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Identification of Amino Acids Important for Substrate Specificity in Sucrose Transporters Using Gene Shuffling*

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Background: Type II sucrose transporters (SUTs) are more selective than type I SUTs.
Results: OsSUT1 was modified using gene shuffling. Variants were selected for the ability to transport the fluorescent β-glucoside esculin.
Conclusion: Replacement of five amino acids conferred substrate specificity typical of type I SUTs.
Significance: The first loop and top of TMS2 and TMS5 are involved in controlling substrate specificity in SUTs.

Plant sucrose transporters (SUTs) are H^+-coupled uptake transporters. Type I and II (SUTs) are phylogenetically related but have different substrate specificities. Type I SUTs transport sucrose, maltose, and a wide range of natural and synthetic α- and β-glucosides. Type II SUTs are more selective for sucrose and maltose. Here, we investigated the structural basis for this difference in substrate specificity. We used a novel gene shuffling method called synthetic template shuffling to introduce 62 differentially conserved amino acid residues from type I SUTs into OsSUT1, a type II SUT from rice. The OsSUT1 variants were tested for their ability to transport the fluorescent coumarin β-glucoside esculin when expressed in yeast. Fluorescent yeast cells were selected using fluorescence-activated cell sorting (FACS). Substitution of five amino acids present in type I SUTs in OsSUT1 was found to be sufficient to confer esculin uptake activity. The changes clustered in two areas of the OsSUT1 protein: in the first loop and the top of TMS2 (T80L and A86K) and in TMS5 (S220A, S221A, and T224Y). The substrate specificity of this OsSUT1 variant was almost identical to that of type I SUTs. Corresponding changes in the sugarcane type II transporter ShSUT1 also changed substrate specificity, indicating that these residues contribute to substrate specificity in type II SUTs in general.

Sucrose is the main carbohydrate that is transported long distances in plant vascular tissue. Proton-coupled sucrose uptake transporters (SUTs)[2] are especially important for loading sucrose into the phloem (vascular tissue) and for sucrose uptake into some sink tissues, such as seeds, that are symplasmically isolated (not connected by plasmodesmata). The physiological functions of SUTs have been reviewed recently (1–3). SUTs are encoded by small gene families in plants. Biochemical analysis of SUT transport activity has revealed differences in substrate specificity between SUTs, and these differences follow the SUT phylogeny (4). This presents an opportunity to discover how evolutionary changes in SUTs have produced differences in substrate specificity.

SUTs belong to the glycoside-pentoside-hexuronide:cation symporter family, which is distantly related to the major facilitator superfamily (5). SUT homologs exist in fungi and animals (6). In humans, mutations in the SUT homolog SLC45A2 (MatP) cause type IV oculocutaneous albinism (7). Changes in SLC45A2 are also associated with diversity in normal skin pigmentation and with susceptibility to malignant melanoma (8). Plant SUTs are divided into three clades (types I, II, and III) by phylogenetic analysis (9). Type I SUTs are localized to the plasma membrane and are only found in eudicots. They have a broad substrate specificity that includes sucrose, maltose, and a variety of α- and β-glucosides (10, 11). Type I SUTs appear to have evolved from type III SUTs that also have a broad substrate specificity (12); are found in all land plants including bryophytes, lycophytes, monocots, and eudicots (4); and are localized to the vacuolar membrane in monocots and eudicots (13, 14). Type II SUTs are also found in all land plants and have a much more restricted substrate specificity (15–17). Of particular interest for this study is the ability of type I SUTs to transport the fluorescent β-glucosides esculin and fraxin (11, 18) that do not serve as substrates for type II SUTs (17).

Little information exists concerning amino acids in SUTs that influence substrate binding or specificity. A conserved His (His-65 in AtSUC1) at the interface of the first extracellular loop and the second transmembrane span was identified as the target for diethyl pyrocarbonate inhibition of the transporters (19). SUT inhibition by diethyl pyrocarbonate is substrate-protectable (20), and therefore His-65 is considered to be involved in or physically close to a substrate-binding site. Analysis of chimeras generated between the type I sucrose transporter StSUT1 and the lower affinity type II AtSUT2 indicated that the N-terminal domain was important for substrate affinity (21). We hypothesized that conserved residues in type II SUTs that are different in type I SUTs produce the difference in substrate specificity that has been observed. To test this experimentally, we used a synthetic version of rice type II sucrose transporter OsSUT1 (Os03g07480) in which 62 amino acid positions were changed to their differentially conserved counterpart present in

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[2] The abbreviations used are: SUT, sucrose uptake transporter; STS, synthetic template shuffling; TMS, transmembrane span.

type I SUTs. We used gene shuffling (22, 23) of the synthetic coding sequence and a wild-type *OsSUT1* coding sequence to produce a library of recombinants and screened these by expression in yeast for the ability to take up esculin.

EXPERIMENTAL PROCEDURES

Yeast Culture and Transformation—Yeast strain SEY6210 (*MATα leu2-3,112 ura3-52 his3-D200 lys2-801 trpl-D901 suc2-D9*) (24) was grown on YPD (1% yeast extract, 2% peptone, 2% glucose) at 30 °C. To select transformants, cells were grown on SD-URA (1.7 g/liter yeast nitrogen base, 5 g/liter ammonium sulfate, 20 g/liter glucose plus addition of the required supplements; for plates, 20 g/liter Bacto agar was added). Yeast transformations were routinely performed using the frozen cell method (25). However, for the library transformations, the lithium acetate/PEG protocol (26) was used.

Synthetic Template Shuffling (STS)—Based on an alignment of 43 plant sucrose transporters, 62 amino acid residues were identified that were differentially conserved in type I or type II SUTs. The coding sequence of *OsSUT1* was modified to encode the corresponding 62 amino acids present in type I SUTs. One additional amino acid change was made to introduce a SacI site at about the same position as a SacI site in the central loop of StSUT1. This resulted in the change of a Val to a Leu so that a total of 63 amino acid changes was made. The coding sequence was synthesized (Blue Heron Biotechnology, Bothell, WA) and called *OsSUT1-m63*.

To perform gene shuffling, both *OsSUT1* and *OsSUT1-m63* were cloned into Gateway (Invitrogen) entry vectors (*OsSUT1* into pCR8 and *OsSUT1-m63* into pENTR221). A set of primers corresponding to regions outside of the recombination sites was used to amplify the SUT ORFs plus the flanking recombination sites (F1, 5′-GCATGGATGTTTTCCCAGTCAC; R1, 5′-GAGATTTTGAGACACGGGCCAG). Equal amounts (2 μg) of the PCR products were mixed and partially digested for 15 min at room temperature with 0.8 unit of DNase I Turbo (Ambion). The resulting fragments were separated on a 1% agarose gel, and fragments sized between 100 and 700 bp were extracted and purified using the Qiaquick gel extraction kit (Qiagen). Shuffling and reassembly of the *OsSUT1* variants was performed in two steps. First, 1 μg of purified DNA fragments (final concentration, 20 ng/μl) was subjected to 45 cycles of primerless reassembly PCR. Second, an aliquot of the reassembled product was used as template in 35 cycles of an amplification PCR that included a set of primers nested just within the primers used to amplify the original ORFs (F2, 5′-GTAAAACGACGGCCAGTCTTAAGCTC; R2, 5′-CAGGAAACAGCTATGACCATGTAATAC). The resulting assembled *OsSUT1* variants were therefore flanked by the attL1 and attL2 recombination sites.

Following the two steps of PCR, the reassembled *OsSUT1* variants were recombined *in vitro* with the Gateway host vector pDR196/GW (27) using LR clonase II (Invitrogen). The recombination products were used to transform *Escherichia coli* strain DH5α. The number of clones resulting from the transformation, *i.e.* the size of the library, was determined. To analyze the quality of the library, 10 clones were picked at random, the plasmids were isolated, and the inserts were sequenced. The remaining clones were washed off the plates, and the plasmid DNA was isolated in bulk using the Qiagen Plasmid Maxi kit (Qiagen).

OsSUT1 variants were expressed in yeast to test for the ability of the encoded proteins to transport esculin, and functional clones were selected by FACS. Yeast strain SEY6210 was transformed with 1 μg of the shuffled library, and transformants were selected on SD-URA. After 3 days of growth at 30 °C, yeast colonies were washed off the plates with liquid SD-URA medium and pooled. A diluted aliquot of the cells (4×10^8 cells/ml) was spun down, resuspended at the same density with 1 mM esculin in 25 mM sodium phosphate buffer (pH 4), and incubated with shaking at 30 °C for 3 h. Cells were then spun down, washed with 25 mM sodium phosphate buffer, and resuspended with the same buffer at a density of 10^7 cells/ml. Fluorescent cells were selected by FACS performed on a BD Biosciences FACSVantage DiVa cell sorter (UV laser; excitation, 350 nm; emission, 450/50 nm detector configuration). Thresholds for sorting were set by using yeast cells transformed with the empty vector (pDR196) as a negative control and yeast cells expressing *StSUT1* as a positive control. PBS (pH 7) was used as running and collection buffers. The sorted cells were plated on SD-URA solid medium and cultured at 30 °C.

Site-directed Mutagenesis—OsSUT1-m9 and *OsSUT1-m5* were amplified and cloned into the Gateway entry vector pCR8/GW (Invitrogen). The correct sequence was confirmed by sequencing. For expression in oocytes, the constructs were recombined with the oocyte expression vector pOO2/GW (17). To create the variants of OsSUT1-m5, each of the five altered positions in OsSUT1-m5 was individually changed back to OsSUT1 wild-type sequence using the QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) and pCR8/GW-OsSUT1-m5 as the template. The OsSUT1-m2 and OsSUT1-m3 variants were made in the same way but using pDR196/GW-OsSUT1-m4C as the template. To create the ShSUT1-m5 version of the sugarcane sucrose transporter ShSUT1, the five amino acid changes were introduced in two steps by site-directed mutagenesis (QuikChange II site-directed mutagenesis kit, Agilent Technologies) of ShSUT1 in the oocyte expression vector pGEMHE (16). In a first step, Thr-60 and Ala-66 were mutated followed by mutation of Ser-200, Ser-201, and Tyr-204 in the second step. All primers used for site-directed mutagenesis are listed in Table 1 with mutated bases in lowercase. In all cases, the desired mutation was confirmed by sequencing.

Fluorometry—The ability of yeast cells to accumulate esculin was assayed as described previously (18) using a 96-well SYNERGYMx spectrofluorometer (BioTek, Winooski, VT) at an excitation wavelength of 382 nm and an emission wavelength of 454 nm. Cells were grown overnight on a shaker at 30 °C in the wells of a 96-well microtiter plate with 200 μl of SD-URA liquid medium. Cells were then spun down, the medium was removed, and the cells were resuspended in 200 μl of 25 mM sodium phosphate buffer (pH 4) with 1 mM esculin and incubated at 30 °C with shaking. For each plasmid, four independent yeast transformants were tested. The cultures were then spun down and washed with 200 μl of 25 mM sodium phosphate buffer (pH 4). After centrifugation, the buffer was removed, and

TABLE 1

Primers used to change single amino acid positions in OsSUT1-m5 back to wild type and to mutate ShSUT1

Introduced mutations are shown in lowercase.

Template	Change	Forward	Reverse
OsSUT1-m5	L80T	5′-CACCCCCTACGTCCAGacACTCGGACTGTCGCAT	5′-ATGCGACAGTCCGAGTgtCTGGACGTAGGGGGTG
OsSUT1-m5	K86A	5′-ACTCGGACTGTCGCATgctCTTACTTCATTCATG	5′-CATGAATGAAGTAAGagcATGCGACAGTCCGAGT
OsSUT1-m5	A220S	5′-GAAACATCCTAGGATACtCCGCTGGTTCCTATAAC	5′-GTTATAGGAACCAGCGGaGTATCCTAGGATGTTTC
OsSUT1-m5	A221S	5′-CATCCTAGGATACGCCtCTGGTTCCTATAACAA	5′-TTGTTATAGGAACCAGaGGCGTATCCTAGGATG
OsSUT1-m5	Y224T	5′-GATACGCCGCTGGTTCCacaAACAATTGGCACAAGTG	5′-CACTTGTGCCAATTGTTgtGGAACCAGCGGCGTATC
OsSUT1-m4C	A221S-m4C	5′-CATCCTAGGATACTCCtCTGGTTCCTATAACAATTG	5′-CAATTGTTATAGGAACCAGaGGAGTATCCTAGGATG
OsSUT1-m4C	Y224T-m4C	5′-GATACTCCGCTGGTTCCacaAACAATTGGCACAAGTG	5′-CACTTGTGCCAATTGTTgtGGAACCAGCGGAGTATC
OsSUT1-m4C	A221S,Y224T-m4C	5′-CATCCTAGGATACTCCtCTGGTTCCacaAACAATTGGCACAAGTGG	5′-CCACTTGTGCCAATTGTTgtGGAACCAGaGGAGTATCCTAGGATG
ShSUT1	T60L,A66K	5′- GACGCCCTACGTGCAGctTCTGGGGCTTTCACATaaaCTCACTTCATTCATG	5′- CATGAATGAAGTGAGtttATGTGAAAGCCCCAGAagCTGCACGTAGGGCGTC
ShSUT1	S200A,S201A,T204Y	5′- CACTTGTGCCAGTTGTTataGGAACCAGcGGcATAGCCTAGGATGTTTTC	5′- GAAACATCCTAGGCTATgCCgCTGGTTCCtatAACAACTGGCACAAGTG

the cells were resuspended with 200 μl of fresh buffer and transferred to a black microtiter plate for the determination of the fluorescence. An aliquot of each sample was then removed and diluted appropriately to determine the A_{600}. This value was used to calculate the relative fluorescence for each sample per OD.

Xenopus Oocyte Expression and Two-electrode Voltage Clamping—Oocytes were prepared, and two-electrode voltage clamping was performed as described previously (12). Oocytes were bathed in modified sodium Ringer solution (115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM MES-Tris at pH 5 or 5.6 (as indicated in the figure legends). Recording pipettes were filled with 1 M KCl and showed tip resistances of 1.5–3 megaohms. Currents were measured using a Dagan TEV 200A amplifier (Dagan Corp., Minneapolis, MN). Currents were filtered on line at 200 Hz and digitized at 2000 Hz using pClamp 5.5.1 (Axon Instruments, Inc., Union City, CA). The holding potential was −40 mV, and voltage pulses from −137 to 38 mV were applied for 200 ms. Mean steady-state currents are presented. Substrate-dependent currents were obtained by subtracting an average of background currents before and after substrate application. Substrates were added at 10 mM except for esculin, which was added at 5 mM, the limit of its solubility.

Sucrose Uptake into Yeast—The uptake of radiolabeled [^{14}C]sucrose into yeast cells was determined essentially as described previously (6). Briefly, yeast strain SEY6210 transformed with OsSUT1 or OsSUT1 variants in pDR196/GW (empty vector pDR196 was used as a control) was grown to midlog phase in SD-URA. Cells were harvested by centrifugation, washed with 25 mM sodium phosphate buffer (pH 4), and resuspended at an A_{600} of 20. For uptake assays, the cells were incubated at 30 °C in the presence of 10 mM glucose and ^{14}C-labeled sucrose at a final concentration of 1 mM for 5 min, then collected onto glass fiber filters by vacuum filtration, and washed with 20 ml of ice-cold 10 mM sucrose. The filters containing the collected cells were counted in a scintillation counter, and the amount of sucrose taken up was calculated.

RESULTS

Esculin Uptake in Yeast via StSUT1—Type I and type II SUTs differ strongly in their substrate specificity. Type I SUTs are less selective for sucrose and transport a broad range of α- and β-glucosides including the coumarin β-glucoside esculin (11). The ability of type I SUTs to transport esculin is the basis for a fluorescent assay of type I SUT activity (18). The type I SUT StSUT1 from potato (28) and the type II SUT OsSUT1 from rice (9) were expressed in yeast, and the cells were incubated in pH 4.0 buffer containing 1 mM esculin, washed, and observed under a fluorescence microscope. Yeast transformed with the empty pDR196 vector were not fluorescent. Consistent with electrophysiological analyses of type I and II SUTs (11, 17), yeast expressing StSUT1 were highly fluorescent, whereas yeast expressing OsSUT1 were not more fluorescent than the vector control (Fig. 1). Previous experiments demonstrated that OsSUT1 is functional when expressed in yeast (17); therefore, the lack of fluorescence of yeast cells expressing OsSUT1 was due to a lack of esculin transport rather than a non-functional transporter.

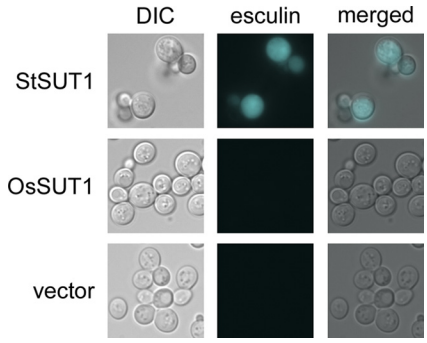

FIGURE 1. **Yeast cells expressing a type I SUT are able to take up esculin.** Yeast cells transformed with *StSUT1*, *OsSUT1*, or the empty vector were incubated with esculin and then imaged with a Nikon E800 fluorescence microscope. The images shown were taken at 1000× magnification. *DIC*, differential interference contrast; *esculin*, using a Nikon cyan fluorescent protein filter cube (excitation filter, 426–446 nm; 455-nm LP dichroic mirror; 460–500 nm emission filter); *merged*, merging both images. The exposure time was the same for all fluorescence images.

Selection of Differently Conserved Residues in Type I and Type II SUTs—The sequence identity between coding regions of *StSUT1* and *OsSUT1* at the nucleotide level is ~53%, which is not high enough for optimal gene shuffling (29). Using an alignment of 43 SUTs (22 type I, 12 type II, and nine type III), 62 amino acid positions were chosen that are differentially conserved in type I and II SUTs. We hypothesized that a small subset of these positions was likely to control the differences in substrate specificity between type I and II SUTs. The resulting protein sequence encoded by the synthetic cDNA is shown in Fig. 2*A*.

The approach that we call STS utilizes a synthetic template with multiple mutations relative to wild type. The synthetic template is similar enough to the wild-type template to allow gene shuffling and in this case was likely to encode the difference in activity observed between type I and type II SUTs. The synthetic clone *OsSUT1-m63* is 94% identical to wild-type *OsSUT1* at the nucleotide level. Several different assays indicated that *OsSUT1-m63* did not encode a functional protein. When expressed in yeast, it did not transport [^{14}C]sucrose or esculin, and it did not allow the yeast strain SuSy7 (30) to grow on sucrose. In *Xenopus* oocytes, it did not produce sucrose-dependent currents. By comparison, *OsSUT1* is functional when expressed in both yeast and oocytes (17).

STS—A diagram of the gene shuffling method is presented in Fig. 2*B*. The wild type *OsSUT1* coding region in pCR8 and the synthetic *OsSUT1-m63* in pENTR221 were amplified by PCR such that the flanking Gateway attL1 and attL2 recombination sites were included. After DNase I digestion, the fragments were reassembled by primerless PCR followed by PCR using nested primers. The resulting products retained the Gateway recombination sites so that the library of shuffled plasmids could be generated in the Gateway-compatible yeast expression vector pDR196/GW (27).

The library contained 26,000 independent *OsSUT1* variants. Randomly sequenced library clones encoded an average of 37 ± 16 amino acid differences compared with the wild-type OsSUT1 (Fig. 3*A*). The average number of crossovers apparent in the library clones was 4 ± 2. In the randomly selected clones, the parental genotypes were not found. A few mutations introduced by PCR errors were also found (Fig. 3*A*, in *parentheses*). The shuffled library was used to transform yeast strain SEY6210, and the transformants were incubated with esculin. In total, 97,600 yeast colonies were screened this way in bulk. Yeast cells that accumulated esculin and became fluorescent were selected by FACS. Of 19 million cells sorted, 41 yeast colonies were recovered. Of those, four encoded esculin uptake activity, and the plasmids were isolated and sequenced. Three of the clones were unique. The clone with the least number of changes, nine changes compared with the wild type, was designated *OsSUT1-m9* (Fig. 3*B*) and chosen for further analysis.

Although the 63 amino acid differences between OsSUT1 and OsSUT1-m63 were evenly distributed across the entire protein, the nine changes found in OsSUT1-m9 were clustered in TMS2 (three), TMS5 (three), TMS6 (one), and TMS7 (two). To determine whether all of these changes were required to enable esculin transport, the *OsSUT1-m9* clone was used in a second round of gene shuffling with *OsSUT1*. This method to remove neutral mutations is called backcrossing (31). In this round, a library consisting of 24,000 independent clones was constructed. Ten randomly selected clones were sequenced (Fig. 3*B*). The average number of amino acid differences compared with the wild-type OsSUT1 was 4 ± 1.5, and the average number of crossovers was 2 ± 1. The library was used to transform yeast strain SEY6210. A total of 80,000 yeast colonies was obtained and incubated in bulk with esculin, and fluorescent cells were selected using FACS. A total of 7.7 million cells was sorted by FACS, and 176 yeast colonies were recovered. Of those, 15 clones were confirmed to be able to take up esculin. Isolation of the plasmids and sequencing their inserts revealed that 10 unique combinations were represented (one clone was recovered three times, and another was recovered four times). None of the recovered clones was identical to the initial clone *OsSUT1-m9*. One clone encoded all the amino acid changes of *OsSUT1-m9* with one additional change introduced by PCR. The clone with the fewest amino acid changes compared with *OsSUT1* encoded five amino acid changes and was named *OsSUT1-m5*.

Five Amino Acid Changes in OsSUT1 Are Sufficient to Allow Esculin Transport—To probe the relative importance of the five amino acid changes in OsSUT1-m5, a series of mutants was constructed with subsets of these mutations (Fig. 4*A*). Yeast cells expressing either *OsSUT1-m9* or *OsSUT1-m5* showed similar fluorescence after incubation with 1 mM esculin for 1 h (Fig. 4*B*). The yeast cells transformed with *OsSUT1* or the empty vector were not fluorescent. Each of the five mutations was individually mutated back to wild type (constructs OsSUT1-m4A through -E). Changing either of the two residues at the top of TMS2 (Leu-80 and Lys-86) had a strongly negative effect on esculin uptake, indicating that these two changes are both important. The mutations in TMS5 (Ala-220, Ala-221, and Tyr-224) appear to have more complicated effects. Changing either Ala-220 or Ala-221 did not decrease esculin uptake (Fig. 4*B*). However, changing both back to wild type decreased esculin uptake as shown for the triple mutant OsSUT1-m3B. Therefore, either Ala-221 or Ala-220 is required for esculin uptake. Tyr-224 appears to be important for esculin uptake

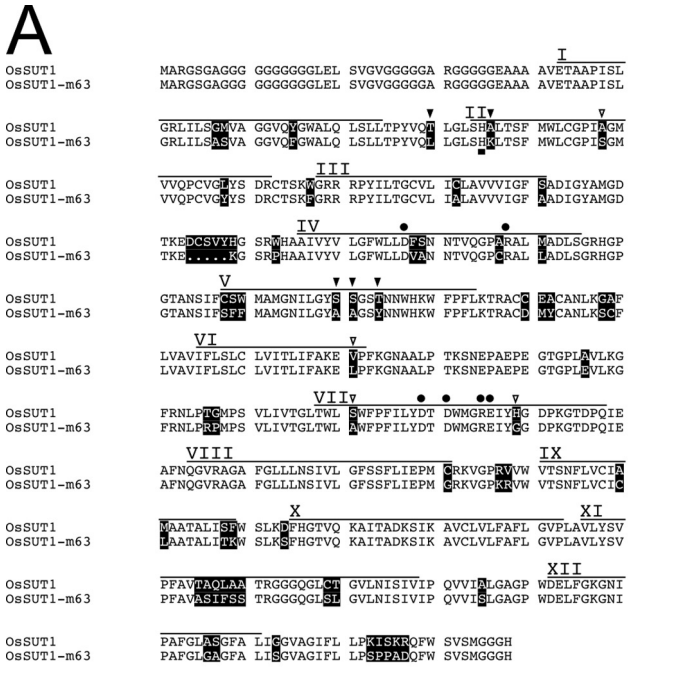

FIGURE 2. **Shuffling of *OsSUT1*.** *A*, alignment of the protein sequences of OsSUT1 and the OsSUT1-m63 variant. Amino acids differing between the two proteins are highlighted. The putative transmembrane spans are indicated by *lines* and *roman numerals* above the sequences. Amino acid differences between OsSUT1 and the OsSUT1-m5 (*closed triangles*) and OsSUT1-m9 (*closed* and *open triangles*) variants are marked above the alignment. Conserved charged residues within the TMS (35) are marked by *closed circles*. His-85, the equivalent of His-65 in AtSUC1 (19), is *underlined* with a *black square*. *B*, strategy used for gene shuffling experiment. Templates for shuffling were prepared by PCR amplification of ORFs plus flanking attL1 and attL2 recombination sites. Equal amounts of templates were mixed and digested with DNase I, and fragments were assembled by primerless PCR followed by PCR using nested primers. The shuffled *OsSUT1* variants contained attL1 and attL2 recombination sites. Through *in vitro* recombination with a Gateway-compatible yeast expression plasmid (pDR196/GW), a yeast expression library of shuffled *OsSUT1* clones was created. *F1* and *R1*, primers for substrate preparation; *L1* and *L2*, attL1 and attL2 recombination sites, respectively; *F2* and *R2*, primers for amplification of shuffled ORFs.

activity as all constructs with the wild-type Thr at this position produced low fluorescence in yeast.

To rule out the possibility that low esculin uptake of the mutants was due to low transporter expression or activity, we tested [^{14}C]sucrose uptake activity of yeast expressing the same set of constructs. All of the OsSUT1 mutants had higher sucrose uptake activity than wild-type OsSUT1 (Fig. 4*C*). Therefore, we conclude that the set of mutations of Leu-80, Lys-86, Tyr-224, and either Ala-220 or Ala-221 can allow OsSUT1 to transport esculin.

Expression in Xenopus Oocytes—Wild-type *OsSUT1* and the *OsSUT1-m9* and *OsSUT1-m5* variants were expressed in *Xenopus* oocytes and subjected to two-electrode voltage clamping. As an example of a type I SUT, the same analysis was performed using *StSUT1*. The wild-type OsSUT1 protein was able to transport sucrose but not esculin (Fig. 5*A*). In contrast, OsSUT1-m9 and OsSUT1-m5 were both able to transport sucrose as well as esculin (Fig. 5, *B* and *C*). At a membrane potential of −40 mV, sucrose and esculin induced similar currents in *OsSUT1-m9*-expressing oocytes (Fig. 5*B*). In oocytes expressing *OsSUT1-m5*, esculin-induced current was significantly smaller than that induced by sucrose. StSUT1 transported both sucrose and esculin (Fig. 5*D*), typical of other type I SUTs (11). On average, the sucrose-induced currents were about 10-fold smaller for the two mutants than for the wild-type OsSUT1.

Altered Substrate Specificity—The substrate specificity of OsSUT1, the two variants OsSUT1-m5 and OsSUT1-m9, and StSUT1 was determined using two-electrode voltage clamping in *Xenopus* oocytes. The two shuffled OsSUT1 variants showed drastically altered substrate specificity compared with the wild-type transporter. OsSUT1 was very selective for sucrose as reported previously (17) and only transported a few other glucosides, maltose, the β-glucoside salicin, and α-phenyl glucoside (Fig. 6). In contrast, the substrate specificity of StSUT1 was typical of type I SUTs that transport a broad range of plant β-glucosides such as salicin, arbutin, fraxin, and esculin as well as several synthetic α- and β-phenyl glucosides (Fig. 6). The substrate specificity of the two OsSUT1 variants was very similar to that of StSUT1 and other type I SUTs with slight differences. OsSUT1-m9 and OsSUT1-m5 showed small currents induced by glucose and trehalose, which are not transported by any wild-type SUT. No current was induced by galactose or

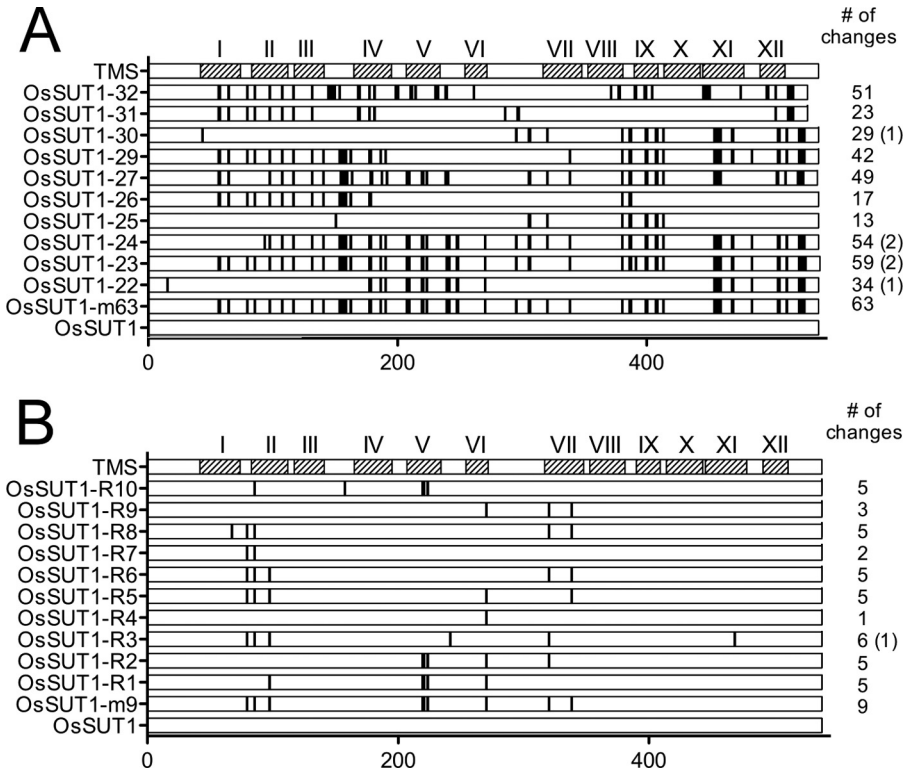

FIGURE 3. **Sequence comparison of 10 randomly selected library clones from two shuffling libraries.** *A*, library resulting from shuffling of *OsSUT1* with *OsSUT1-m63*. *B*, library resulting from shuffling of *OsSUT1* with *OsSUT1-m9*. *Black bars* indicate the position of amino acid changes compared with wild-type OsSUT1. The total number of amino acid changes is given on the *right*, and the number of PCR errors is given in *parentheses*. The *top row* indicates the predicted positions of TMS1–12 (*I–XII*) (35).

raffinose for any of the SUTs. The OsSUT1 variants showed currents several times larger than those induced by sucrose for several substrates such as salicin, helicin, and the α-phenyl glucosides.

Conversion of Sugarcane Type II ShSUT1—ShSUT1 is a type II SUT from sugarcane that is 83.2% identical to OsSUT1. Typical of type II SUTs, it is very selective and only transports sucrose, maltose, salicin, α-phenyl glucoside (16), and helicin (11). Sucralose was identified as a non-transported competitive inhibitor of ShSUT1 (16). ShSUT1 does not transport esculin (11). To test whether the five amino acid changes that convert OsSUT1 to type I substrate specificity can be generally applied to other type II SUTs, we made the corresponding mutations in ShSUT1. Consistent with previous reports, when ShSUT1 was expressed in oocytes, sucrose induced an inward current, whereas esculin and sucralose did not (Fig. 7*A*). Five mutations were made in ShSUT1 (T60L, A66K, S200A, S201A, and T204Y) to produce ShSUT1-m5. When this mutated transporter was expressed in oocytes, inward currents were induced by application of sucrose, esculin, and sucralose (Fig. 7*B*). As for OsSUT1-m5, transport rates for sucrose were lower for the mutant compared with wild type. The results indicate that this set of mutations can be applied in general to convert selective type II SUTs to substrate specificity similar to that of type I SUTs.

DISCUSSION

Plants utilize several mechanisms to transport sucrose into phloem cells of the vasculature (32). The apoplasmic phloem loading mechanism used by herbaceous plants such as most crop species involves export of sucrose into the wall space in the vicinity of the sieve element-companion cell complex followed by uptake into the sieve element-companion cell complex via SUTs. The hypothesis that apoplasmic phloem loading evolved separately in eudicots and monocots (4) is based on two observations. 1) Monocots and eudicots utilize phylogenetically different SUTs for phloem loading: eudicots use type I SUTs, whereas monocots use type II SUTs. 2) Type I SUTs appear to have evolved from vacuolar type III SUTs (4) rather than type II SUTs. Representatives of early land plants such as bryophytes and lycophytes contain type II and III SUTs. Type I SUTs appeared more recently in eudicots, and based on phylogeny and substrate specificity, they are more similar to type III SUTs. Type I SUTs that function in loading sucrose into the phloem in eudicots have a different substrate specificity than the type II SUTs that perform the same function in monocots. Here, we identified five amino acid positions that control differences in substrate specificity between type I and II SUTs. This is the first step toward being able to engineer the specificity of phloem loading and the specificity of sugar uptake into sinks.

In previous work, type II SUTs were found to be more selective for sucrose than type I or III SUTs as summarized by Reinders *et al.* (4). In particular, type I SUTs transport several plant β-glucosides including the fluorescent coumarin β-glucosides esculin and fraxin, whereas type II SUTs do not (11). We exploited this difference in activity to select type II mutants that have a wider substrate specificity. A gene shuffling strategy

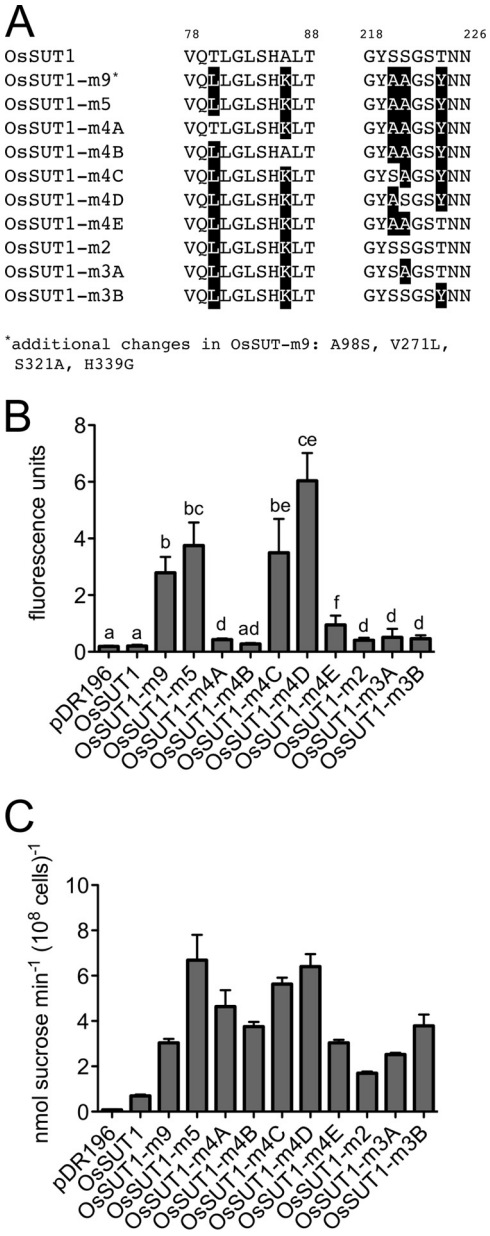

FIGURE 4. **Effect of each of five mutations on esculin and sucrose transport by OsSUT1 transport activity.** *A*, diagram of OsSUT1 and variants used in this study. The amino acids differing from the wild-type sequence are highlighted with *black*. *B*, esculin uptake by yeast cells expressing *OsSUT1* and *OsSUT1* variants. Yeast strain SEY6210 expressing *OsSUT1*, *OsSUT1* variants, or the empty vector was incubated with 1 mM esculin in sodium phosphate buffer at pH 4 for 1 h, and the relative fluorescence of the cells was determined. Data are presented as mean ± S.D. of relative fluorescence normalized to A_{600} for four independent transformants. The *letters* above the *columns* indicate significant differences based on one-way analysis of variance performed on values converted to their $\log_{10}$ (Tukey's multiple comparison test; $p < 0.05$). The five OsSUT1-m4A–E variants were derived from OsSUT1-m5 by converting each of the five amino acid changes individually back to the corresponding OsSUT1 residue. In OsSUT1-m2, three of the five amino acids were converted to the corresponding OsSUT1 residues, and in OsSUT1-m3A and OsSUT1-m3B, two of the five amino acids were converted to the corresponding OsSUT1 residues (see *A* for details). *C*, sucrose transport activity in yeast cells expressing OsSUT1 and OsSUT1 variants. Yeast cells were incubated with 1 mM [^{14}C]sucrose for 5 min in the presence of 10 mM glucose, aliquots were filtered, and the amount of radioactivity taken up was determined. The results presented are the mean ± S.D. of three independent experiments.

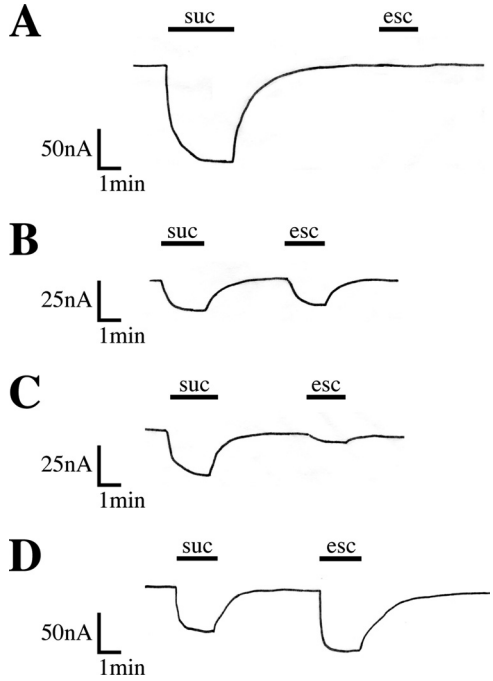

FIGURE 5. **Transport activity of OsSUT1, the two OsSUT1 variants selected by gene shuffling, and StSUT1.** *Xenopus* oocytes expressing *OsSUT1* (*A*), *OsSUT1-m9* (*B*), *OsSUT1-m5* (*C*), or *StSUT1* (*D*) were voltage-clamped at −40 mV, and currents were recorded. The oocytes were perfused with modified sodium Ringer at pH 5.6 (*A–C*) or pH 5 (*D*), and at the times indicated by the *lines* above the traces, 10 mM sucrose (*suc*) or 5 mM esculin (*esc*) was applied in the same buffer. Downward deflection of the recorded trace indicates an inward coupling ion current.

was designed to introduce amino acids that are conserved in type I SUTs into a type II SUT. After selection in yeast for the ability to transport esculin, a variant of the type II transporter OsSUT1 containing only five amino acid changes was found to have a substrate specificity typical of type I SUTs as shown by electrophysiological analysis in *Xenopus* oocytes.

The five amino acid positions identified in this study are not likely to be directly involved in SUT binding to sugar substrates. These positions are more likely to determine whether substrate binding leads to translocation. We know that the glucosyl moiety of the sugar substrate is required for binding to SUTs (10) and that glucosyl hydroxyls at positions 2, 3, 4, and 6 are important for binding (33, 34). An Arg residue (Arg-188 in TMS4 of OsSUT1) that is fully conserved in all SUTs has been hypothesized to interact with glucosyl hydroxyls (35), similar to the situation in LacY in which Arg-144 interacts with galactosyl hydroxyls (36). The five mutations occur in two clusters. Thr-80 and Ala-86 are in the first extracellular loop and at the top of TMS2, respectively, very close to His-85. His-85 in OsSUT1 is equivalent to His-65 in AtSUC1 that is the site of inhibitory diethyl pyrocarbonate modification (19). Substrate binding to AtSUC1 protects the protein from inhibition by diethyl pyrocarbonate. Ser-220, Ser-221, and Thr-224 are in TMS5, and no previous mutations have been analyzed in this helix. In LacY, TMS5 contains important residues for substrate binding; in particular, Trp-151 and Arg-144 directly bind the galactosyl moiety. In the present study, all of the positions important for substrate specificity were found in the N-terminal half of the protein, and this is similar to the situation in LacY

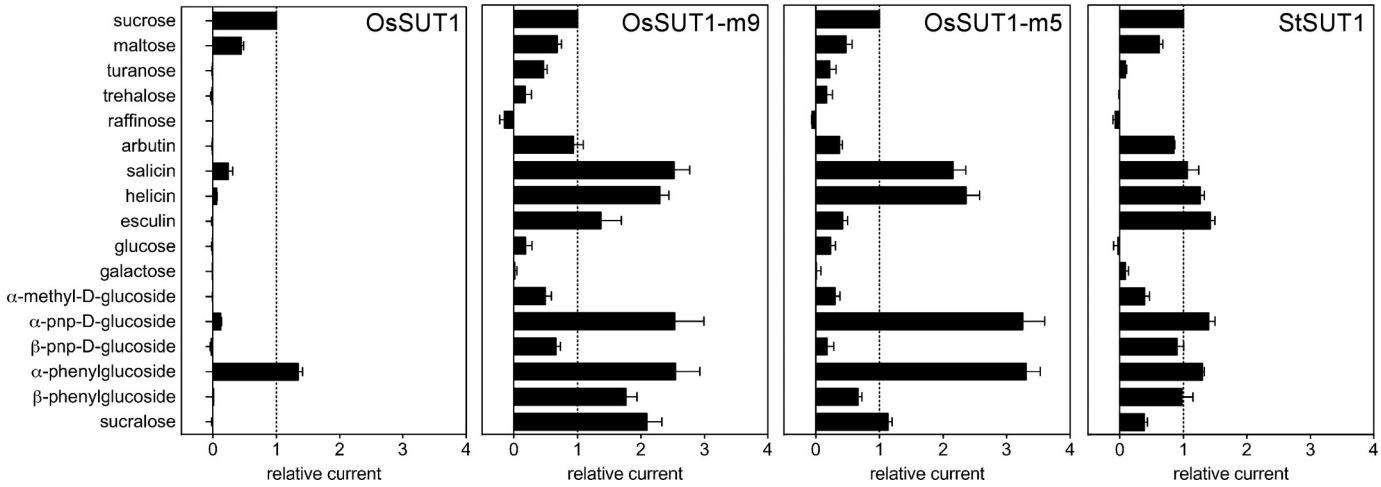

FIGURE 6. **Substrate specificities of OsSUT1, the two variants selected by gene shuffling (OsSUT1-m5 and OsSUT1-m9), and StSUT1.** Currents were recorded at −121.5 mV at pH 5.6 (OsSUT1, OsSUT1-m5, and OsSUT1-m9) or pH5 (StSUT1) and normalized to the current induced by 10 mM sucrose to account for different expression levels between oocytes. All substrates were added at a concentration of 10 mM except for esculin, which was added at 5 mM, the limit of its solubility. The results shown are average ± S.E. (n = 3–9). The average ± S.E. sucrose current at −121.5 mV was −361.8 ± 23.7 nA for OsSUT1 (n = 18), −38.7 ± 5.1 nA for OsSUT1-m9 (n = 16), −41.1 ± 5.0 nA for OsSUT1-m5 (n = 29), and −103.7 ± 12.0 nA for StSUT1 (n = 25). *pnp*, *para*-nitrophenyl.

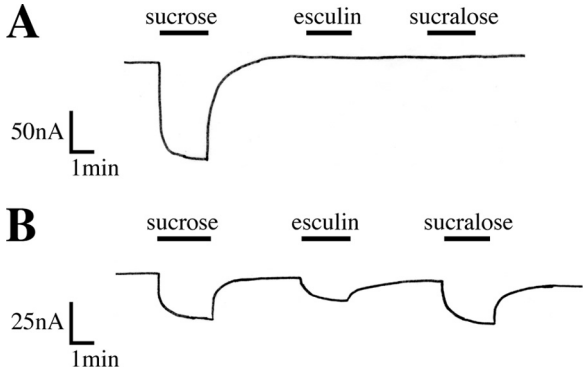

FIGURE 7. **Transport activity of ShSUT1 and ShSUT1-m5.** *Xenopus* oocytes expressing *ShSUT1* (*A*) or *ShSUT1-m5* (*B*) were voltage-clamped at −40 mV, and currents were recorded. The oocytes were perfused with modified sodium Ringer at pH 5.6, and at the times indicated by the *lines* above the traces, 10 mM sucrose, 5 mM esculin, or 10 mM sucralose was applied in the same buffer. Downward deflection of the recorded trace indicates an inward coupling ion current.

where the N-terminal half is involved in sugar substrate binding and the C-terminal half is involved in H^+ translocation (36). Although we selected only for the ability to transport esculin, the OsSUT1-m5 and OsSUT1-m9 variants transport the broad range of substrates typical of type I SUTs. We know that type II SUTs interact with a broader range of glucosides than they transport. For example, β-*para*-nitrophenyl glucoside is transported by type 1 SUTs (10) but is a competitive inhibitor of the type II SUT HvSUT1 (15). Similarly, sucralose is transported by type I SUTs (11), whereas the type II ShSUT1 does not transport sucralose but is competitively inhibited by it (16). We show here that ShSUT1-m5 transports sucralose. Therefore, the five amino acid changes identified here determine whether substrate binding leads to translocation.

The OsSUT1 Thr-80 position is 100% conserved in all type II SUTs in both monocots and eudicots. All type I SUTs have a Leu at this position, and type III SUTs contain either a Gln or Glu. Therefore, this position should also be considered a candidate for controlling the difference in substrate specificity between type I and III SUTs as well. In general, type I and III SUTs have a similar substrate specificity (4), but type III SUTs do not transport esculin or fraxin. The OsSUT1 Ala-86 position is well conserved in type II SUTs with a few exceptions; OsSUT5 has a Ser at this position, and differences in substrate specificity were found between OsSUT1 and OsSUT5 (17). For example, OsSUT5 transports the plant β-glucoside arbutin, whereas OsSUT1 does not. Type I SUTs have either a Thr, Lys, or Arg at this position, whereas most type III SUTs have an Ala at this position, but LjSUT4, the only type III SUT for which we have analyzed substrate specificity (12), has a Gln at this position.

Three amino acid positions, Ser-220, Ser-221, and Thr-224, are closely clustered in TMS5 and underlined in the following sequence from OsSUT1: SSGST. Ser-220 is well conserved in type II SUTs; one exception is OsSUT5, which has a Leu at this position. The sequence of OsSUT5 in this region, LSGAD, is quite different from OsSUT1 and other monocot type II SUTs and may explain why OsSUT5 shows differences in substrate specificity compared with other type II SUTs. All type I and III SUTs have an Ala at the position corresponding to Ser-220. Ser-221 is well conserved in monocot type II SUTs; however, type II SUTs from eudicots as well as all type I and type III SUTs have an Ala at this position. This indicates that the substrate specificity of type II SUTs from eudicots may be different from that of monocot type II SUTs, although this has not been tested directly. Position Thr-224 of OsSUT1 is either a Ser or Thr in type II SUTs with a few exceptions such as Asp in OsSUT5 as noted above. However, in type I and III SUTs, this position is predominantly Tyr, but there are a few examples of either Phe or Ile at this position. Therefore, the consensus sequence for type I SUTs in this region is AAGSY; for type III, the consensus sequence is ATGAY; and for type II, it is S(S/A)G(S/A)(S/T).

Several of the methods used in this study may be generally useful. The STS method has some advantages over other shuffling methods for analyzing the structural basis for differences in activity between related proteins especially if the sequences

do not have high identity. Here, STS was used to limit the changes introduced to differentially conserved positions in type I and II SUTs. Backcrossing and site-directed mutagenesis was used to identify the five positions that are sufficient to confer type I substrate specificity on a type II SUT. Gateway cloning to produce the shuffled library may also be generally useful because it tends to produce full-length shuffled products containing both 5′- and 3′-terminal recombination sites. Finally, the fluorescent substrate uptake assay used in conjunction with FACS (18) should be useful for identifying novel transporters using fluorescent substrates.

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