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The Arabidopsis SWEET1 and SWEET2 uniporters recognize similar substrates while differing in subcellular localization

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Sugars Will Eventually be Exported Transporters (SWEETs) are central for sugar allocation in plants. The SWEET family has approximately 20 homologs in most plant genomes, and despite extensive research on their structures and molecular functions, it is still unclear how diverse SWEETs recognize different substrates. Previous work using SweetTrac1, a biosensor constructed by the intramolecular fusion of a conformation-sensitive fluorescent protein in the plasma membrane transporter SWEET1 from *Arabidopsis thaliana*, identified common features in the transporter's substrates. Here, we report SweetTrac2, a new biosensor based on the Arabidopsis vacuole membrane transporter SWEET2, and use it to explore the substrate specificity of this second protein. Our results show that SWEET1 and SWEET2 recognize similar substrates but some with different affinities. Sequence comparison and mutagenesis analysis support the conclusion that the differences in affinity depend on nonspecific interactions involving previously uncharacterized residues in the substrate-binding pocket. Furthermore, SweetTrac2 can be an effective tool for monitoring sugar transport at vacuolar membranes that would be otherwise challenging to study.

Sugars are the main products of plant photosynthesis, and efficiently mobilizing them from chloroplasts to other organs and tissues is crucial for many biological processes. Members of the SWEET family are uniporters found in both plasma and vacuolar membranes that play crucial roles in plant growth and development, pathogen susceptibility, and stress tolerance (1, 2). Consequently, extensive research is being conducted to capitalize on these proteins for crop improvement since their discovery in 2010 (3, 4).

SWEETs are also small, making them good molecular models for exploring sugar recognition mechanisms. Most eukaryotic SWEETs have seven transmembrane domains, while the bacterial homologs, the SemiSWEETs, have only three transmembrane domains and form dimers to complete the sugar translocation path (5–7). SWEETs are smaller than other sugar transporters with available crystal structures, such as the human GLUT1, the *Escherichia coli* LacY, and the *Vibrio parahaemolyticus* SGLT (8, 9). The minimal size of SWEETs facilitates cloning, purification, heterologous expression, and mutagenesis studies. In fact, mutations of 13% of the amino acids in the Arabidopsis SWEET1 (AtSWEET1, 27 kDa) protein have been characterized so far (2), a large fraction compared with most other transporters.

Unlike plasma membrane transporters like AtSWEET1, vacuolar membrane transporters are considerably more difficult to characterize. Most studies on vacuolar proteins start with the isolation of vacuoles, which are very fragile and require careful handling (10, 11). Purifying and testing the transporters afterward is even more challenging since they only represent about 1% of the total cell protein and must go through labor-intensive solubilization and reconstitution steps (12). Apart from isolating vacuoles, generating cell-free artificial lipid vesicles to incorporate purified vacuolar transporters can also be used to characterize this type of proteins (6). When sufficient amounts of transporters are successfully embedded into such artificial vesicles, radiolabeled substrates are commonly used to test uptake and efflux. If sugar translocation is accompanied by the movement of charged ions (*e.g.*, H^+), sophisticated methods such as solid-supported membrane–based electrophysiology could be used instead (13). Despite the complications associated with studying vacuolar sugar transporters, they should not be neglected, as the vacuole is the main sugar storage organelle and is an important regulator of sugar dynamics in plant cells (14).

An alternative to these traditional characterization methods is the use of transporter biosensors, which are chimeras of transporters and fluorescent proteins that translate the conformational changes of the transporter into a fluorescence response (15). These biosensors can be a powerful tool to study transporter activity in real-time, and a few have been successfully engineered for plant proteins, including SweetTrac1, a biosensor based on AtSWEET1 (16–19). We previously proposed a kinetic model that describes the dynamic fluorescence response of SweetTrac1 to D-glucose (16). More recently, we employed SweetTrac1 and cheminformatics to investigate the substrate specificity of AtSWEET1, enabling us to find potential substrates of AtSWEET1 easily without the need for radiolabeled chemicals or individual intracellular sensors for each chemical (20). Specifically, we tested the binding of 182 natural and artificial carbohydrates to SweetTrac1 using fluorescence as a proxy, confirmed the transport of six substrates by the transporter (including D-glucose, D-

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mannose, and D-fructose), and identified nine new other potential substrates.

In this work, we convert the vacuole membrane localized AtSWEET2 (26 kDa) uniporter into the biosensor SweetTrac2 and characterize its substrate specificity. We show that while differing in subcellular localization, AtSWEET1 and AtSWEET2 recognize similar substrates. Lastly, sequence comparison of AtSWEET1 and AtSWEET2 and mutagenesis analysis of SweetTrac1 identified three residues in AtSWEET1 responsible for tuning its affinity for some substrates. These results illustrate the potential of using biosensors to accelerate transporter characterization and protein engineering.

Results

Generating and photophysically characterizing SweetTrac2

AtSWEET2 is a vacuole transporter that facilitates sugar storage in roots (21, 22). AtSWEET1 and AtSWEET2 are both classified as clade I SWEETs that prefer hexoses as substrates (23). Given the 44% sequence identity of these two proteins (Fig. 1*A*), we hypothesized that transferring the circularly permutated, superfolded GFP and linkers of SweetTrac1 into the same position in AtSWEET2 would result in another successful biosensor. As predicted, this approach generated the new biosensor SweetTrac2, which responded to the addition of D-glucose in a similar manner to SweetTrac1 when expressed in yeast (Fig. 1*B*).

The subcellular localization of both c-terminus tagged AtSWEET1 and AtSWEET2 in yeast cells mirrors that of the natural transporters *in planta* (21, 24). Similarly, SweetTrac1 localized to the plasma membrane (16), while SweetTrac2 localized to the vacuole (Fig. 1*C*).

Spectra analysis of SweetTrac2 revealed two excitation maxima—a major peak from the deprotonated chromophore at a wavelength of ∼490 nm and a minor peak from the protonated chromophore at a wavelength of ∼410 nm. A single emission maximum was observed at a wavelength of ∼515 nm (Fig. 1*B*). The peak fluorescence intensity increased with D-glucose addition, and no shift in excitation and emission maxima was observed (Fig. 1*B*).

AtSWEET2 can recognize 14 chemicals transported by AtSWEET1

In our previous work (20), we expressed SweetTrac1 in yeast, screened a custom library of sugar and sugar analogs, and performed a cheminformatics analysis of the results. In total, we tested the binding of 182 natural and synthetic carbohydrates to SweetTrac1, consisting predominantly of sugar acids (15%), amino sugars (12%), disaccharides (9%), sugar alcohols (8%), sugar phosphates (8%), aldoses (5%), and ketoses (3%). Several L- (5%), methyl (3%), deoxy (3%), alkyl (2%), thio (2%), and thio ester (2%) sugars, as well as cyclitols (2%), were also present in the custom library. We identified 15 chemicals capable of inducing a fluorescence response by SweetTrac1, suggesting they can bind the transporter's substrate-binding pocket (20). Three of these hits (D-glucose, D-fructose, and D-mannose) were known substrates of AtSWEET1, and we confirmed their cellular uptake using radiolabeled versions of these sugars. We also confirmed that AtSWEET1 could mediate the cellular uptake of three other hits (1-deoxynojirimycin, voglibose, and

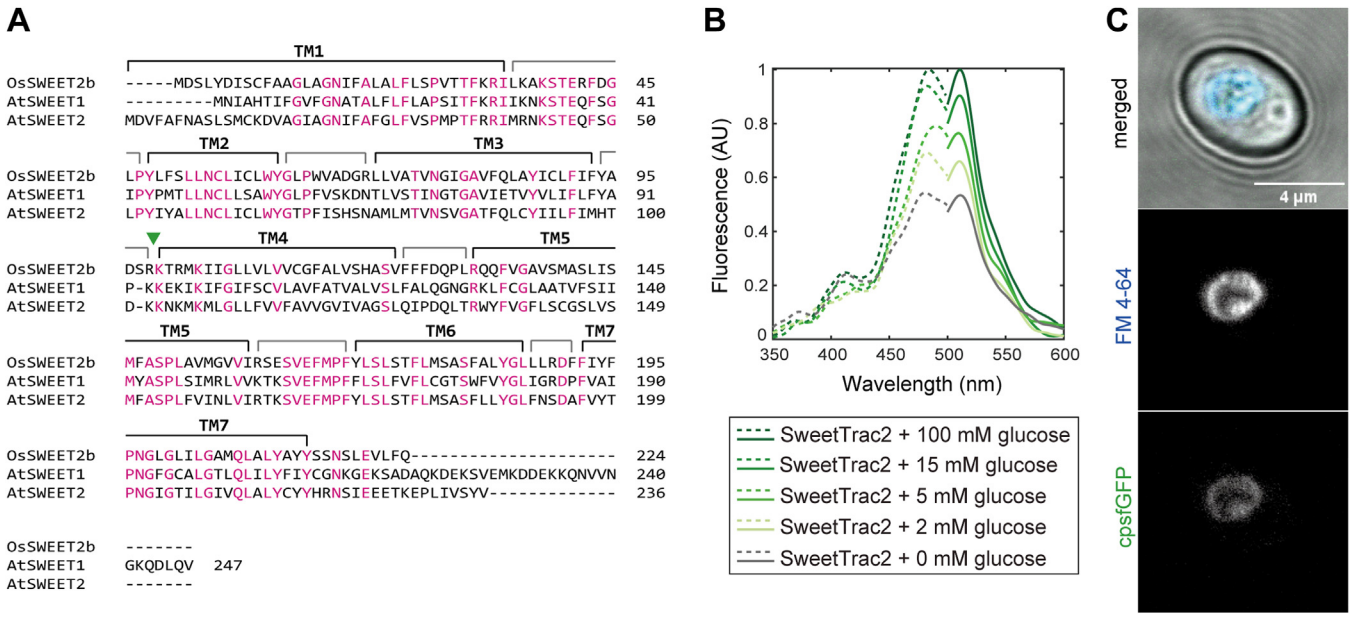

Figure 1. Characterization of the SweetTrac2 biosensor. *A*, multiple sequence alignment of OsSWEET2b, AtSWEET1, and AtSWEET2. Highlighted in *magenta* are amino acids that are conserved in the three proteins. Transmembrane (TM) domains based on an alignment with OsSWEET2b are marked above the sequence. *Green arrowhead* indicates the positions where the linkers and cpsfGFPs were inserted for the construction of SweetTrac1 and SweetTrac2. *B*, normalized fluorescence excitation and emission spectra of SweetTrac2 (455 nm excitation, 530 nm emission) at increasing concentrations of D-glucose. *Dashed lines* illustrate excitation, and *solid lines* illustrate emission. *C*, localization of SweetTrac2 to the vacuolar membrane in yeast cells. FM 4-64 was used to stain the vacuolar membrane. The scale bar represents 4 µm. cpsGFP, circularly permutated, superfolded GFP; SWEET, Sugars Will Eventually be Exported Transporter.

1-thio-D-glucose), which have adverse effects on yeast growth (20). Unfortunately, this previous study did not clarify whether the remaining nine hits were substrates of AtSWEET1 or competitive inhibitors (capable of binding the transporter but not translocated to the cytosolic side of the membrane), as radiolabeled versions of them are not commercially available and they did not hinder cell growth.

Given the relatively high sequence identity of AtSWEET1 and AtSWEET2 compared to other Arabidopsis SWEETs, we hypothesized that AtSWEET1 and AtSWEET2 would recognize similar substrates. SweetTrac2, our proxy to AtSWEET2, is localized in the vacuole and can only respond to intracellular sugars. Thus, when coexpressed with AtSWEET1, the SweetTrac2 biosensor could help investigate whether the nine remaining hits identified in our previous work are *bona fide* substrates of AtSWEET1.

To this end, we first generated a yeast strain where AtSWEET1 is the sole hexose transporter on the plasma membrane. To achieve that, we integrated the *AtSWEET1* coding sequence under the control of the constitutive GPD promoter into the genome of the EBY4000 strain, which lacks all endogenous hexose transporters. Subsequent expression of SweetTrac2 using a multicopy plasmid in the vacuole of these modified cells allowed us to test if any chemicals could be taken up *via* AtSWEET1 (Fig. 2*A*).

SweetTrac2 showed increased fluorescence in response to 14 out of the 15 hits that were discovered in our previous work (Fig. 2), confirming that AtSWEET1 can mediate the cellular uptake of the majority of the chemicals capable of inducing a fluorescence response in SweetTrac1 (20). The only molecule that did not produce a fluorescence response was 1-deoxy-1-morpholino-D-fructose, suggesting that this modified sugar may be a competitive inhibitor of AtSWEET1 rather than a substrate or that it may not be able to bind AtSWEET2.

Next, we measured the fluorescence response of SweetTrac2 to different concentrations of the 14 chemicals using our engineered strain. However, modifications to our protocol were required to quantify the affinity of SweetTrac2 for D-glucose, D-fructose, and D-mannose, as the catabolism of these sugars produced variability in fluorescence measurements. We reasoned that increasing the amount of *AtSWEET1* template DNA using a multicopy plasmid would result in higher levels of protein and higher rates of sugar uptake, allowing cytosolic and extracellular concentrations to equilibrate faster and offsetting the consumption of these sugars by glycolysis, consistent with previous observations that the use of multicopy plasmids results in higher transgenic protein levels (25). Indeed, protein levels of AtSWEET1 were higher in plasmid-transformed cells than in the genome-integrated lines (Fig. S1*A*), which also correlated with higher influx of D-glucose into the plasmid-transformed cells as suggested by their higher rates of sugar utilization and cell growth (Fig. S1*B*). As expected, the overexpression of *AtSWEET1* resulted in less variability in the fluorescence responses of SweetTrac2 to different concentrations of D-glucose, D-fructose, and D-mannose (Fig. 2, *B–D*). The remaining 11 chemicals did not display obvious signs of being metabolized and were tested in the engineered strain where *AtSWEET1* is integrated into the genome (Fig. 2, *E–O*).

The affinity of SweetTrac2 for the different chemicals that produced a fluorescence response can be quantified using an equilibrium exchange constant (KR_0/R), which we previously defined as the concentration of substrate that would saturate half of the biosensor at a steady state when the intracellular concentration of a substrate had reached equilibrium with the extracellular one (16). SweetTrac2 displayed the highest affinity for D-glucose (KR_0/R = 3 ± 1 mM, Fig. 2*B*) and the lowest for D-fructose (KR_0/R = 122 ± 38 mM, Fig. 2*D*), intimating that D-glucose is the preferred substrate of AtSWEET2.

We point out that the results for D-turanose need to be interpreted cautiously (Fig. 2*J*). Both EBY4000 cells expressing AtSWEET1 and cells transformed with an empty vector grow in the presence of D-turanose (Fig. S2). This suggests that there may be other transporters capable of mediating the uptake of D-turanose in the EBY4000 strain. Moreover, D-turanose is broken down by α-glucosidase in yeast cells into D-glucose and D-fructose (26). Hence, the steady-state response of SweetTrac2 to D-turanose is likely a composite response to the multiple sugars rather than only the disaccharide. This may explain why the KR_0/R value for D-turanose is remarkably close to the value for D-glucose.

Investigating the basis of substrate specificity using SweetTrac1

We were surprised to discover the differences in KR_0/R between SweetTrac1 and SweetTrac2 (Table 1). Among the 14 chemicals that could bind SweetTrac2, we noticed that D-mannose, D-glucopyranosyl amine, D-turanose, 1-amino-2,5-anhydro-1-deoxy-D-mannitol, and *sn*-glycerol 3-phosphate (Fig. 2, *C*, *I–K*, *O*) showed KR_0/R values that were at least 3-fold lower than previously reported for SweetTrac1, while the KR_0/R values for the other nine chemicals were closer in value for both biosensors (Table 1). We reasoned that the differences in KR_0/R may be associated with differences between the substrate binding pocket of AtSWEET1 and AtSWEET2. Therefore, we decided to investigate this idea experimentally.

Given our results and ample mutagenesis studies performed on AtSWEET1 (2), we propose that substrate recognition by SWEETs relies on a combination of specific and nonspecific interactions. The specific interactions consist of hydrogen bonds formed between key hydroxyl groups in the substrates and conserved residues in the transporters, such as N73 and N192 in AtSWEET1 (2). We previously showed that mutating N73 and N192 abolished the fluorescence response of SweetTrac1 (16). Nonspecific interactions are likely mediated by hydrophobic residues that determine the size and tortuosity of the binding pocket and may better explain the subtle differences in affinities between the two biosensors (Table 1). The role of the binding pocket size has been previously demonstrated for bacterial SemiSWEETs and the disaccharide transporter AtSWEET13 (7, 27, 28).

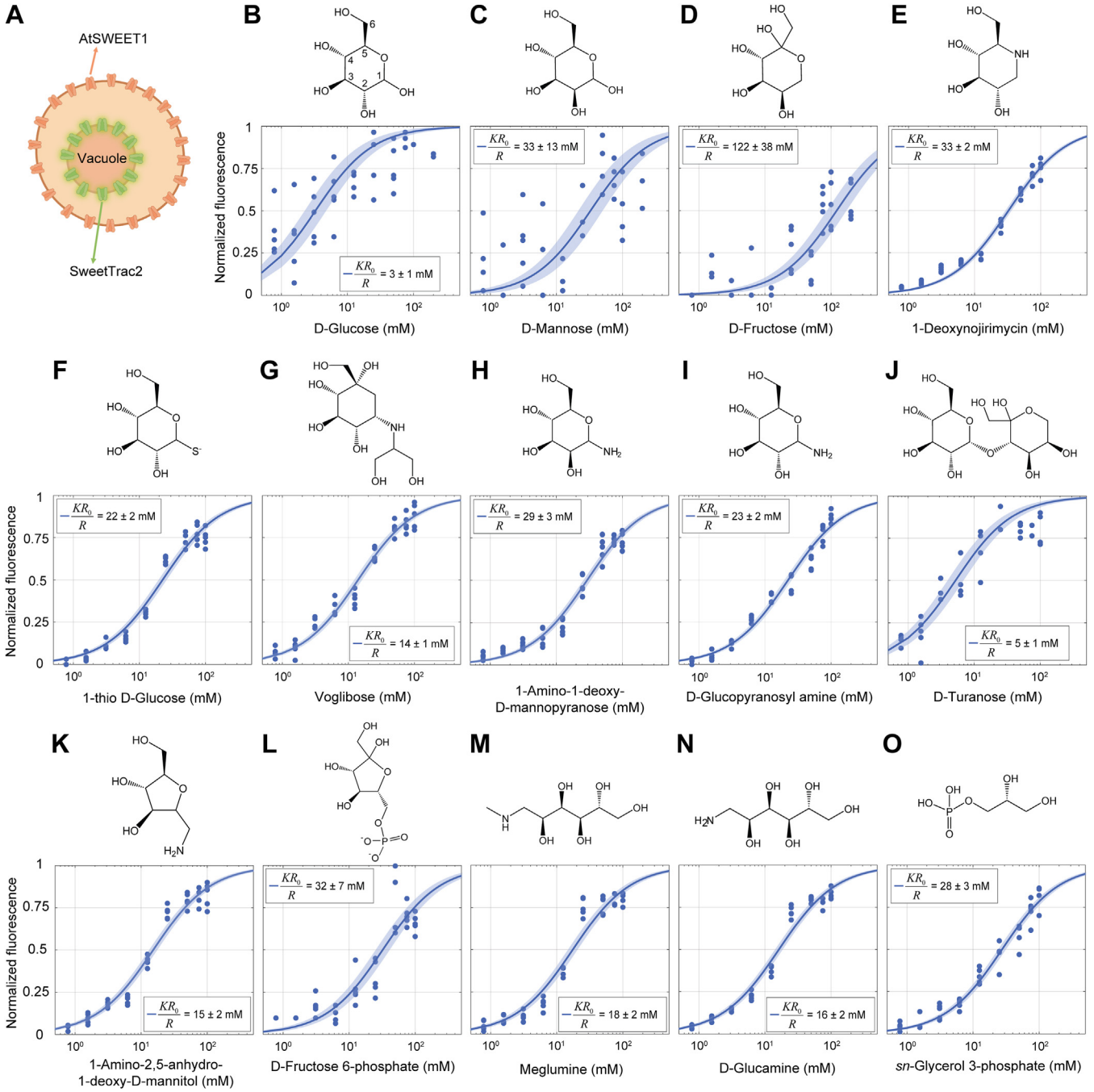

Figure 2. SweetTrac2 recognizes 14 compounds transported by AtSWEET1. *A, cartoon* describing the yeast cell that was used to collect the steady-state response of SweetTrac2. AtSWEET1 (*orange*) is the solo hexose transporter on the plasma membrane, and SweetTrac2 (*green*) is expressed on the vacuolar membrane of EBY4000 cells. *B–O*, SweetTrac2's steady-state response to sugar and sugar analogs transported by AtSWEET1. The carbons are numbered for D-glucose. The sugar concentrations listed correspond to the levels in the extracellular media. *Blue solid lines* represent uniporter model fit as described in Park *et al.* 2022 (16), and the *shaded areas* represent 95% confidence intervals. Equilibrium exchange constants are reported as estimated ± 95% confidence intervals (n = 5). All chemical structures are depicted in their most probable conformation in aqueous solution. SWEET, Sugars Will Eventually be Exported Transporter.

From molecular docking simulation results using the available crystal structure of the rice (*Oryza sativa*) SWEET2b in the inside-open conformation (6) and D-glucose, D-fructose, and D-mannose, we selected three hydrophobic residues (V73, V76, and I193) in the binding pocket that are most likely to be directly involved in the interaction with the three sugars (Fig. 3). This corresponds to residues V69, I72, and V188 in AtSWEET1.

Next, we performed alanine, isoleucine, and leucine substitutions of the V69, I72, and V188 residues of SweetTrac1 (numbering based on the sequence on AtSWEET1) (Fig. 1). Except for I72A, all other mutant biosensors correctly

Table 1
Steady-state response of SweetTrac1 and SweetTrac2 to different chemicals

Chemicals	SweetTrac1 (mM)	SweetTrac2 (mM)
D-Glucose	8 ± 1	3 ± 1
D-Mannose	**107 ± 13**	**33 ± 13**
D-Fructose	274 ± 42	122 ± 38
1-Deoxynojirimycin	20 ± 2	33 ± 2
1-Thio-D-glucose	17 ± 2	23 ± 2
Voglibose	19 ± 4	14 ± 1
1-Amino-1-deoxy-D-Mannopyranose	28 ± 3	29 ± 3
1-Glucopyranosyl amine	**120 ± 10**	**23 ± 2**
D-Turanose	**33 ± 5**	**5 ± 1**
1-Amino-2,5-anhydro-1-deoxy-D-mannitol	**121 ± 10**	**15 ± 2**
D-fructose 6-phosphate	25 ± 3	32 ± 7
Meglumine	17 ± 3	18 ± 2
D-Glucamine	29 ± 3	16 ± 2
***sn*-Glycerol 3-phosphate**	**102 ± 42**	**28 ± 3**

Comparison of the equilibrium exchange constants measured for SweetTrac2 and those for SweetTrac1 reproduced from Park *et al.* 2023 (20). Values reported as estimated ± 95% confidence intervals (n = 5 for SweetTrac2). Chemicals with more than 3-fold difference in equilibrium exchange constant values are emphasized in bold.

localized to the plasma membrane (Fig. S3) and showed a concentration-dependent change in fluorescence intensity (Figs. S4–S6).

Mutation of the less conserved V188 that made the binding pocket smaller (V188I and V188L) increased the biosensor's affinity for D-glucose, D-mannose, and D-fructose (Table 2). In contrast, mutations that made the binding site bigger (V18A) had the opposite effect, albeit to different extents (Table 2).

Notably, some mutations of the more conserved V69 and I72 residues differentially affected the binding of D-glucose, D-mannose, and D-fructose. Specifically, V69L and I72L worsened the binding of D-glucose but facilitated that of D-fructose, while I72V had the opposite effect. All three mutations decreased the affinity of SweetTrac1 for D-mannose (Table 2). These results suggest that V69 and I72 may affect the shape of the binding pocket, which reduces steric hindrance for some substrates but increases it for others.

Discussion

Due to their role in cellular energy and carbon storage, many studies have been conducted on vacuolar membrane sugar transporters, including the three vacuolar SWEETs (21, 29–32). In this work, we generated SweetTrac2, a new vacuolar biosensor that reports the activity of AtSWEET2 *in vivo*. SweetTrac2 localized to the vacuolar membrane in yeast cells (Fig. 1*A*), mimicking the location of the natural transporter *in planta* (21). The ability to quantify the activity of transporters localized to intracellular membranes is especially significant since they cannot be studied in whole cells with radiotracer uptake or growth assays.

Since AtSWEET1 and AtSWEET2 share a considerable level of sequence identity, we used the same linkers and sfcpGFP in SweetTrac1 to convert AtSWEET2 into SweetTrac2. The success of this approach suggests that using plasma membrane transporters as proxies to convert intracellular membrane transporters into biosensors may be a viable method that bypasses the need to isolate organelles and reconstitute vesicles. However, we note that the approach may be limited to homologs with high sequence identity.

We explored the substrate specificity of AtSWEET2 using SweetTrac2 and discovered 14 sugars and sugar analogs capable of binding to the biosensor. Overall, it appears that the stereochemistry of the hydroxyl groups at the C3, C4, and C6 positions of the sugars (numbered according to D-glucose, Fig. 2*B*) are crucial for the recognition of substrates by AtSWEET2 as was previously suggested for AtSWEET1 (20). We noticed that the affinity of SweetTrac2 for some of these chemicals is distinct from SweetTrac1 (Table 1) and identified three hydrophobic residues in the binding pocket of AtSWEET1 (V69, I72, and V188) that contribute to the differences. It is worth emphasizing that the values of the equilibrium exchange constants reported here were calculated based on the extracellular concentrations of substrates, and thus inaccuracies may exist for substrates that are metabolized by the yeast cells. While we have tried to reduce such artifacts by optimizing the timing at which fluorescence measurements were taken (Fig. S7), we cannot rule out an effect on our results. On the other hand, the reported equilibrium exchange constants for unmetabolizable compounds are likely accurate as intracellular concentrations would closely match extracellular ones at steady state.

Lastly, our work demonstrated the use of biosensors like SweetTrac1 to tune the specificity and selectivity of

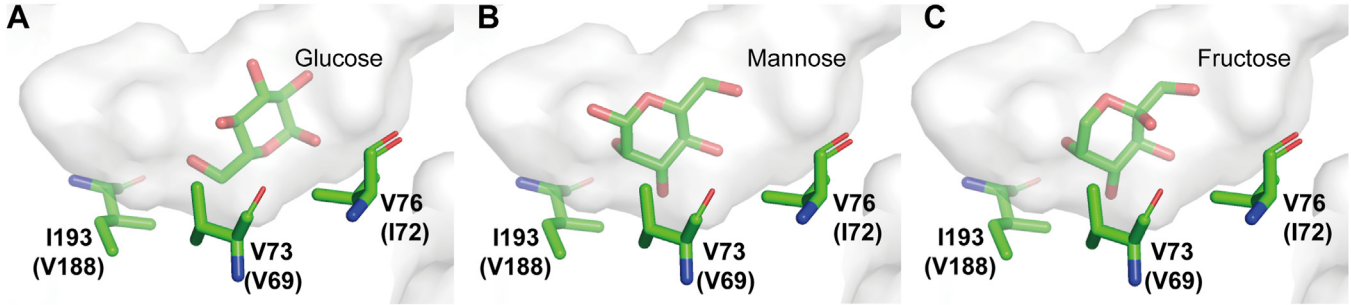

Figure 3. Molecular docking simulation results performed on the inside-open conformation of OsSWEET2b with different sugars. *A*, D-glucose, (*B*) D-mannose, and (*C*) D-fructose. The substrate binding pocket is shown in *light gray*, and sugars and key amino acids in the binding pocket of OsSWEET2b are shown as *sticks*. Corresponding amino acids in AtSWEET1 are listed in *parentheses*. Mutations in V69, I72, and V188 altered the affinity of SweetTrac1 for D-glucose, D-mannose, and D-fructose. Molecular docking performed with AutoDock Vina (Version 1.2.5). Image created with PyMOL Molecular Graphics System (Version 2.0 Schrödinger, LLC) from Protein Data Bank ID 5CTG. SWEET, Sugars Will Eventually be Exported Transporter.

Table 2
Steady-state response of SweetTrac1 mutants to three different sugars

Constructs	D-Glucose (mM)	D-Mannose (mM)	D-Fructose (mM)
SweetTrac1	8 ± 1	107 ± 13	274 ± 42
V69A	14 ± 2	559 ± 95	411 ± 122
V69I	2 ± 0	23 ± 5	72 ± 11
V69L	11 ± 2	312 ± 57	97 ± 17
I72V	6 ± 1	219 ± 30	334 ± 44
I72L	17 ± 3	173 ± 45	197 ± 36
V188A	21 ± 3	1977 ± 467	851 ± 255
V188I	7 ± 1	69 ± 13	174 ± 24
V188L	5 ± 1	29 ± 6	29 ± 5

Mutagenesis analysis of SweetTrac1. Equilibrium exchange constant reported as estimate ± 95% confidence intervals (n = 4). Plotted curve fits are available in Figs. S3–S5. Mutant SweetTrac1^{I72A} was nonfunctional despite proper plasma membrane localization (Fig. S2).

transporters and potentially other small molecule binding proteins. Mutagenesis analysis of the conserved V69 and I72 residues in the binding pocket of SweetTrac1 decreased affinity for D-glucose while increasing it for D-fructose, or vice versa. Future work combining several of these mutants or identifying new ones could help make D-fructose the preferred substrate of AtSWEET1, resulting in a high-capacity transporter that could, for example, improve D-fructose utilization in winemaking (33, 34). Another potential application of our approach includes the isolation of mutants that allow D-xylose transport, by mutating the binding pocket of AtSWEET1 to resemble that of the D-xylose transporter AtSWEET7 (35). Such an engineered transporter could be beneficial for obtaining higher biofuel yields from hemicellulose fermentation (36).

Experimental procedures

Full experimental procedures can be found in the Supporting information.

Data availability

All data are available within the article or Supporting information.

Supporting information—This article contains Supporting information.

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Conflicts of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviation—The abbreviations used are: SWEET, Sugars Will Eventually be Exported Transporter.

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