose by STP6 (black circles) or empty plasmid (empty circles) into yeast cells.

C) Time dependent uptake of D-galactose by STP6 (black circles) or empty plasmid (empty circles) into yeast cells.

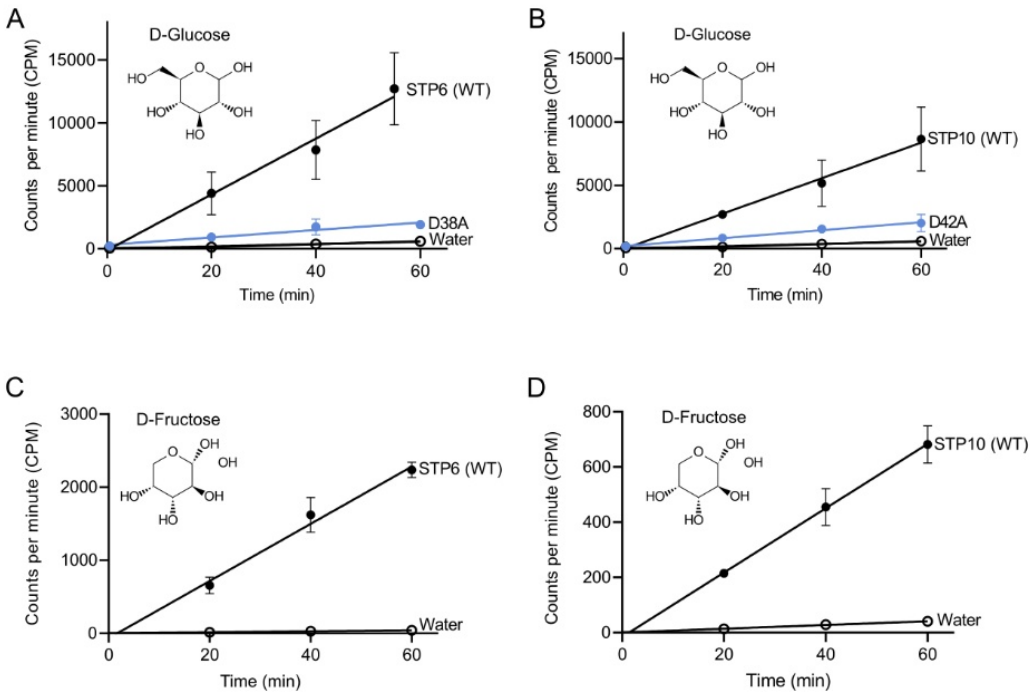
D) Time dependent uptake of D-mannose by STP6 (black circles) or empty plasmid (empty circles) into yeast cells. All of the above experiments were carried out at pH 5.



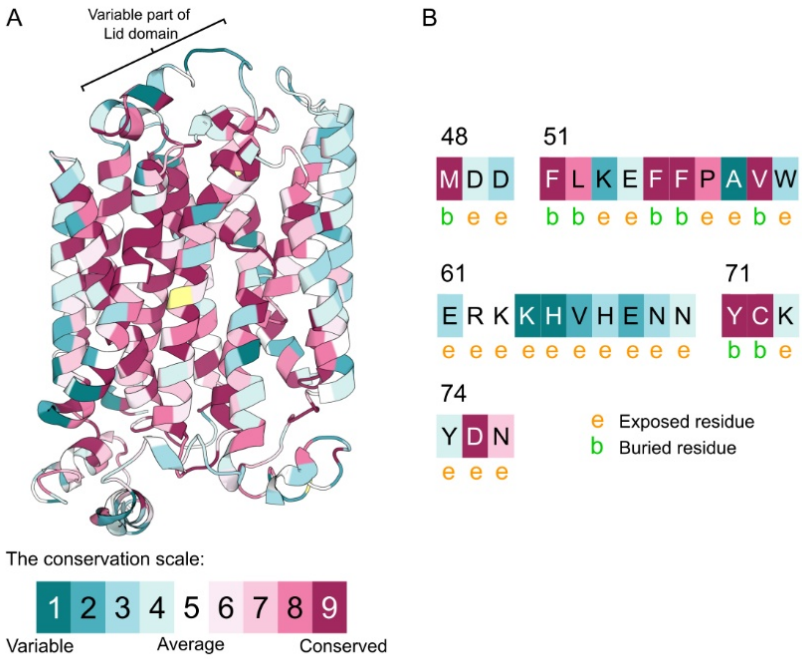
Supplementary Figure 7. Transport activity of STP6 and STP10 determined by SSM-based electrophysiology

A) Raw traces of currents obtained by SSM-based electrophysiology when titrating STP6 containing proteoliposomes with a range of D-glucose (0.156 – 10 mM).

B) Raw traces of currents obtained when titrating STP10 containing proteoliposomes with a range of D-glucose (0.156 – 10 mM). Traces show data obtained from a representable experiment performed on one sensor with three technical replicates. Experiments were performed at a symmetrical pH of 7.4.



Supplementary Figure 8. Transport activity tested in *Xenopus oocytes* for STP6 and STP10
A) Time dependent uptake of 10 µM D-glucose into *Xenopus oocytes* expressing WT STP6 (black circles), STP6 D38A (blue circles) or water-injected oocytes (empty circles).
B) Time dependent uptake of 10 µM D-glucose into *Xenopus oocytes* expressing WT STP10 (black circles), STP10 D42A (blue circles) or water injected oocytes (empty circles).
C) Time dependent uptake of 10 µM D-fructose into *Xenopus* oocytes expressing WT STP6 (black circles) or water-injected oocytes (empty circles).
D) Time dependent uptake of 500 µM D-fructose into *Xenopus* oocytes expressing STP10 (black circles) or water injected oocytes (empty circles). All experiments were performed at pH 5 and data points represent mean ± SD of n = 3-5 biologically independent experiments.



Supplementary Figure 9. Sequence conservation analysis of *A. thaliana* STPs

A) Sequence conservation analysis of the 14 members of the *A. thaliana* STP family represented on the crystal structure of STP6 colored from teal (variable) to bordeaux (conserved). Yellow coloring indicates regions where the data provided was insufficient to calculate a conservation score (region present in < 10% of the data provided).

B) Sequence conservation of the STP6 Lid domain along with the predicted exposure of each residue (e = exposed residue, b = buried residue). Exposed residues are seen to be highly variable. Conservation analysis was performed utilizing ConSurf (Ben Chorin *et al*, 2020).

Supplementary Table 1: Data collection and refinement statistics.

Name	**STP6**
State	Inward occluded
Data Collection	
Space group	P 3_2 2 1
Cell dimensions	
a. b, c (Å)	122.22 122.22 139.09
alpha, beta, gamma (deg)	90 90 120
Monomers per asym. unit.	1
Wavelenght (Å)	0.9686
Number of reflections measured	38.166
Number of unique reflections	19.452 (2813)[a]
Resolution (Å)	84.23-3.20 (3.31-3.20)
Rmeas (%)	14.8 (92.2)
Mean I/σ(I)	7.6 (1.3)
CC(1/2)	98.5 (34.6)
Completeness (%)	95.91 (99.08)
Redundancy	2.0 (1.3)
Refinement	
Resolution (Å)	84.23-3.20 (3.31-3.20)
No. reflections (work/free)	19,451 / 922
Rwork (%)	22.65
Rfree (%)	25.16
Twin fraction	0.47
Twin law	-h,-k,l
No. of Atoms	
Protein	3708
Ligands	117
Average B Factors (Å)	
Overall	69.1
Protein	69.1
Ligands	68.3
RMSD	
Bond lenghts (Å)	0.0047
Bond angles (deg)	0.87
Validation	
MolProbity score	1.63
Clashscore	7.61
Rotamer outliers (%)	0.00
Ramachandran Plot Statistics	
Favored regions	96.62
Allowed regions	3.38
Disallowed regions	0.00
Deposited model (PDB ID)	9G11

[a] Highest resolution shell is shown in parenthesis.

Supplementary Table 1. Data collection and refinement statistics