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Research Article

Exploring the Substrate Specificity of a Sugar Transporter with Biosensors and Cheminformatics

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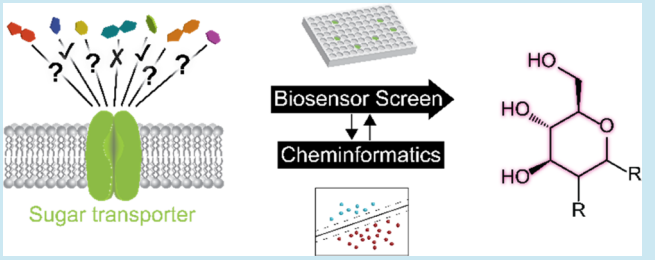
ABSTRACT: Sugars will eventually be exported transporters (SWEETs) are conserved sugar transporters that play crucial roles in plant physiology and biotechnology. The genomes of flowering plants typically encode about 20 SWEET paralogs that can be classified into four clades. Clades I, II, and IV have been reported to favor hexoses, while clade III SWEETs prefer sucrose. However, the molecular features of substrates required for recognition by members of this family have not been investigated in detail. Here, we show that SweetTrac1, a previously reported biosensor constructed from the Clade I *Arabidopsis thaliana* SWEET1, can provide insight into the structural requirements for substrate recognition. The biosensor translates substrate binding to the transporter into a change in fluorescence, and its application in a small-molecule screen combined with cheminformatics uncovered 12 new sugars and their derivatives capable of eliciting a response. Furthermore, we confirmed that the wild-type transporter mediates cellular uptake of three of these species, including the diabetes drugs 1-deoxynojirimycin and voglibose. Our results show that SWEETs can recognize different furanoses, pyranoses, and acyclic sugars, illustrating the potential of combining biosensors and computational techniques to uncover the basis of substrate specificity.



INTRODUCTION

Sugars will eventually be exported transporters (SWEETs) are conserved in all kingdoms of life. Plant SWEETs mediate phloem loading, grain filling, pollen nutrition, and pathogen susceptibility, making them prime targets for crop improvement.[1−3] Humans have a single member of this family, which has been suggested as a biomarker for breast cancer,[4] while fungi SWEETs have been singled out as valuable tools for biotechnology applications. For example, the construction of chimeras between the *Neocallimastix californiae* SWEET1 and other fungal SWEETs facilitated the novel coconsumption of glucose and xylose in *Saccharomyces cerevisiae*. Transporters that allow simultaneous cofermentation of glucose and xylose (like NcSWEET1, its engineered chimeras, or the *Arabidopsis thaliana* SWEET7) could improve the fermentation of lignocellulosic hydrolysates with a high amount of D-xylose.[5,6]

The substrates of SWEETs are usually identified by transgenic expression of the transporter in yeast knockout mutant strains lacking sugar transporters, as well as mammalian cell lines and *Xenopus* oocytes with a negligible expression of endogenous sugar transporters. Intracellular accumulation of sugars is subsequently detected using cell growth, radiolabeled sugars, or intracellular biosensors that are specific for different sugars.[7]

Activity biosensors have been proposed as valuable tools to determine if a transporter is active or idle directly. These biosensors are often constructed by inserting circularly permutated fluorescent proteins into full-length transporters.[8,9] Similar biosensors can also be created using Förster resonance energy transfer (FRET) or for membrane receptors and enzymes.[10,11] Recently, we reported the design and quantitative characterization of SweetTrac1, a chimera where a circularly permutated, superfolded green fluorescent protein (cpsfGFP) was inserted into the second intracellular loop of the Clade I AtSWEET1. When tested in yeast cells, SweetTrac1 displays an increase in fluorescence intensity in the presence of D-glucose, the canonical substrate of AtSWEET1.[1,3,12] To explain this result, we proposed a model where the substrate-bound form of the biosensor was bright, while the apoforms were dim. We then showed that this mechanism captured the concentration-dependent dynamic behavior of the biosensor, which had comparable D-glucose influx and efflux affinities to the wild-type transporter.[9]

Herein, we employed SweetTrac1 to discover new substrates of AtSWEET1. Using a combination of small-molecule screening and cheminformatics, we identified multiple chemicals capable of binding AtSWEET1, many of which have never been associated with the SWEET family. Comparison of these

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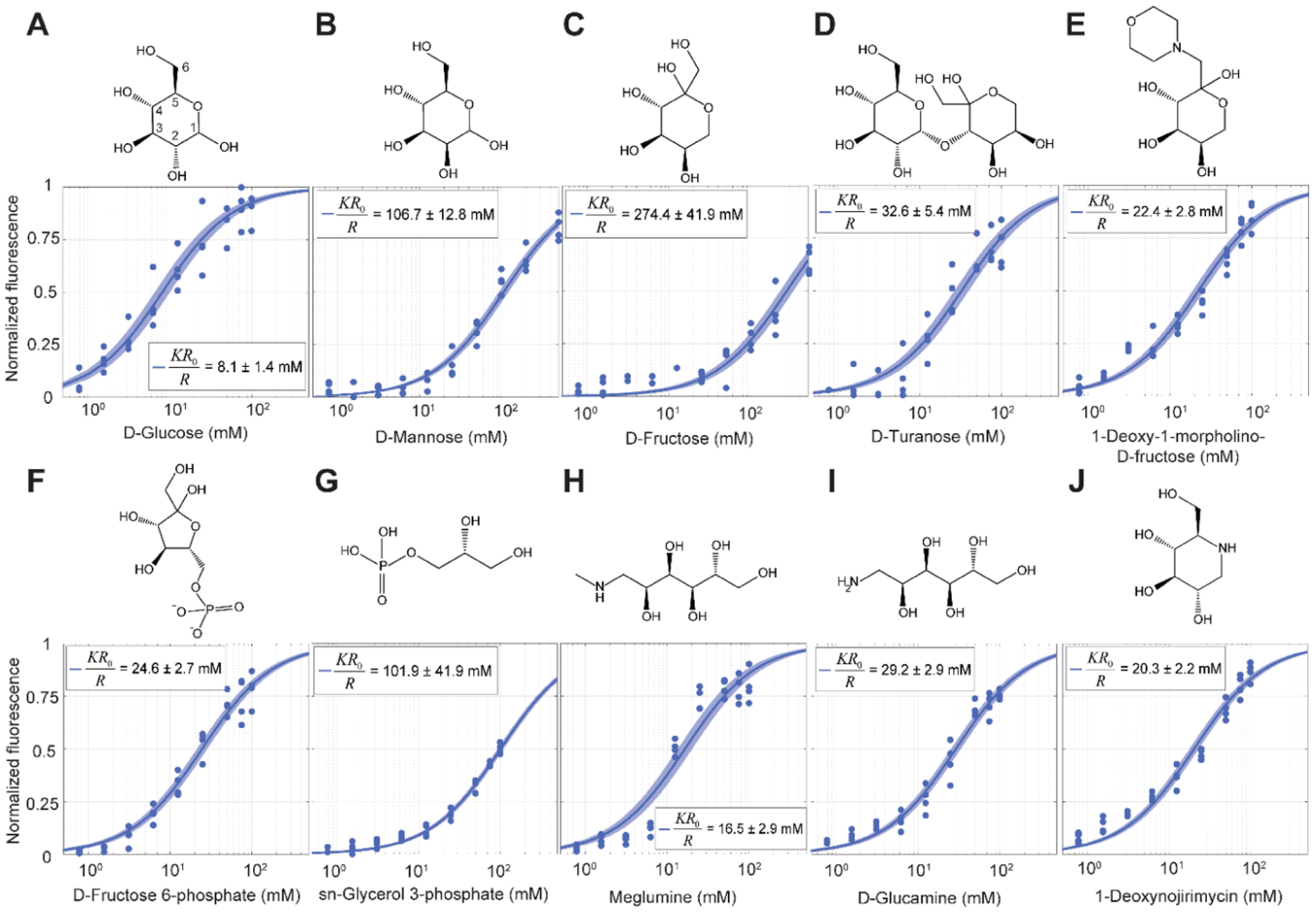

Figure 1. Potential molecules recognized by AtSWEET1 identified through chemical screening. (A–H) SweetTrac1's steady-state response to chemicals considered positive hits in our primary screen. The carbons are numbered for D-glucose. (I–J) SweetTrac1's response to chemicals considered positive hits in our secondary screen. Blue solid lines represent uniporter model fit, and the shaded areas represent 95% confidence intervals (equilibrium exchange constants are reported as estimated ±95% confidence intervals, n = 4). All chemical structures are depicted in their most probable conformation in solution.

molecules provided insights into the sugar recognition mechanism for the membrane transporter.

RESULTS

Identifying Potential Substrates for AtSWEET1. To study the substrate specificity of AtSWEET1, we expressed SweetTrac1 in yeast and screened a custom-made library of 162 sugars and their derivatives (Supporting Table). The Z'-factor for the assay was 0.24, less than the preferred score range of 0.5–1.0.[13] Consequently, we adopted a stringent cut-off criterion and only considered chemicals that produced a significant increase in fluorescence (p < 0.01) when compared to that of a negative control, D-sorbitol.[9]

The screen identified eight positive hits that generated a concentration-dependent fluorescence response when retested (Figure 1A–G). The positive predictive value of the screen was 0.72.[14] All of the positive hits were D-enantiomers, despite the presence of 17 L-enantiomers in our chemical library (e.g., L-glucose and L-mannose).

Three positive hits, D-glucose, D-mannose, and D-fructose, are known to be transported by AtSWEET1 based on growth complementation and [^{14}C]-radiolabeled substrate uptake assays in EBY4000 (Figure 2), a yeast strain lacking endogenous hexose transporters that requires transformation with hexose transporters to allow the uptake of this type of sugars.[3] These

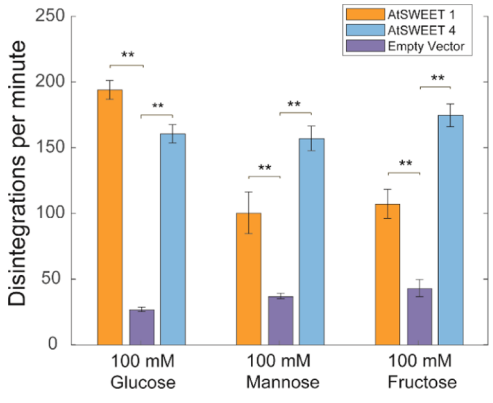

Figure 2. Uptake of [^{14}C]-glucose, [^{14}C]-fructose, and [^{14}C]-mannose mediated by AtSWEET1 in yeast. The D-mannose and D-fructose transporter AtSWEET4 serves as a positive control[18] (reported as mean ± S.E.; n = 3; ** represents p-values less than 0.01).

results support the effectiveness of our approach, although we found discrepancies with two other reported substrates of AtSWEET1: D-galactose did not induce a significant fluorescence response (p = 0.20), while 2-deoxy-D-glucose decreased the fluorescence intensity of SweetTrac1. The effect

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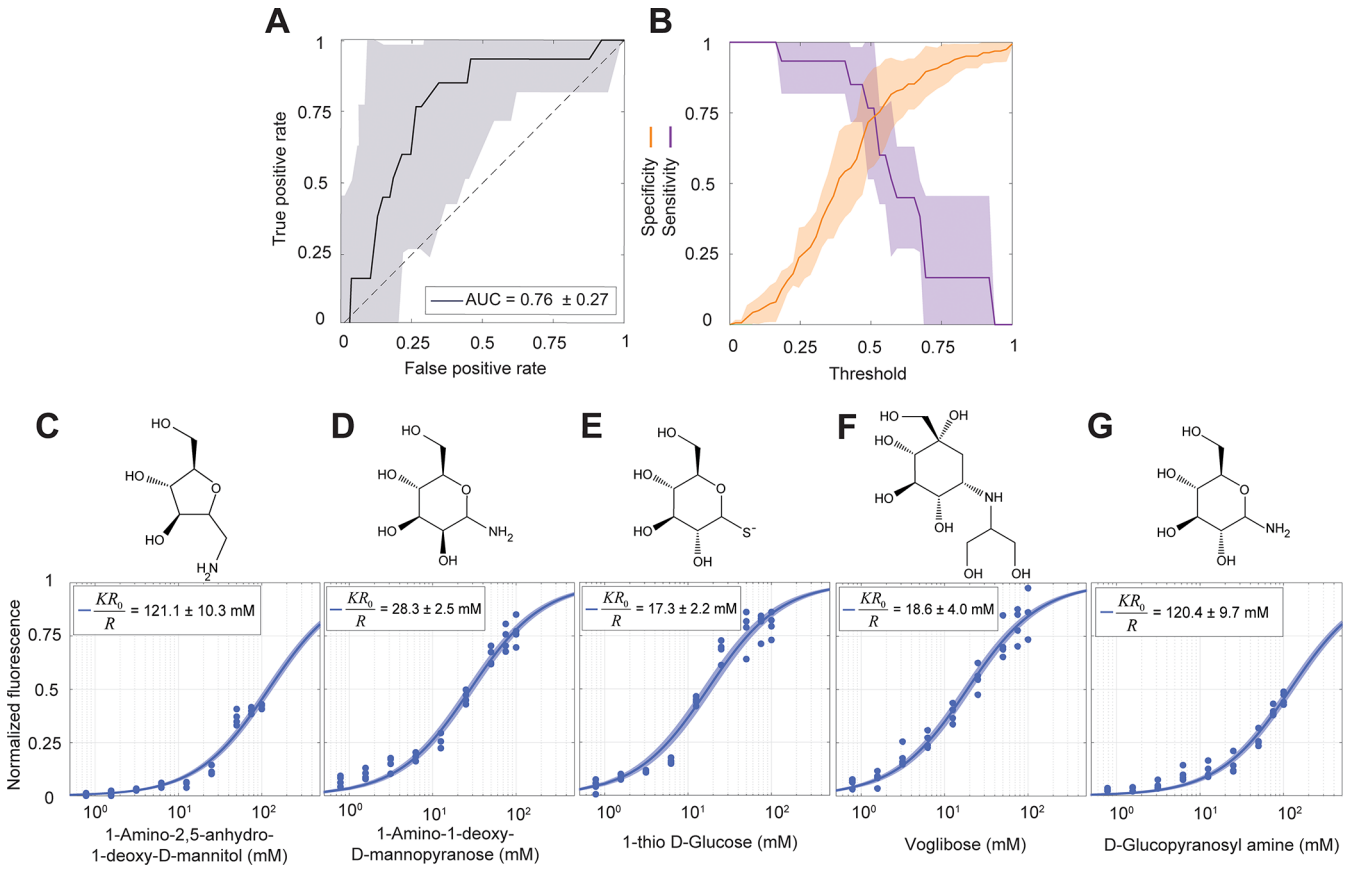

Figure 3. Potential molecules recognized by AtSWEET1 identified through cheminformatics analysis. (A) Receiver operating characteristic (ROC) plot evaluating model performance after training on chemicals from the primary and secondary screens. Dark gray solid lines represent the mean of a 3-fold cross-validation, and shaded areas represent one standard deviation. (B) Sensitivity and specificity of the model. Purple and orange lines represent the mean of a 3-fold cross-validation, while shaded areas represent one standard deviation. (C−G) SweetTrac1's steady-state response to chemicals considered true positive hits from the model-guided tertiary screen. Blue solid lines represent uniporter model fit, and the shaded areas represent 95% confidence intervals (equilibrium exchange constants are reported as estimates ±95% confidence intervals, $n = 4$). All chemical structures are depicted in their most probable conformation in solution.

of 2-deoxy-D-glucose may be caused by the well-documented cell toxicity of this analogue.[15−17]

The affinity of SweetTrac1 for different sugars can be quantified using an equilibrium exchange constant (KR_0/R), which we previously defined as the concentration of the substrate that would generate half of the maximum fluorescence response at steady state. Since higher values indicate a less favorable interaction between SweetTrac1 and its substrate, KR_0/R is inversely correlated with affinity.[9] Of the eight positive hits of the screen, the affinity of the biosensor was the highest for D-glucose and the lowest for D-fructose, suggesting that D-glucose is the preferred substrate of AtSWEET1. The trend in affinities between D-glucose, D-mannose, and D-fructose correlated with the uptake of these substrates in EBY4000 cells expressing AtSWEET1 (Figure 2).

Comparing D-glucose (Figure 1A) with the other positive hits and 2-deoxy-D-glucose suggested that the hydroxyl groups at the C3 and C4 positions may be critical for recognition. Moreover, the effect is stereospecific, as D-galactose (the C4 epimer of D-glucose) did not produce a fluorescence response ($p = 0.20$). To follow up on these observations, we performed a smaller secondary screen with nine new chemicals (Supporting Table). Among them, D-allose (the C3 epimer of D-glucose) did not produce a fluorescence response ($p = 0.15$), confirming the stereospecificity for the hydroxyl group at the C3 position.

We also noticed that meglumine, a derivative of D-sorbitol in which a methylamino group replaces the hydroxyl group in the C1 position, produced a fluorescence response in our primary screen (Figure 1H). Thus, we included three nitrogen-containing sugar analogous in our secondary screen and found two more positive hits: D-glucamine (an amino sugar similar to meglumine but without the *N*-methyl group) and 1-deoxynojirimycin (an iminosugar where nitrogen replaces the ring oxygen that also lacks the hydroxyl group at the C5 position) (Figure 1I,J). Overall, it appears that certain amine substitutions (meglumine and D-glucamine) and dehydroxylations (1-deoxynojirimycin) are better tolerated than negatively charged phosphate substitutions (D-glucose 1-phosphate).

Cheminformatics Search for New Candidates. To systematically identify more molecules capable of binding SweetTrac1, we trained a binary classification model on the results from our screens. Specifically, we used the open-source packages RDKit to extract molecular features and Scikit-learn for classification.[19,20] The model assigns a probability of binding SweetTrac1 to each chemical based on features corresponding to the presence or absence of substructures. The area under the receiver operating characteristic (ROC) curve for the classifier was 0.76 ± 0.27 (Figure 3A), indicating that the model was able to classify sugars and their derivatives as hits based solely on their molecular features.

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We then used the model to score a list of carbohydrates available from a supplier of biochemicals for research. We selected 12 chemicals (Supporting Table) with a predicted probability of binding above 0.65 that were not present in the training data, had a molecular weight under 400 gr/mol, and were affordable and water-soluble for testing. We chose a predicted probability threshold of 0.65 to balance sensitivity and specificity (Figure 3B). Using this cut-off, we identified five more true positive hits that produced a concentration-dependent increase in fluorescence ($p < 0.05$; Figure 3C−G).

Testing Transport by AtSWEET1. SweetTrac1 is likely to report the binding of small molecules to the biosensor but not necessarily the translocation of those molecules to the cytosolic side of the plasma membrane. It could be possible, for instance, for some of our new hits to act as competitive inhibitors, binding and eliciting a fluorescence response in SweetTrac1 without being transported. Thus, competition assays using known radiolabeled substrates (e.g., [^{14}C]-glucose, Figure 2) would not help differentiate whether the new candidates are bona fide substrates or competitive inhibitors. Moreover, radiolabeled versions of the new hits were not commercially available, preventing us from measuring their cellular uptake directly.

However, we noticed that two new hits, 1-deoxynojirimycin and voglibose, are α-glucosidase inhibitors used to treat diabetes.[21,22] Thus, we reasoned that we could detect their cellular uptake by AtSWEET1 and intracellular accumulation using a growth inhibition assay in EBY4000. Because this mutant yeast strain lacks all hexose transporters and relies on maltose as a carbon and energy source, AtSWEET1-mediated uptake of 1-deoxynojirimycin or voglibose could inhibit maltose catabolism and hinder growth. For comparison, the toxic sugar analogue 2-deoxy-D-glucose inhibits glycolysis and impairs the growth of EBY4000 cells expressing AtSWEET1 but not the cells expressing an empty vector control ($p = 2.6 \times 10^{-5}$, Figure 4).[15−17]

As expected, cells expressing AtSWEET1 showed significant growth inhibition in the presence of 10 mM 1-deoxynojirimycin or voglibose after 8 hours (Figure 4). Inhibition was stronger for 1-deoxynojirimycin ($p = 3.3 \times 10^{-4}$) than for voglibose ($p = 6.6 \times 10^{-3}$) when compared to the empty vector. The stronger growth defect observed with 1-deoxynojirimycin may result from its secondary inhibition of cell wall synthesis, not only on maltose metabolism.[23]

Moreover, we found that another positive hit identified by our cheminformatics analysis, 1-thio-D-glucose, is also transported by AtSWEET1. The growth of cells expressing AtSWEET1 was significantly lower than that of cells transformed with an empty vector control ($p = 3.7 \times 10^{-4}$). However, we noticed that even cells transformed with the empty vector showed reduced growth, suggesting that other transporters still present in EBY4000 may also contribute to the uptake of 1-thio-D-glucose.

The remaining new hits of our screens did not significantly hinder the growth of EBY4000 (Figure 4). One explanation for the lack of effect could be that the tested chemicals can be recognized but not transported by AtSWEET1 and thus act as competitive inhibitors. Another reason could be that they are transported by AtSWEET1 and accumulate cytosolically but do not affect growth.

DISCUSSION

Our results show that AtSWEET1 recognizes pyranoses, furanoses, and acyclic carbohydrates. Sugars can undergo mutarotation in aqueous solutions, leading to a mixture of

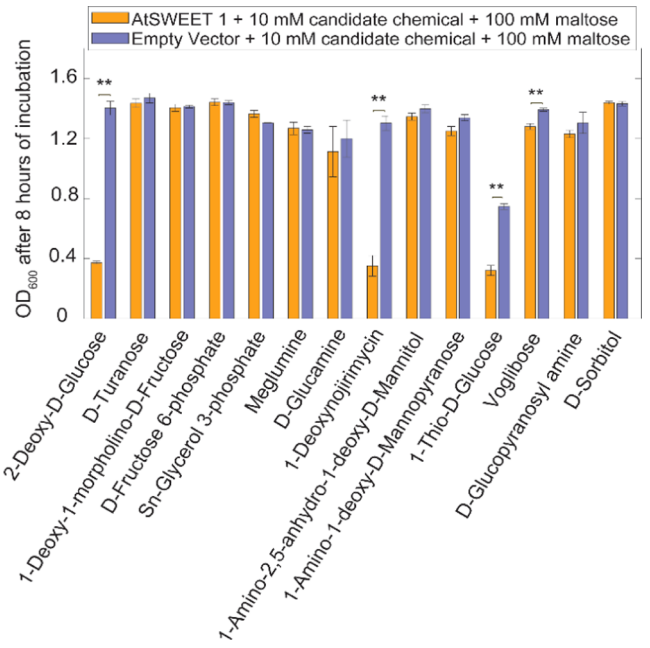

Figure 4. Transport by AtSWEET1 and subsequent growth inhibition of positive hits in the yeast strain EBY4000. Cells were supplemented with 100 mM of maltose and 10 mM of the listed chemicals (optical density at 600 nm (OD_{600}) reported as mean ± S.E.; $n = 3$; ** represents p-values less than 0.01). 2-Deoxy-D-glucose was used as the positive control. Likewise, 1-deoxynojirimycin, 1-thio-D-glucose, and voglibose produced a significant growth reduction.

tautomers, which are cyclic and acyclic structures of various stereochemistries. In solution, the 6-ring pyranoid form is favored over the 5-ring pyranoid form, while acyclic tautomers typically exist in trace amounts at equilibrium.[24−27] Before this study, AtSWEET1 was known to recognize D-glucose and D-mannose,[3] which almost entirely exist in cyclic pyranoid forms in solution.[27] Previous data from growth assays suggesting that AtSWEET1 transports D-fructose was debatable,[3] but our results using SweetTrac1 and [^{14}C]-D-fructose uptake confirm that conclusion. In solution, about 72−75% of D-fructose is in the pyranoid form.[25,28] Overall, 10 of the 16 positive hits primarily exist as pyranoses in solution.

Our results also show that SweetTrac1 responded to D-fructose-6-phosphate and 1-amino-2,5-anhydro-1-deoxy-D-mannitol (Figures 1F and 3C). The former exists in the cyclic furanoid form in solution,[29] and the latter is likely to be a furanose, too, as its analogue 2,5-anhydro-D-mannitol is known to be in a cyclic furanoid form in solution.[30] The presence of furanoses among our hits was not a surprise, given that AtSWEET13 was previously shown to bind 2′-deoxycytidine 5′-monophosphate when crystallized, which has a 5-ring structure at its center.[31] Although we could not confirm the transport of D-fructose-6-phosphate and 1-amino-2,5-anhydro-1-deoxy-D-mannitol (Figure 4), the response of SweetTrac1 does indicate that AtSWEET1 is likely to recognize furanoses as well.

Interestingly, SweetTrac1 responded to polyols like *sn*-glycerol 3-phosphate, meglumine, and D-glucamine (Figure 1G−I), suggesting that AtSWEET1 may also recognize acyclic compounds. Plant sugar carriers that can recognize both acyclic and cyclic carbohydrates are not uncommon. For instance, the celery mannitol transporter AgMaT1 has been shown to transport both D-glucose and D-mannose,[32] while the apple

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sorbitol transporter MdSOT6 is inhibited by the presence of D-glucose as well as D-fructose.[33]

SweetTrac1 did not recognize D-allose or D-galactose, indicating that the stereochemistries of the C3 and C4 positions (Figure 1A) are critical for recognizing aldohexoses. Moreover, none of the positive hits were D-allose nor D-galactose derivatives, while several of the positive hits were D-glucose, D-fructose, and D-mannose derivatives. The specificities for the C3 and C4 positions are extended to ketohexoses as SweetTrac1 also did not recognize D-psicose nor D-tagatose, the C3 and C4 epimers of D-fructose, respectively. Removal of the hydroxyl group at the C6 position (6-deoxy-D-glucose) and substitutions with nonamine and nonhydroxyl groups (D-glucose 6-phosphate) caused loss of recognition by SweetTrac1, indicating that the C6 position may also be important for hydrogen bonding.

The hydroxyls in the C1, C2, and C5 positions of the ring (Figure 1A), however, tolerated substitutions and removal more readily. For example, compounds with substitutions in the C1 (1-thio-D-glucose, D-glucopyranosyl amine, 1-deoxynojirimycin) and C5 (1-deoxynojirimycin and voglibose) positions could still be recognized by SweetTrac1 (Figures 1 and 3). It is worth noting that SweetTrac1 recognized D-glucopyranosyl amine, which strongly favors the equatorial position due to steric and "reverse anomeric" effects.[34,35] Furthermore, the C2 position tolerates dehydroxylation as 2-deoxy-D-glucose can still be transported by AtSWEET1.

We note that these conclusions should be interpreted with some caution. First, our work did not consider anomers. Second, we assumed that our positive hits are recognized by AtSWEET1 in their most prevalent 5- or 6-ring cyclical form when information was available in the literature,[25−29,36,37] while the 6-ring form was assumed for hits without data. Still, we cannot rule out that SweetTrac1 may recognize the less prevalent tautomers of the positive hits.

A question left open in this work is whether our measured equilibrium exchange constants are within the concentration ranges of the natural metabolites in plants. The affinity constant of SweetTrac1 for glucose, for example, is within the low millimolar concentrations reported in tissues affected by mutations in SWEET genes,[38−43] albeit no physiological function has been reported for AtSWEET1 yet. The equilibrium exchange constant of fructose does not seem to be within reported ranges,[38,40,42,43] and hardly any information is available for the other natural positive hits (D-mannose, D-fructose 6-phosphate, D-turanose, *sn*-glycerol 3-phosphate, and 1-deoxynojirimycin), making it difficult to determine if they interact with AtSWEET1 at physiological levels. Nevertheless, the information generated in this work could still serve as the basis for engineering new transporters, given that promiscuity is often exploited in directed evolution.[44] Of particular interest among the confirmed substrates of AtSWEET1 is 1-deoxynojirimycin, a natural product from mulberry trees and bacteria with applications in the treatment of diabetes and obesity.[45] Our results suggest that AtSWEET1 can translocate this iminosugar and thus could be engineered for improving its bioproduction.

The work presented here offers the most comprehensive analysis of the substrate specificity of any SWEET protein thus far and outlines a strategy for exploring the substrate specificity of transporters in general. SweetTrac1 provided an easier and faster approach to screen for potential substrates compared to synthesizing radiolabeled versions of the chemicals in our library or constructing individual biosensors to detect the intracellular accumulation of each chemical. SweetTrac1 also allowed us to gain new molecular insights into the substrate recognition mechanism of AtSWEET1 and to develop a predictive model that can help screen for more substrates *in silico*. The approach and the data set reported here would facilitate the characterization of sugar transporters, which are some of the most challenging proteins to study, given their indispensability and need to be embedded in a membrane to function.

■ ASSOCIATED CONTENT

Data Availability Statement

The classification model and accompanying data are available at https://github.com/Ryan-Abramowitz/MoleculeBinding

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.2c00571.

Methods: DNA constructs; yeast transformation; chemical library screen design and evaluation; fluorometric analyses and parameter estimation; influx assays; growth inhibition assay, and model training and predictions (PDF)

Screened chemicals and their model scores (XLS) (XLSX)

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Notes

The authors declare no competing financial interest.

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