

Investigating polymorphisms in membrane-associated transporter protein *SLC45A2*, using sucrose transporters as a model

ANKE REINDERS and JOHN M. WARD

Department of Plant Biology, University of Minnesota, St. Paul, MN 55108, USA

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Abstract. Solute carrier family 45 member 2 encodes the melanosomal membrane protein, membrane-associated transporter protein (MATP), of unknown function, that is required for normal melanin synthesis. The present study analyzed the effects of two human MATP mutations, D93N, which causes oculocutaneous albinism 4 (OCA4), and L374F, which is correlated with light pigmentation in European populations. Corresponding mutations were produced in the related and well-characterized sucrose transporter from rice, OsSUT1, and transport activity was measured by heterologous expression in *Xenopus laevis* oocytes, in addition to ^{14}C -sucrose uptake in yeast. The mutation corresponding to D93N resulted in a complete loss of transport activity. The mutation corresponding to L374F resulted in a 90% decrease in transport activity, although the substrate affinity was unaffected. The results indicated that the D93N mutation causes OCA4 as a result of loss of MATP transport activity, and that the F374 allele confers significantly lower transport activity than L374.

Introduction

Melanin is the main pigment responsible for skin, eye and hair pigmentation in animals. It is produced in melanocytes, in lysosome-derived organelles, termed melanosomes. Melanin is synthesized from tyrosine by the enzyme tyrosinase (monophenol, L-dopa: oxygen oxidoreductase, EC 1.14.18.1). The gene solute carrier family 45 member 2 (*SLC45A2*) encodes the membrane-associated transporter protein (MATP) that is hypothesized to be localized to the melanosome membrane and to regulate tyrosinase activity by controlling melanosome pH (1). MATP is an integral membrane protein that contains twelve predicted membrane spans and belongs to

the glycoside-pentoside-hexuronide (GPH):cation symporter family, which is related to the major facilitator superfamily (MFS) (2). Although no transport function has so far been identified for MATP, plant sucrose transporters, which also belong to the GPH family, have been characterized in detail. Plant sucrose transporters are localized to the plasma membrane or the vacuolar (lysosomal) membrane and catalyze the symport of H^+ and sucrose, as well as that of other glucosides (3-5).

Loss of function mutations in *SLC45A2* result in a lack of melanin pigmentation. In humans, mutations in *SLC45A2* are the underlying cause of oculocutaneous albinism 4 (OCA4) (6). Similarly, *SLC45A2* mutations in other animals result in hypopigmentation (7,8). Evidence that MATP regulates the pH of the melanosome lumen includes the ability of bafilomycin A1 (BafA1), an inhibitor of V-type ATPases, to reverse the albino phenotype of zebrafish carrying a mutation in *SLC45A2* (1). Therefore, the transport activity of MATP may involve H^+ cotransport similar to that of plant sucrose transporters. V-type ATPases acidify the lumen of membrane-bound organelles, such as lysosomes, in animal cells or vacuoles in plants. Sucrose transporters in plants utilize this proton gradient to transport sucrose into the cytoplasm (4,9). In melanosomes, MATP may employ the same mechanism, cotransporting an unknown substrate with a proton in order to raise the pH of the melanosome lumen. The activity of tyrosinase is strongly dependent on the luminal pH of the melanosome; it is most active at neutral pH and is inhibited by an acidic pH (10). FM3 and B16-G4F melanoma cell lines are amelanotic and lack tyrosinase activity. Treatment of these cells with BafA1 leads to rapid melanin synthesis, which is independent of *de novo* synthesis of tyrosinase (11), indicating that melanosome pH controls tyrosinase activity and melanin synthesis.

The c.1122G>C mutation (L374F) in MATP is associated with skin color variation in humans. The ancestral form, L374, is nearly fixed in Africa and Japan (12,13). The L374F mutation is thought to have occurred only once, and the F374 allele is associated with light-colored skin and predominates in the European population (14,15). A previous study using cultured melanocytes revealed that homozygous F374 melanocytes have less melanin and lower tyrosinase activity compared with L374 cells (16). This indicates that MATP influences tyrosinase biosynthesis or stability, in addition to possibly regulating tyrosinase activity by controlling melanosome pH. A previous study in the mouse underwhite (uw) mutant suggested that MATP may also have a role in melanosome development (17).

Correspondence to: Professor John M. Ward, Department of Plant Biology, University of Minnesota, 250 Biological Sciences Center, 1445 Gortner Avenue, St. Paul, MN 55108, USA
E-mail: jward@umn.edu

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It is difficult to investigate the effects of mutations on MATP function directly, as the transported substrates of this protein have not been identified to date. Therefore it may be helpful to utilize related transporters as a model with which to study MATP activity. The closest homologs to MATP with known transport function are sucrose transporters from plants. The substrate specificity of sucrose transporters has been analyzed (18); they transport a wide range of glucosides including sucrose. Amino acid positions that contribute to substrate specificity have also been investigated (19). In the current study, two important polymorphisms in SLC45A2 that result in single amino acid changes in MATP were studied. The effects of these changes on MATP function are not known. The corresponding mutations were created in a sucrose transporter and the effects on transport activity were analyzed.

Materials and methods

Constructs. Site-directed mutagenesis of OsSUT1 in Gateway entry vector pCR8/GW (Invitrogen Life Technologies, Carlsbad, CA, USA) was performed using the QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). The following primers were used: Forward: 5'-catgcgtgggtctatacagtAataggtgcacctc-3' and reverse: 5'-gaggtgcacctatTactgtatagacccacgcctg-3' for OsSUT1_D111N (to change Asp111 to Asn) and forward: 5'-cagggcaggtgcatttggcTtCtactgaattcgatttg-3' and reverse: 5'-cacaatcgaattcagtagGaAgccaaatgcacctgccctg-3' for OsSUT1_L363F (to change Leu363 to Phe). Upper case letters denote the changes introduced into the primer sequences. All introduced DNA sequence changes were verified by sequencing the entire insert. Sequencing services were provided by ACGT, Inc. (Wheeling, IL, USA). For oocyte expression, the constructs in the entry vector were recombined with pOO2/GW (20). cRNA for oocyte injection was prepared from templates linearized with MluI, using the SP6 mMessage mMachine kit (Invitrogen Life Technologies). Each oocyte was injected with 50 ng of RNA. For yeast expression, entry clones were recombined with pDR196/GW (21).

Preparation of oocytes and two-electrode voltage-clamping (TEVC). *Xenopus laevis* oocytes were prepared and TEVC was performed as described previously (4). Oocytes were bathed in modified sodium Ringer solution (115 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂ and 5 mM MES-Tris, at pH 5.6). Recording pipettes were filled with 1 M KCl and showed tip resistances of 1.5 to 3 megaohms. Currents were measured using a Dagan TEV 200A amplifier (Dagan Corp., Minneapolis, MN, USA). Currents were filtered online at 200 Hz and digitized at 2,000 Hz using pClamp 5.5.1 (Axon Instruments, Inc., Union City, CA, USA). The holding potential was -40 mV and voltage pulses from -142 to +62 mV were applied for 200 ms. Mean steady-state currents are presented. Sucrose-dependent currents were obtained by subtracting an average of background currents prior to and following sucrose application. Sucrose in modified sodium Ringer solution was added at the concentrations indicated in the figure legends.

Yeast ¹⁴C-sucrose uptake assay. The uptake of radio-labeled ¹⁴C-sucrose into yeast cells was determined as described

previously (22). Briefly, yeast strain, SEY6210 (MAT α leu2-3,112 ura3-52 his3- Δ 200 lys2-801 trp1- Δ 901 suc2- Δ 9) (23), transformed with OsSUT1, OsSUT1_D111N or OsSUT1_L363F in pDR196/GW (empty vector pDR196 was used as control) was grown to mid-log phase in SD-URA [1.7 g/l Difco™ yeast nitrogen base (Becton Dickinson, Franklin Lakes, NJ, USA), 5 g/l ammonium sulfate, 20 g/l glucose and addition of the required supplements]. Cells were harvested by centrifugation for 5 min at 2,000 x g, washed with 25 mM Na-phosphate buffer (pH 4) and resuspended at an OD₆₀₀ of 20. For uptake assays the cells were incubated at 30°C in the presence of 10 mM glucose and ¹⁴C-labeled sucrose (final concentration 1 mM) for 5 min, then collected onto glass fiber filters (type A/C; Pall Corporation, Ann Arbor, MI, USA) by vacuum filtration and washed with 20 ml 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.

Statistical analysis. Means and standard deviations were calculated using Microsoft Excel 2011 (Microsoft Corp., Redmond, WA, USA). GraphPad Prism software version 5.0a (GraphPad Software, La Jolla, CA, USA) was used for non-linear regression analyses of transporter kinetics and to perform one-way analysis of variance followed by Tukey's multiple comparison test. P<0.05 was considered to indicate a statistically significant difference.

Results

Conserved positions in MATP and sucrose transporters. More than 50 alleles of SLC45A2 have been identified in patients with OCA4. Many of them are predicted to result in amino acid changes in the MATP protein. MATP is distantly related to plant sucrose transporters, with ~25% similarity at the amino acid level between plant sucrose transporters and MATP. Sequence identity between MATP and sucrose transporters is predominantly within the membrane-spanning domains. A number of the mutations that are known to lead to OCA4 in humans result in amino acid changes at positions conserved with plant sucrose transporters, and those are also located primarily within membrane-spanning domains (Fig. 1). Table I summarizes a list of known OCA4 alleles and the corresponding amino acids in the rice sucrose transporter OsSUT1 (24). Of the identified amino acids, thirteen are perfectly conserved between humans and plants, and five are similar amino acids (Table I).

Changing Asp111 to Asn in OsSUT1 inactivates sucrose transport activity. The D93N allele of SLC45A2 has been identified in Turkish (25) and Korean (26) patients with OCA4, and due to their strong phenotype, it is likely that this change abolishes function of MATP. D93 is conserved in animal MATP homologs and in plant sucrose transporters. This position corresponds to D111 in the OsSUT1 sucrose transporter. Site-directed mutagenesis of Asp to Asn (D111N) was performed, and the mutated protein was subsequently expressed in *Xenopus* oocytes. While the wild-type OsSUT1 induced large currents in the presence of applied sucrose, as a result of the transport of the co-transported H⁺ (Fig. 2) (20), the OsSUT1_D111N mutant did not induce any currents under the

Table I. AA positions in MATP causing OCA4 or pigmentation phenotypes, which are conserved in plant sucrose transporters.

A, OCA4 alleles with identical residues in OsSUT1

OCA4 allele	OsSUT1 residue	Reference(s)
G44R	G62	38
P58A/S	P76	36,37
G64S	G82	44
G89R	G107	45
D93N	D111	25
G100S	G118	38
R101C	R119	38
G110R	G127	35
L151P	L175	35
G198D	G222	38
G349R	G340	35
G473D	G467	45
A511E	A506	25

B, OCA4 alleles with similar residues in OsSUT1

OCA4 allele	OsSUT1 residue	Reference
Y49C	W67	45
G188D	A212	46
G188V	A212	37
T440A	S449	45
A477T	G471	36

C, Non-OCA polymorphism

OCA4 allele	OsSUT1 residue	Reference
L/F374	L363	6

Corresponding positions in the rice sucrose transporter, OsSUT1, are shown. MATP, membrane-associated transporter protein; OCA, oculocutaneous albinism; AA, amino acids.

same conditions (Fig. 2). This is consistent with an inactivation of the transporter due to the introduced amino acid change.

Changing Leu363 to Phe in OsSUT1 reduces sucrose transport activity but does not affect the apparent substrate affinity. One polymorphism that is not implicated in OCA4, although it is known to be correlated with differences in pigmentation in human populations, is L/F374. Since loss-of-function mutations in SLC45A2 result in OCA4, a lower activity of MATP is thought to result in reduced pigmentation. In accordance with this, mutations in SLC45A2 that lower transcription result in a reduction in pigmentation (27). The F374 allele is generally found in individuals with lighter skin and hair (12,14,15,28). In plants, the corresponding position contains a Leu (L363), and is conserved in all plant sucrose transporters.

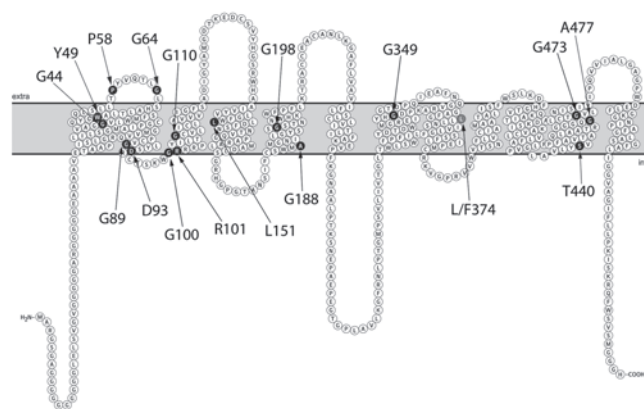


Figure 1. Position of known OCA4 alleles conserved between MATP and rice sucrose transporter, OsSUT1. The drawing, made using Protter (47), shows the OsSUT1 protein with its predicted transmembrane spans according to Sun *et al* (40). Arrows indicate the position of conserved residues (in black) associated with OCA or pigmentation phenotypes. The amino acid numbers given are for MATP. For the corresponding position in OsSUT1, see Table I. The L363 residue corresponding to the L/F374 polymorphism in MATP is shown in dark gray. OCA4, oculocutaneous albinism 4; MATP, membrane-associated transporter protein.

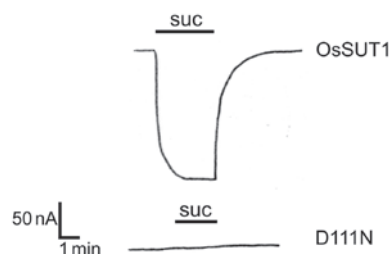


Figure 2. Mutation D111N in the sucrose transporter, OsSUT1, inactivated transport activity. Traces show the current induced by addition of 10 mM sucrose (indicated by the bar above the trace) to oocytes, expressing either wild-type OsSUT1 or the D111N mutant, held at a membrane potential of -40 mV. D111N corresponds to the OCA4 allele D93N. suc, sucrose.

The effect of L374F on activity was examined by replacing L363 with Phe in the rice sucrose transporter, OsSUT1. Transport activity was analyzed in oocytes by TEVC. Compared with the wild-type transporter, the OsSUT1_L363F mutant produced only ~10% of the current in the presence of 10 mM sucrose at a membrane potential of -40 mV (Fig. 3A). The transport activity of OsSUT1_L363F was lower than that of the wild type transporter at all membrane potentials tested (Fig. 3B). However, the apparent affinity of the mutated transporter was not different from that of the wild type OsSUT1. OsSUT1_L363F had a $K_{0.5}$ of 7.46 mM sucrose at pH 5.6 and a membrane potential of -100 mV (Fig. 3C). The $K_{0.5}$ of wild-type OsSUT1 was previously reported to be 7.5 mM at pH 5.6 (20).

In order to confirm the results using an independent heterologous system, OsSUT1 and the two mutants, OsSUT1_D111N and OsSUT1_L363F, were expressed in yeast, and sucrose uptake was determined using ^{14}C -sucrose as a substrate. Consistent with the results from oocytes, the D111N mutant showed no significant sucrose uptake above the background level (empty vector). Sucrose uptake by yeast expressing the L363F mutant was also not statistically different from the vector control (Fig. 4).

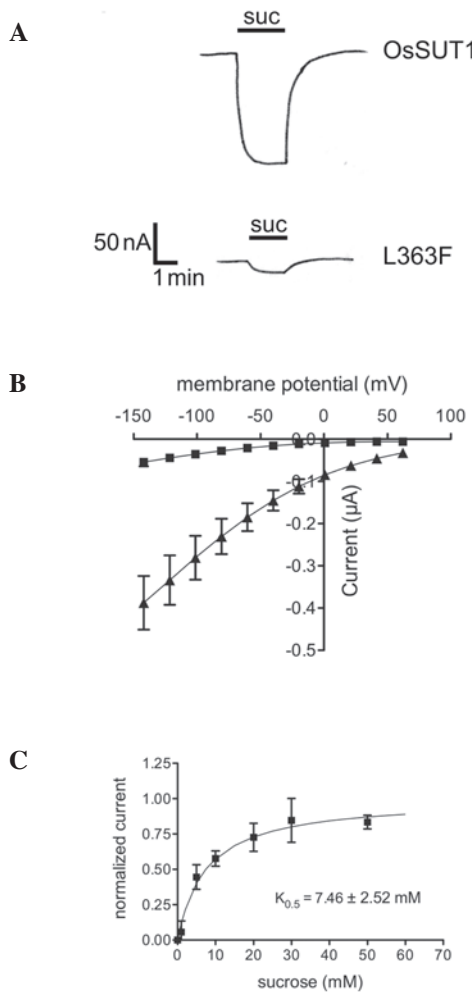


Figure 3. Mutation L363F in OsSUT1 decreased sucrose transport activity. L363F corresponds to the L/F374 allele of MATP, which is correlated with differences in pigmentation: Individuals carrying the L374 allele have dark hair and dark eyes. (A) OsSUT1_L363F exhibited reduced sucrose transport activity. Current induced by addition of 10 mM sucrose (indicated by the bar above the trace) in oocytes, expressing either wild-type OsSUT1 or the L363F mutant, at a membrane potential of -40 mV. (B) Transport activity of the L363F mutant (squares) was reduced compared with the wild-type allele (triangles) at each membrane potential tested. Data are presented as the mean \pm standard deviation of sucrose-dependent currents from three oocytes for each transporter. (C) Changing Leu 363 to Phe did not affect the apparent affinity of OsSUT1. Sucrose-dependent currents at a membrane potential of -100 mV were plotted against sucrose concentration. Data are presented as the mean \pm standard deviation of three oocytes each. $K_{0.5}$ was calculated based on a non-linear regression fit to the Michaelis-Menten equation. The previously determined $K_{0.5}$ for OsSUT1 wild type was 7.5 mM (20). MATP, membrane-associated transporter protein.

Discussion

Worldwide, ~1 in 17,000 people are affected by oculocutaneous albinism (OCA) (29), a heritable autosomal recessive condition that is defined by a lack of pigmentation in hair, skin and eyes. It is projected that 1 in 70 people carry an allele for albinism (30). Besides the visible effects on pigmentation, patients also experience a variety of problems with vision as a consequence of the lack of pigment in the retina. OCA subtypes are differentiated based on underlying mutations. OCA1 is caused by mutations in the tyrosinase gene, encoding an enzyme critical for melanin synthesis. In OCA2, the OCA2 gene is affected,

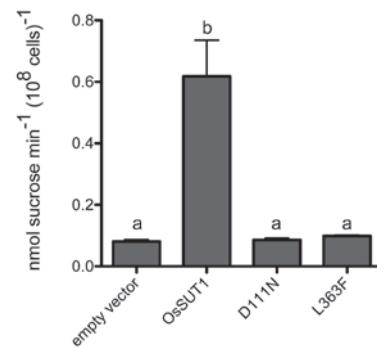


Figure 4. ^{14}C -sucrose uptake activity in yeast expressing OsSUT1 wild type, the D111N or L363F mutant. The yeast strain, SEY6210, transformed with OsSUT1, OsSUT1_D111N, OsSUT1_L363F or the empty vector, was incubated with 1 mM ^{14}C -sucrose at pH 4. Data are presented as the mean \pm standard deviation from three independent transformants. Different letters above the columns (a, b) denote values that were statistically different from each other ($P < 0.05$) based on one-way analysis of variance followed by Tukey's multiple comparison test.

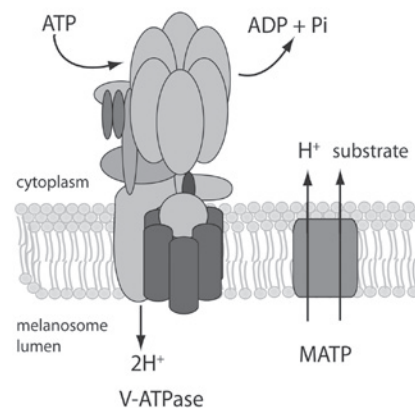


Figure 5. Model for the proposed function of MATP in increasing pH in melanosomes. V-ATPase H^+ -pumping activity in the melanosomal membrane acidifies the melanosome lumen and generates an H^+ gradient across the membrane. MATP is also localized in the melanosome membrane and uses the H^+ gradient to energize symport of H^+ and an unidentified substrate, possibly a sugar, out of the melanosome. This increases the pH of the melanosome and increases the activity of tyrosinase and melanin synthesis. MATP, membrane-associated transporter protein.

which encodes the P-protein, a transporter with homology to bacterial Na^+/H^+ -antiporters. OCA3 is characterized by mutations in the tyrosinase-related protein 1 (TRP1) gene, while OCA4 is caused by mutations in SLC45A2, which encodes a membrane protein that is localized to melanosomes and has homology to plant sucrose transporters. Melanin synthesis occurs in melanosomes, which are specialized organelles of melanocytes. Key steps of melanin synthesis are catalyzed by the enzyme tyrosinase, a copper-dependent glycoprotein with a single transmembrane-spanning domain. Polymorphisms in SLC45A2 produce similar effects in other animals. In horse, D153N in the SLC45A2 homolog MATP is associated with a cream coat color (31). In mouse, an SLC45A2 homolog is encoded by the *uw* gene (6,17), while mutations in the corresponding gene *b* in medaka fish reduce melanin content (7) and in birds, plumage color is also controlled by alleles of the gene encoding MATP (32). In zebrafish, bafilomycin, an inhibitor

of V-ATPase, has been found to rescue melanin synthesis in an SLC45A2 mutant (1). This indicates that MATP activity may increase the luminal pH of melanosomes and thereby stimulate melanin synthesis. A model for V-ATPase and MATP in the melanosome membrane is presented in Fig. 5. In this model, V-ATPase acidifies the melanosome lumen, inhibiting tyrosinase activity, and MATP functions to stimulate tyrosinase by increasing luminal pH. However, transport activity has not been assayed for an MATP homolog from any animal, and the substrates and transport mechanisms remain unknown. SLC45A2 has been expressed in *Xenopus* oocytes and in yeast, without detection of sucrose transport activity (Ward and Reinders, unpublished result). The closest homologs to SLC45A2 with known transport function are sucrose transporters (SUTs) from plants (18). SUTs are H⁺-coupled uptake transporters (symporters) that transport sucrose and H⁺ into the cytoplasm. They are localized to the plasma membrane, with the exception of type III sucrose transporters, which are found in the vacuole membrane (4,33).

Certain mutations in SLC45A2, isolated from patients with OCA4, produce a truncated MATP protein that is likely to be non-functional (6,25,34-39). Polymorphisms that produce single amino acid substitutions in MATP, and that lead to OCA4 or result in pigmentation phenotypes, are more difficult to interpret. For these alleles, it may be useful to have a biochemical assay for the MATP protein in order to determine whether transport activity is altered and whether V_{max} or substrate affinity, or other activities are affected. In the absence of a direct assay of MATP, this group used transport assays of the related plant sucrose transporters as a model to test the effect of SLC45A2 mutations. A number of the point mutations that cause OCA4 result in changes in amino acids that are conserved between animal and plant homologs (Table I). In the present study, the effects of two SLC45A2 mutations, D93N that causes OCA4, and L374F that is related to light skin color in Europeans were tested.

When the MATP D93N mutation was reproduced in OsSUT_D111N, sucrose transport activity was not detectable (Fig. 2). A 3D model of OsSUT1 locates D111 at the cytoplasmic edge of TMS2 (40). D111 is conserved in all plant sucrose transporters, and the lack of activity of the D111N mutant indicates that it may possess an important function. This result suggests that the D93N MATP mutant, which causes OCA4, lacks transport activity. The L374F mutation in SLC45A2 is hypothesized to have occurred recently and to have undergone positive selection during the last 5,000 years (41,42). Selection for lighter pigmentation may have been driven by a requirement for greater exposure to sunlight, necessary for vitamin D biosynthesis, at locations distant from the equator.

The results indicate that although sequence identity is only 25% between MATP and sucrose transporters, the conserved residues may be important for transport function in each of these transporters. Therefore, information on the structure-function relation of sucrose transporters may be useful in predicting the effects of mutations on the function of MATP. The sucrose transporter 3D structure has been modeled (40), based on the crystal structure of lactose permease and other members of the major facilitator superfamily obtained from *E. coli*. This produced a refinement in the transmembrane model for sucrose transporters as presented in Fig. 1. As in lactose

permease, conserved charged amino acids within membrane spans in the sucrose transporter, OsSUT1, were found to be important for transport function (40). Further studies may be conducted in order to determine whether other mutations corresponding to OCA alleles affect the function of sucrose transporters. Conversely, the effects of numerous sucrose transporter mutations on transport activity have been investigated in detail (19,40,43) and these may be used to predict the effect of corresponding mutations in SLC45A2.

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