# Impaired Function of the Tonoplast-Localized Sucrose Transporter in Rice, *OsSUT2*, Limits the Transport of Vacuolar Reserve Sucrose and Affects Plant Growth[1][W]

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Physiological functions of sucrose (Suc) transporters (SUTs) localized to the tonoplast in higher plants are poorly understood. We here report the isolation and characterization of a mutation in the rice (*Oryza sativa*) *OsSUT2* gene. Expression of *OsSUT2-green fluorescent protein* in rice revealed that OsSUT2 localizes to the tonoplast. Analysis of the *OsSUT2* promoter::*β-glucuronidase* transgenic rice indicated that this gene is highly expressed in leaf mesophyll cells, emerging lateral roots, pedicels of fertilized spikelets, and cross cell layers of seed coats. Results of Suc transport assays in yeast were consistent with a $H^+$-Suc symport mechanism, suggesting that OsSUT2 functions in Suc uptake from the vacuole. The *ossut2* mutant exhibited a growth retardation phenotype with a significant reduction in tiller number, plant height, 1,000-grain weight, and root dry weight compared with the controls, the wild type, and complemented transgenic lines. Analysis of primary carbon metabolites revealed that *ossut2* accumulated more Suc, glucose, and fructose in the leaves than the controls. Further sugar export analysis of detached leaves indicated that *ossut2* had a significantly decreased sugar export ability compared with the controls. These results suggest that *OsSUT2* is involved in Suc transport across the tonoplast from the vacuole lumen to the cytosol in rice, playing an essential role in sugar export from the source leaves to sink organs.

The disaccharide Suc is a major product of photosynthesis produced in source leaves and is the main carbohydrate transported in the vascular tissue of many plants. The orchestrated production, transport, and storage of Suc, therefore, is essential for normal plant growth and development (Lalonde et al., 2004; Lim et al., 2006; Kühn and Grof, 2010). Suc transport occurs at both the whole plant and intracellular levels, and this sugar is loaded over a short distance to the phloem either apoplastically by the plasma membrane sucrose transporters (SUTs) or symplastically through plasmodesmata, depending on the plant species (Rennie and Turgeon, 2009). Subsequent to phloem loading, Suc is transported over a long distance to sink tissues (ap Rees and Hill, 1994; Williams et al., 2000; Lalonde et al., 2004; Lim et al., 2006; Sauer, 2007). Within cells, Suc is partitioned into organelles, especially vacuoles, for short-term or long-term storage.

SUTs are encoded by small gene families in plants that have been divided into five major clades, SUT1 to SUT5 (Kühn and Grof, 2010). The SUT1 clade represents dicot SUTs, which have the highest substrate affinities. Members of SUT1 and monocot-specific SUT3 clades are localized at the plasma membrane and are responsible for phloem loading or Suc import into sink tissues (Riesmeier et al., 1994; Gottwald et al., 2000; Slewinski et al., 2009). The SUT2 clade members, localized also at the plasma membrane, possess an unusually long N terminus and central loop sequence. Recently, some SUT4 clade members such as Arabidopsis (*Arabidopsis thaliana*) AtSUT4, barley (*Hordeum vulgare*) HvSUT2, *Lotus japonicus* LjSUT4, and poplar

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(*Populus tremula* × *Populus alba*) PtaSUT4 have been assigned to the vacuolar membrane, tonoplast, through proteomic and/or GFP fusion analyses (Endler et al., 2006; Reinders et al., 2008; Payyavula et al., 2011). The SUT5 clade is a largely uncharacterized group of monocot Suc transporters, of which OsSUT5 has a higher affinity for Suc, and its transport activity is less sensitive to pH than transporters in the SUT3 clade (Sun et al., 2010).

The organ- and tissue-specific expression of SUTs suggests that they have distinct functions. In the SUT1 clade, potato (*Solanum tuberosum*) *StSUT1* (Riesmeier et al., 1994), tobacco (*Nicotiana tabacum*) *NtSUT1* (Bürkle et al., 1998), and Arabidopsis *AtSUC2* (Gottwald et al., 2000) are expressed in leaf vascular tissues and are essential for phloem loading. Similarly, members of the SUT3 clade, such as rice (*Oryza sativa*) *OsSUT1* and maize (*Zea mays*) *ZmSUT1*, are also thought to be responsible for phloem loading (Scofield et al., 2007; Slewinski et al., 2009). For both clades, the expression of a number of members is also observed in sink tissues, suggesting additional functions in relation to Suc transport into sink organs. For instance, *AtSUC1* (Stadler et al., 1999; Sivitz et al., 2008), *NtSUT3* (Lemoine et al., 1999), and *Plantago major SUC1* (*PmSUC1*; Lauterbach et al., 2007) are expressed in pollen tubes, suggesting their function in growing pollen tubes. From the SUT3 clade, *OsSUT1* is expressed in pollen and in nucellar projections and aleurone tissues of immature seeds and thus likely functions in apoplastic Suc transport into these sink organs (Furbank et al., 2001; Hirose et al., 2010). *HvSUT1* is also mainly expressed in maternal nucellar projections and in the filial transfer cells of barley (Weschke et al., 2000), suggesting that it has similar functions.

Among the SUT4 clade members that include tonoplast-localized SUTs, *HvSUT2* is expressed in leaves, roots, and pericarps (Weschke et al., 2000) and was further found to be expressed in leaf mesophyll cells (Endler et al., 2006). *AtSUT4*, which functions as a $H^+$-Suc symporter (Weise et al., 2000), is expressed in the companion cells (Schulze et al., 2003) and mesophyll cells of leaves (Endler et al., 2006). Thus, *HvSUT2* and *AtSUT4* have been suggested to function in the transport and vacuolar storage of photosynthetically derived Suc (Endler et al., 2006). *LjSUT4* was demonstrated to be a tonoplast-localized $H^+$-Suc symporter and thus hypothesized to function in Suc transport across the tonoplast from the vacuole lumen to the cytosol (Reinders et al., 2008). Recently, RNA interference (RNAi) transgenic poplar plants with reduced *PtaSUT4* expression showed an increased ratio of leaf to stem biomass, indicating a link between vacuolar transport of Suc and biomass partitioning (Payyavula et al., 2011). However, physiological evidence for the function of tonoplast SUTs has not yet been reported in plant mutants.

It has been previously shown that a number of cereals including rice store relatively high ratios of Suc to transitory starch in their leaves, which differs from other plant species, including Arabidopsis, which primarily store starch (Nakano et al., 1995; Winder et al., 1998; Murchie et al., 2002; Trevanion, 2002; Lee et al., 2008). Considering that Suc is temporarily stored in the vacuoles of photosynthetic assimilatory tissues (Riens et al., 1991; Winter et al., 1993; Martinoia et al., 2007; Neuhaus, 2007; Linka and Weber, 2010), this raises the question of the involvement and function of tonoplast-localized SUTs in plant growth and development in cereals. The rice genome contains five SUTs, *OsSUT1* to *OsSUT5* (Aoki et al., 2003). To date, however, only *OsSUT1* has been characterized in detail (Scofield et al., 2007; Hirose et al., 2010; Sun et al., 2010). We aimed to increase our understanding of Suc storage and transport in rice, an important worldwide crop. In this regard, characterization of the remaining OsSUTs is necessary, as these proteins underpin the process of Suc transport in rice. In this study, *OsSUT2*, a tonoplast-localized SUT, was analyzed by subcellular localization using a GFP fusion protein, via its Suc transport ability in yeast and through its gene expression properties using promoter::*GUS* transgenic rice. Furthermore, an *OsSUT2* mutant allele, *ossut2*, was characterized in terms of morphological phenotype, primary metabolite levels, and sugar export ability. Finally, a possible physiological role of *OsSUT2* is proposed.

## RESULTS

### Identification of the Rice Tonoplast Suc Transporter *OsSUT2*

To compare the amino acid similarity between the five reported OsSUTs (Aoki et al., 2003) and SUTs from other plant species, we constructed an unrooted phylogenetic tree using the neighbor-joining method (Supplemental Fig. S1). This analysis revealed that OsSUT2 (LOC_Os12g44380) belongs to the SUT4 clade of tonoplast-localized SUTs including AtSUT4, HvSUT2, LjSUT4, and PtaSUT4 (Endler et al., 2006; Reinders et al., 2008; Payyavula et al., 2011) and the plasma membrane-localized StSUT4 (Chincinska et al., 2008). For the other members of the SUT4 clade, their localization remains to be determined.

To characterize *OsSUT2*, its full-length cDNA was cloned by reverse transcription (RT)-PCR. This cloned *OsSUT2* gene (GenBank accession no. HQ540307) was from the *japonica* cv Dongjin and found to encode a protein of 501 amino acids with six amino acid differences compared with OsSUT2 from the *japonica* cv Nipponbare (AB091672; Aoki et al., 2003) and a single amino acid difference compared with OsSUT2 from the *indica* cv Minghui86 (DQ072592; Sun et al., 2008). OsSUT2 consists of 12 predicted transmembrane spans, short cytoplasmic N- and C-terminal ends, and a short central cytoplasmic loop between transmembrane spans 6 and 7, which are common characteristics of members of the SUT4 clade (Supplemental

Fig. S2). This substantial structural similarity to AtSUT4, HvSUT2, LjSUT4, and PtaSUT4 further suggests that OsSUT2 is a strong candidate as a tonoplast-localized SUT.

To next determine the subcellular localization of OsSUT2, we generated transgenic rice plants expressing an *OsSUT2-GFP* fusion construct under the control of the cauliflower mosaic virus (*CaMV*) *35S* promoter. The tonoplast localization of OsSUT2 was demonstrated in protoplasts isolated from the mesophyll cells of *OsSUT2-GFP* transgenic rice. The GFP signal was clearly evident at the tonoplast, which is on the inside of and is indented by chloroplasts (indicated by chlorophyll autofluorescence; Fig. 1A). When using a regenerated albino plant expressing *OsSUT2-GFP*, the GFP signal was evident on the inside of the nucleus, thereby confirming that it is on the tonoplast, not the plasma membrane (Fig. 1B). In addition, the leaf mesophyll protoplasts of *OsSUT2-GFP* transgenic rice were stained with FM4-64, which primarily stains the plasma membrane (Uemura et al., 2004). The GFP signals did not overlap with those of FM4-64 (Supplemental Fig. S3), further indicating that OsSUT2 does not localize to the plasma membrane. These results clearly demonstrate that OsSUT2 resides on the tonoplast.

### Expression Pattern of *OsSUT2*

To examine the spatiotemporal expression pattern of *OsSUT2* in rice, we generated transgenic rice plants expressing the *OsSUT2* promoter::*GUS* construct (*pOsSUT2*::*GUS*) and carried out histochemical GUS analysis. There was no significant variation found in *GUS* expression among independent transgenic rice plants expressing *pOsSUT2*::*GUS*. A representative line with the highest GUS intensity was selected for subsequent analysis. In this line, GUS activity was detectable in all examined rice tissues, including leaves, stems, roots, flowers (spikelets), and immature seeds (Fig. 2). In leaf blades of the *pOsSUT2*::*GUS* line, GUS activities were high in mesophyll and bundle sheath cells but no activity was found in the vascular bundles, including the phloem sieve elements and companion cells (Fig. 2, A and B). In epidermal cells, *GUS* expression was barely detectable. The *GUS* expression pattern in the leaf sheaths was similar to that of leaf blades, but higher levels of GUS activity were observed in the bundle sheath cells (Fig. 2C). In roots, *GUS* expression was only detectable in the regions around the emerging lateral roots (Fig. 2D). In spikelets before fertilization, weak GUS activity was detectable at the edges of palea and lemma but not in other floral organs, including the stamens (Fig. 2E). In contrast, following fertilization, the pedicel regions of the spikelets exhibited high GUS activity (Fig. 2F).

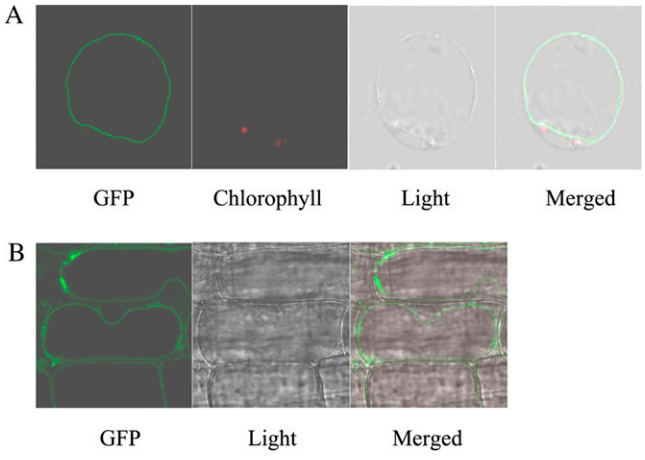


**Figure 1.** Subcellular localization of the OsSUT2-GFP fusion protein. A, Localization of the OsSUT2-GFP fusion protein in leaf mesophyll protoplasts isolated from transgenic rice plants expressing *OsSUT2-GFP*. B, Localization of the OsSUT2-GFP fusion protein in an albino leaf of transgenic rice. Chlorophyll autofluorescence was used as a chloroplast marker in A. GFP signal and chlorophyll autofluorescence are indicated in green and red, respectively.

Seeds are elongated longitudinally with the cellularization and proliferation of endosperm cells during the prestorage phase (1–6 d after fertilization [DAF]) and thickened during the starch-filling milky phase (7–15 DAF) in rice (Hirose et al., 2002; Lim et al., 2006). In developing immature seeds of transgenic *pOsSUT2*::*GUS* lines, high GUS activity was observed in the 7- to 8-DAF seed coat at the early starch-filling stage, while only low activity was observed at 3 to 4 DAF in the prestorage phase seed (Fig. 2, G and H). To further examine *OsSUT2* expression in immature seeds, stained seeds were longitudinally or transversely sectioned. In the 7- to 8-DAF immature seeds, *OsSUT2* was highly expressed in the cross cell layers but not in the main vascular tissues, nucellar projection, or endosperm cells (Fig. 2, I and J). We also evaluated *OsSUT2* expression in immature seeds by real-time PCR. The results confirmed that *OsSUT2* is expressed preferentially in the seed coat and not in the endosperm (Supplemental Fig. S4). In addition, this expression pattern is consistent with previously reported RT-PCR results showing that the Minghui86 *OsSUT2* is highly expressed in leaf blades and in developing caryopses (Sun et al., 2008).

### Suc Transport Activity of OsSUT2 in Yeast

To test whether OsSUT2 has Suc transport activity, we performed assays with yeast cells expressing the rice *OsSUT2* cDNA using radiolabeled [$^{14}$C]Suc at pH 4.0. In this experiment, an expression-optimized synthesized open reading frame of *OsSUT2*, referred to as $OsSUT2_{syn}$, was also tested (Sun et al., 2010). Suc transport into yeast cells expressing the expression-optimized $OsSUT2_{syn}$ was found to be linear between 1 and 5 min and approximately 14-fold higher than the uptake levels into yeast cells transformed with the empty vector after 5 min (Fig. 3A). In contrast, the native *OsSUT2* rice cDNA was found not to be

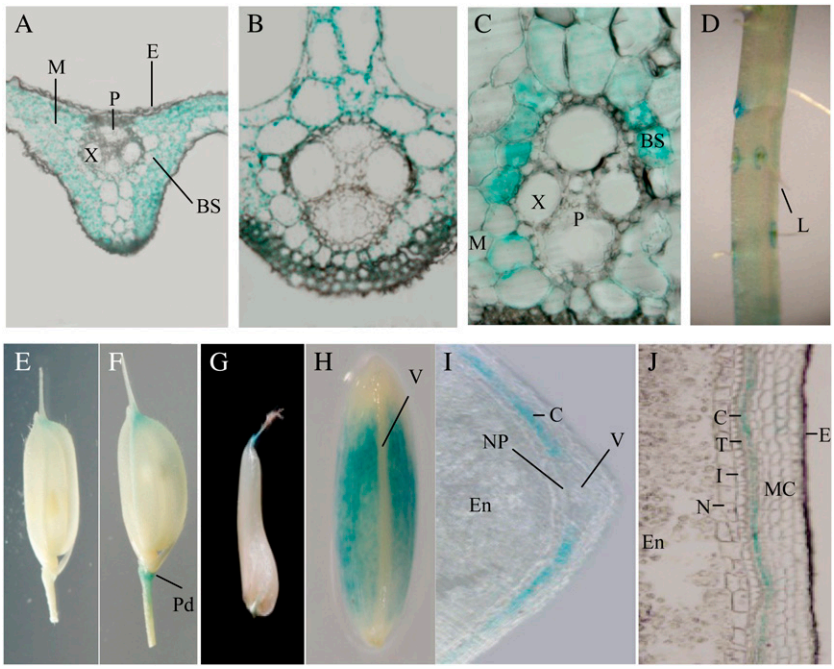


**Figure 2.** Histochemical localization of *GUS* expression in leaves, roots, flowers, and seeds of transgenic rice carrying the *pOsSUT2*::*GUS* construct. A and B, Cross-sections of leaf blades. C, Cross-sections of leaf sheaths. D, Roots. E, Spikelet before fertilization. F, Spikelet after fertilization. G, Immature seed at 3 to 4 DAF. H, Immature seed at 7 to 8 DAF. I, Transverse section of 7- to 8-DAF immature seed. J, Longitudinal section of 7- to 8-DAF immature seed. BS, Bundle sheath cell; C, cross cell layer; E, epidermis; En, endosperm; I, integument; L, lateral root; M, mesophyll cell; MC, mesocarp; N, nucellar epidermis; NP, nucellar projection; P, phloem cell; Pd, pedicel; T, tube cell layer; V, main vascular bundle; X, metaxylem.

functional in yeast, even though it encodes an identical protein to the synthetic, expression-optimized clone (Fig. 3A). Changes in codon usage are thought to improve translation efficiency in heterologous systems by relieving translation-limiting regulation or by modifying mRNA structure (Gustafsson et al., 2004).

Suc transport by OsSUT2 was strongly inhibited by the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Fig. 3B). This indicates that OsSUT2 functions as a $H^+$-Suc symporter. Suc transport by the potato $H^+$-Suc symporter StSUT1 (Schulze et al., 2000) was similarly sensitive to CCCP (Fig. 3B). The kinetics of [$^{14}$C]Suc uptake into yeast cells expressing *OsSUT2* were analyzed; the $K_m$ of OsSUT2 was determined to be 1.86 ± 0.38 mM, and the $V_{max}$ was 2.30 ± 0.06 nmol Suc $min^{-1}$ ($10^8$ cells)$^{-1}$ at pH 4.0 (Fig. 3C).

### Plant Growth Phenotype of the *OsSUT2* Mutant

To examine the in planta function of *OsSUT2*, we isolated a null mutant of this gene from the rice T-DNA mutant population (An et al., 2005a, 2005b; Jeong et al., 2006; http://www.postech.ac.kr/life/pfg/risd/index.html). The isolated mutant allele, *ossut2*, harbors a T-DNA insertion in the fifth exon of *OsSUT2* (Fig. 4A). Using *OsSUT2*-specific and T-DNA-specific primers, mutants that were homozygous for the insertion were isolated from the segregating progeny. RT-PCR analysis indicated that the endogenous *OsSUT2* transcript is absent in *ossut2*, confirming that it is a null mutant (Fig. 4B).

The *ossut2* mutant displayed strong plant growth reduction under greenhouse conditions at 4 weeks after sowing (Fig. 4C). The same effect was observed in mature plants grown in rice paddy fields (Fig. 4D). The growth of *ossut2* was compared with its wild-type segregant control in terms of tiller number, plant height, 1,000-grain weight, and root weight (Fig. 5). The average tiller number of the *ossut2* plants was 6.1, 58.8% lower than that of the wild-type plants (14.8; Fig. 5A). Similarly, the average height was 112.8 cm in the control plants compared with only 91.4 cm in *ossut2*, a decrease of about 19% (Fig. 5B). The average weight of 1,000 grains was 16 and 19.1 g in *ossut2* and control wild-type plants, respectively, a reduction of about 16% in *ossut2* (Fig. 5C). Consistently, *ossut2* mutant grains were visibly smaller than seeds from control plants (Fig. 4E). In addition, the root dry weight was decreased by 54.5% in *ossut2* (35.5 mg per plant) compared with the controls (78.0 mg per plant; Fig. 5D).

To determine whether this plant growth reduction is indeed due to the *ossut2* mutation, we transformed the mutant line with wild-type *OsSUT2* cDNA under the control of the maize *Ubiquitin1* (*Ubi1*) promoter. More than 10 independent transgenic rice plants were obtained, among which two lines, designated Comp-3 and Comp-4, showing high expression of *OsSUT2* were selected and analyzed in subsequent experiments. RT-PCR analysis indicated that these two lines expressed *OsSUT2* at higher levels than the wild-type control (Fig. 4B). In phenotype analysis of Comp-3 and Comp-4, the tiller number, plant height, 1,000-grain weight, grain size, and root dry weight were all found to be restored to levels similar to the control plants (Figs. 4, C–E, and 5). This genetic complementation experiment clearly demonstrated that the *ossut2* mutation is responsible for the growth defect phenotype and strongly suggested that the function of *OsSUT2* is essential for the normal growth and development of rice plants.

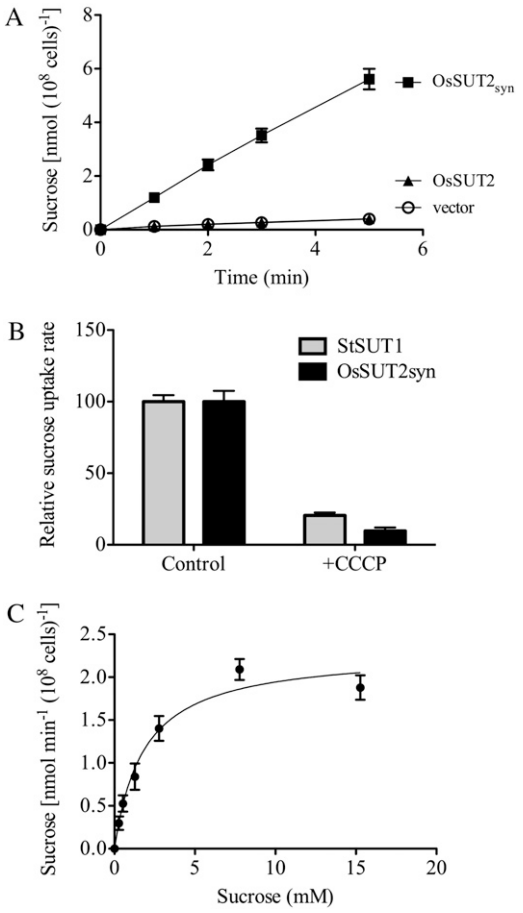


**Figure 3.** Suc transport assay in yeast. A, Time course of Suc uptake in yeast. SEY6210 cells transformed with *OsSUT2*, *OsSUT2*$_{syn}$, or the empty vector were incubated with $^{14}$C-labeled Suc at a final concentration of 1 mM at pH 4.0. Results shown are means ± SD for three independent yeast transformants. B, $H^+$ coupling of Suc transport by OsSUT2$_{syn}$. Yeast cells expressing either *OsSUT2*$_{syn}$ (black bars) or *StSUT1* (gray bars) were incubated in 1 mM [$^{14}$C]Suc with (+CCCP) or without (Control) the protonophore CCCP (10 $\mu$M final concentration). Background uptake rates of cells transformed with the empty vector were subtracted, and the average uptake rate in the absence of CCCP for each transporter was set to 100%. The absolute uptake rates were 51.39 ± 2.35 nmol Suc min$^{-1}$ ($10^8$ cells)$^{-1}$ for StSUT1, 0.96 ± 0.068 nmol Suc min$^{-1}$ ($10^8$ cells)$^{-1}$ for OsSUT2, and 0.059 ± 0.002 nmol Suc min$^{-1}$ ($10^8$ cells)$^{-1}$ for the empty vector. Results shown are means ± SD for three independent yeast transformants. C, Suc uptake kinetics of OsSUT2. Cells were incubated with [$^{14}$C]Suc at pH 4.0, and uptake rates corrected for the uptake rates of cells transformed with the empty vector were determined. The $K_m$ was 1.86 ± 0.38 mM, calculated by a nonlinear regression fit of the Michaelis-Menten equation. The $V_{max}$ was 2.30 ± 0.06 nmol Suc min$^{-1}$ ($10^8$ cells)$^{-1}$. Results shown are based on experiments with three independent yeast transformants. *OsSUT2*$_{syn}$, Synthetic cDNA; *OsSUT2*, native cDNA.

### Primary Carbon Metabolites in the *ossut2* Mutant

To assess whether the levels of Suc, as well as of other soluble sugars, Glc and Fru, and of starch in the source leaves of *ossut2* were altered by the loss of the tonoplast SUT, *OsSUT2*, we analyzed these metabolites in 4-week-old wild-type and mutant plants. Suc was significantly increased in *ossut2* leaves compared with the controls, segregant wild-type and complemented lines (Fig. 6A). In the *ossut2* mutant, the Suc content was about 2-fold higher at the end of the day and about 4-fold higher at the end of the night compared with the controls. Similarly, the levels of Glc and Fru were increased in the *ossut2* mutant compared with the controls (Fig. 6, B and C). In contrast, the transitory starch levels in *ossut2* mutant leaves were not different from the controls (Fig. 6D). The complemented lines all showed similar soluble sugar contents to those of the segregant wild-type plants.

### Sugar Export Activity of the *ossut2* Mutant

To examine whether any change in photosynthetic capacity accompanied the phenotypic manifestations in *ossut2*, we evaluated the rates of net photosynthesis

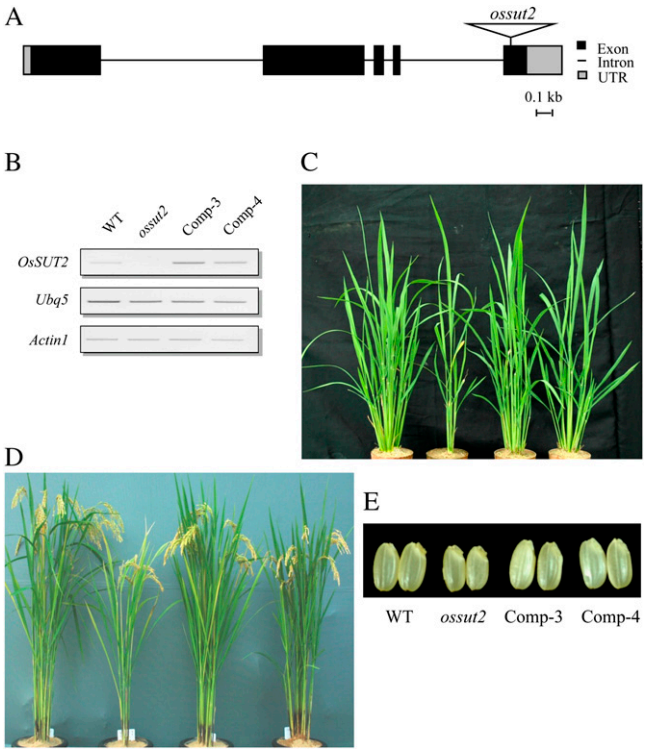


**Figure 4.** Isolation and characterization of the *ossut2* mutant. A, Schematic diagram of the rice *OsSUT2* gene and the insertion position of the T-DNA. In *ossut2*, T-DNA is inserted into the fifth exon. UTR, Untranslated region. B, RT-PCR analysis of *ossut2* and the complemented lines, Comp-3 and Comp-4. *OsSUT2* transcripts are not detectable in the homozygous mutant, while the two complemented lines express *OsSUT2* at higher levels than the wild type. *UBQ5* and *ACTIN1* are PCR controls. C, Four-week-old plants grown in the greenhouse. The leftmost plant is the wild type (WT). The *ossut2* mutant and two complemented lines are shown left to right. D, Mature flowering plants of the wild type, *ossut2*, and complemented lines (left to right) grown in a rice paddy field. E, Husked grains of the same plants shown in D.

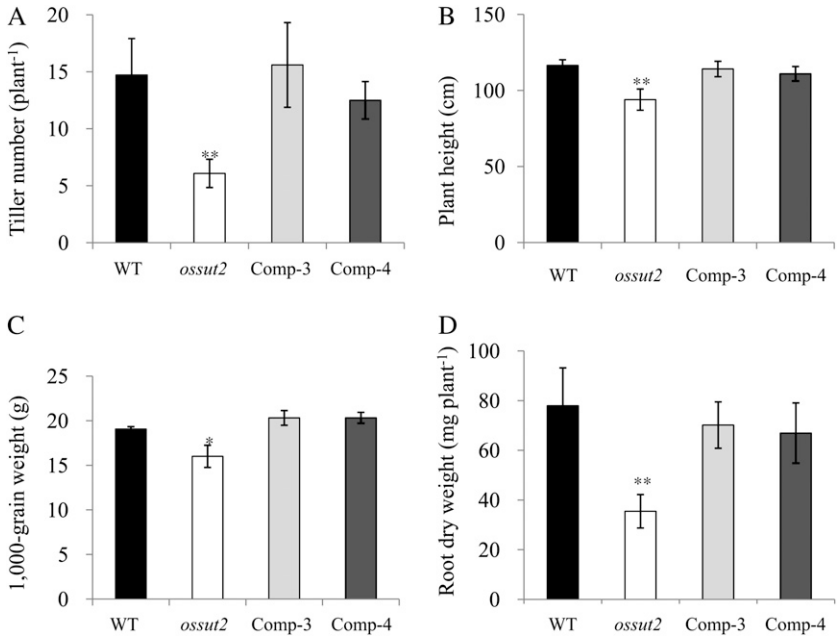


**Figure 5.** Growth phenotype of mature *ossut2* plants grown in a rice paddy field. A, Number of tillers. B, Plant height. C, Weight of 1,000 grains. D, Root dry weight. Each data point represents the mean ± SD from 10 different plants. Black bars, The wild type (WT); white bars, *ossut2*; light gray bars, Comp-3; dark gray bars, Comp-4. The statistical significance of correlation coefficients is indicated by asterisks: * $P < 0.05$, ** $P < 0.01$.

in this mutant by measuring the $CO_2$ consumption from the fully expanded leaves of 8-week-old rice plants. The *ossut2* mutant did not show a significant reduction of net photosynthetic activity compared with wild-type plants at a sequentially changed photon flux density (Supplemental Fig. S5).

We speculated whether the accumulation of sugars in the source leaves may lead to a decreased transfer of Suc to the sink organs, since grain size and weight and root weight, as well as plant height and tiller number, were reduced in *ossut2*. To quantify the sugar export ability in this mutant, we detached mature leaf blades of 4-week-old plants at the end of day, incubated these for 16 h under dark conditions, and determined the soluble sugar levels (total of Suc, Glc, and Fru). During this dark phase, detached wild-type leaves exported 25.1 $\mu$mol C6 units $g^{-1}$ fresh weight, whereas *ossut2* mutant leaves showed about a 54% reduction (11.6 $\mu$mol C6 units $g^{-1}$ fresh weight) in sugar export. Both complemented lines exhibited a similar sugar export activity level (27.4 and 31.1 $\mu$mol C6 units $g^{-1}$ fresh weight of Comp-3 and Comp-4, respectively) to that of the wild type (Fig. 7).

## DISCUSSION

### *OsSUT2* Encodes a Tonoplast $H^+$-Suc Symporter in Rice

This study provides several lines of evidence that OsSUT2 is a functional tonoplast Suc transporter. First, OsSUT2 belongs to the SUT4 clade that includes the previously identified tonoplast-localized SUTs, AtSUT4, HvSUT2, LjSUT4, and PtaSUT4 (Endler et al., 2006; Reinders et al., 2008; Payyavula et al., 2011; Supplemental Fig. S1). Second, OsSUT2-GFP localizes to the tonoplast in rice mesophyll protoplasts and plants (Fig. 1). Third, Suc transport by *OsSUT2* expressed in yeast was inhibited by CCCP, a protonophore (Fig. 3). These findings indicate that OsSUT2 functions as a tonoplast $H^+$-Suc symporter. Fourth, we found that the loss of function of *OsSUT2* in rice resulted in higher accumulation of Suc in leaves compared with the controls (Fig. 6) and led to a significantly reduced sugar export ability (Fig. 7). This is most likely due to reduced Suc transport from the vacuole to the cytosol, resulting in higher Suc accumulation in the vacuole lumen. Our results are consistent with the observation that *PtaSUT4* RNAi transgenic poplar had elevated Suc content in source leaves (Payyavula et al., 2011). Considering that OsSUT2 functions as a $H^+$-Suc symporter in yeast as well as the normal pH gradient across the tonoplast, our data indicate that *OsSUT2* functions in Suc transport across the tonoplast from the vacuole lumen to the cytosol.

Suc transport in *AtSUT4*-expressing yeast cells was previously found to be stimulated by Glc but inhibited by addition of the protonophore CCCP (Weise et al., 2000). Suc transport into *HvSUT2*-expressing yeast cells was also previously reported to be inhibited by treatments with the protonophore CCCP and by the Suc transport inhibitor *p*-chloromercuribenzoylsulfonic acid (Weschke et al., 2000). Similarly, Suc transport into *Xenopus* oocytes by LjSUT4 was found to be inhibited by CCCP, and the extracellular application of Suc induced membrane depolarization in *LjSUT4*-expressing *Xenopus* oocytes (Reinders et al., 2008). Hence, our results here are consistent with the contention that all currently identified tonoplast Suc transporters function as $H^+$-Suc symporters rather than

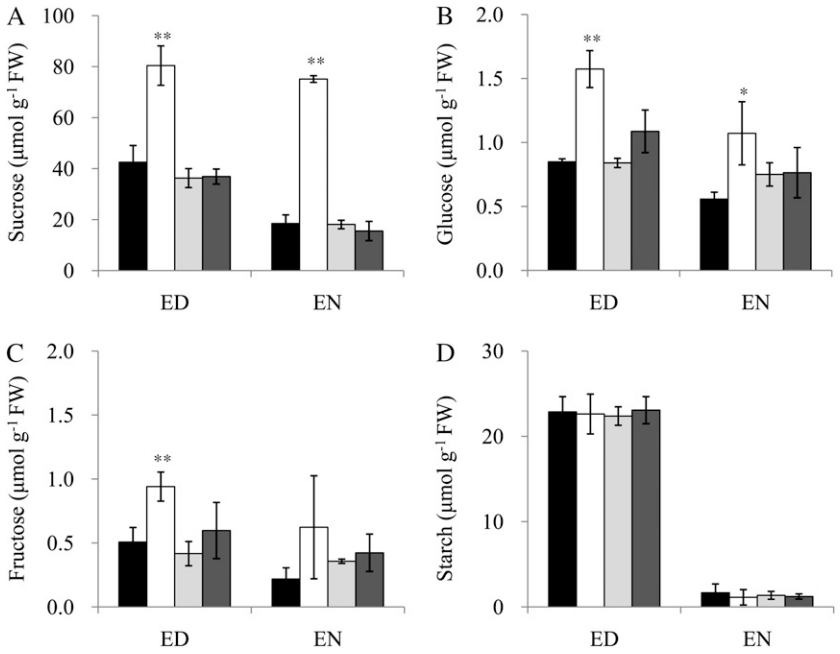


**Figure 6.** Leaf soluble sugar and starch contents in *ossut2* mutant plants grown for 4 weeks in the greenhouse. The levels of Suc (A), Glc (B), Fru (C), and starch (D) are shown. Each data point represents the mean ± SD from five different plants. ED, End of the day; EN, end of the night; FW, fresh weight. Black bars, The wild type (WT); white bars, *ossut2*; light gray bars, Comp-3; dark gray bars, Comp-4. The statistical significance of correlation coefficients is indicated by asterisks: * $P < 0.05$, ** $P < 0.01$.

antiporters and support the previous hypothesis that they function in Suc transport from the vacuole to the cytosol (Neuhaus, 2007; Reinders et al., 2008). It is likely that the Suc transport activity of OsSUT2 in yeast is due to mistargeting of the overexpressed protein to the plasma membrane in the heterologous system.

Our kinetic analysis in yeast indicated that the $K_m$ of OsSUT2 is about 1.9 mM at pH 4.0 (Fig. 3). Compared with other tonoplast SUTs, including LjSUT4, this represents a relatively high affinity for Suc (Weise et al., 2000; Weschke et al., 2000; Reinders et al., 2008). Analysis of [$^{14}$C]Suc uptake into yeast is thought to underestimate $K_m$ because SUT activity depletes the proton motive force and membrane potential is not controlled (Chandran et al., 2003). This tends to decrease the $V_{max}$ measured and therefore decreases the apparent $K_m$. Using voltage clamping to measure $K_m$ is more accurate; however, OsSUT2 did not show activity when expressed in *Xenopus* oocytes (Sun et al., 2010). Of the tonoplast SUTs, only LjSUT4 has been studied by expression in *Xenopus* oocytes and electrophysiology (Reinders et al., 2008).

### *OsSUT2* Functions Mainly in Photosynthetic Assimilatory Tissues

In dicot species, Arabidopsis *AtSUT4* was found to be expressed predominantly in the minor veins in source leaves in promoter::*GUS* fusion experiments (Weise et al., 2000). Subsequently, *AtSUT4* was confirmed to be expressed in the companion cells of minor veins (Schulze et al., 2003), thus suggesting a role in Suc loading into the phloem. In monocot species, barley *HvSUT2* expression was reported to be highest in growing leaves and also to be at considerable levels in other tissues such as roots and caryopses, while that of *HvSUT1*, a member of the SUT3 clade, was highest in developing caryopses. Thus, *HvSUT2* has been suggested to play a general housekeeping role (Weschke et al., 2000). In further studies, RT-PCR analysis revealed that both *AtSUT4* and *HvSUT2* transcripts are abundant in leaf mesophyll cells. In poplar, in situ hybridization was used to show that *PtaSUT4* is expressed in leaf mesophyll cells and minor veins (Payyavula et al., 2011). The recent finding that all

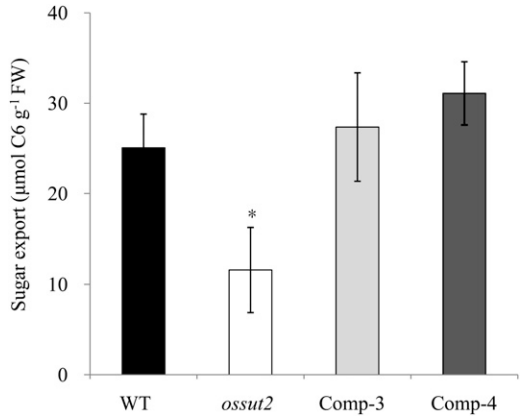


**Figure 7.** Quantification of sugar export activity of the *ossut2* mutant. Histograms show the amount of soluble sugars (total of Suc, Glc, and Fru) detected in the mature leaf blades of 4-week-old plants detached at the end of day and incubated in 15 mM EDTA solution for 16 h under dark conditions. FW, Fresh weight. Black bars, The wild type (WT); white bars, *ossut2*; light gray bars, Comp-3; dark gray bars, Comp-4. The indicated data are means ± SD from three separate experiments. The statistical significance of correlation coefficients is indicated by the asterisk ($P < 0.05$).

these transporters are localized to the tonoplast suggests that they may be involved in vacuolar transfer of photosynthetically derived Suc.

Our histochemical GUS assays revealed that *OsSUT2* is highly expressed in leaf mesophyll cells but not in the vascular bundle itself (Fig. 2A). Interestingly, in developing caryopses, we found that *OsSUT2* expression was detected preferentially in the cross cell layer of seed coats (Fig. 2J). It is known that this cross cell layer contains some chloroplasts and functions in supplying photoassimilates to the endosperm (Cochrane and Duffus, 1979; Ebenezer et al., 1990). The high expression of *OsSUT2* in seed coats was confirmed by real-time PCR analysis (Supplemental Fig. S4). We were not able to observe any significantly altered expression of *OsSUT2* in response to environmental stress stimuli such as drought, cold, or salt treatment (data not shown), suggesting that *OsSUT2* does not likely function in stress responses. Previously reported results have shown that *HvSUT2* is strongly expressed in pericarps (Weschke et al., 2000). In rice, barley, and wheat (*Triticum aestivum*), the pericarp supplies photoassimilates to the endosperm (Cochrane and Duffus, 1979). This suggests that cereals have a high demand for Suc transporters to facilitate the transport of reserve Suc across the tonoplast in photosynthetic assimilatory cells. It is noteworthy that the regions around emerging lateral roots (Fig. 2D) and the pedicel parts of spikelets after fertilization (Fig. 2F) also exhibited high GUS activity. This suggests that *OsSUT2* function is required for efficient transport of reserve Suc in tissues with a high metabolic turnover.

The tonoplast monosaccharide transporters (TMTs) have been characterized in Arabidopsis (Wormit et al., 2006; Wingenter et al., 2010) and rice (Cho et al., 2010) and are thought to function in the transport of monosaccharides, Glc and Fru, from the cytosol to the vacuole lumen using a $H^+$-sugar antiport mechanism. Based on their expression patterns, the Arabidopsis TMTs are thought to play a major role in osmotic stress responses, whereas rice TMTs mainly function in sugar retrieval during sugar translocation. Our results here indicate that OsSUT2 functions as a $H^+$-symporter rather than an antiporter. Tonoplast $H^+$-Suc antiporter activity has been characterized previously (Briskin et al., 1985; Greutert and Keller, 1993), but the corresponding genes remain to be identified. It is likely that Suc symporters and antiporters function together to control Suc flux across the tonoplast and that a regulatory system must be involved to avoid a futile cycle of $H^+$ and Suc transport.

### *OsSUT2* Function Is Essential for the Normal Growth of Rice Plants

Suppression of *PtaSUT4* in RNAi transgenic poplar had no obvious effect on plant growth but led to altered biomass partitioning, with a higher ratio of leaf area to stem volume compared with wild-type plants. Thus, *PtaSUT4* appeared to modulate Suc efflux and utilization (Payyavula et al., 2011). However, no null mutant for a tonoplast SUT has been described to date. Here, we characterize a rice *ossut2* mutant in detail and describe its function in planta. Rice plants lacking *OsSUT2* display severe growth defects such as reduced tiller number, plant height, and grain and root weights compared with the wild type and *OsSUT2*-complemented lines (Figs. 4 and 5). Hence, *OsSUT2* function is necessary for normal plant growth in rice. In the *ossut2* mutant, the level of Suc was found to be increased by about 2-fold in the source leaves (Fig. 6), mostly because of the loss of Suc transport ability across the tonoplast to the cytosol. Thus, it is probable that the accumulation of sugar in *ossut2*, due to the decreased transport of Suc from the vacuole lumen to the cytosol, reduces the metabolic flux, thereby interfering with efficient energy supply for normal plant growth. In addition, we found that *ossut2* has a significantly reduced sugar export ability (Fig. 7), which indicates that the reduced transport of Suc to the cytosol in *ossut2* likely also interferes with Suc translocation to sink organs, thus affecting plant growth.

It is noteworthy that the net photosynthetic activity of the *ossut2* mutant was not altered (Supplemental Fig. S5). In this regard, the higher levels of Glc and Fru could be the result of the hydrolysis of accumulated Suc by vacuolar invertase (Sturm and Tang, 1999; Ji et al., 2007). Given that soluble sugar levels in the cytosol control photosynthesis (Rolland et al., 2002), considerable amounts of Glc and Fru might be accumulated in the vacuole lumen in *ossut2*, which thereby do not affect photosynthetic capacity.

In summary, we have characterized the expression and function of the rice tonoplast Suc transporter *OsSUT2* in detail. Our results demonstrate that *OsSUT2* is most likely to be involved in Suc transport across the tonoplast from the vacuole to the cytosol, a process that is required to support normal plant growth in rice.

## MATERIALS AND METHODS

### Plant Materials

Wild-type rice (*Oryza sativa* 'Hwayoung'), the *OsSUT2* mutant *ossut2*, and complemented transgenic rice lines were used in these experiments. Rice plants were grown in a greenhouse at 30°C during the day and 20°C at night in a light/dark cycle of 14 h/10 h or in an experimental field plot of Kyung Hee University under natural environmental conditions during the summer. Leaves were sampled from 4-week-old plants grown in the greenhouse to analyze primary carbon metabolites and sugar export ability. Roots were collected from 4-week-old plants grown in the greenhouse to determine dry weights. Plant height, tiller number, and 1,000-grain weight were analyzed from mature plants grown in a rice paddy field.

### Cloning of the *OsSUT2* Full-Length cDNA Clone

The full-length cDNA of *OsSUT2* was isolated by RT-PCR from the leaves of 4-week-old rice (cv Dongjin) plants using gene-specific primers encompassing the translation start codon and 3′ untranslated region: 5′-CTCTTCTGAACTAACCCAAAGAT-3′ and 5′-AACTCCTGCAACTTTTATTCACTA-3′. The cDNA was then subcloned into the pGEM-T Easy vector (Promega) and confirmed by sequencing. The *OsSUT2* cDNA sequence has been deposited in

the National Center for Biotechnology Information database with the accession number HQ540307.

## Sequence Alignment and Phylogenetic Tree Construction

The deduced amino acid sequence of the *OsSUT2* gene was aligned with previously reported *SUT* genes from other plant species using the ClustalW program (Thompson et al., 1994). A phylogenetic tree was constructed using MEGA software version 4.0 (Tamura et al., 2007) via the neighbor-joining method.

## Construction of the *OsSUT2-GFP* Fusion Vector and Subcellular Localization Analysis

To examine the subcellular localization of OsSUT2 in rice, the entire open reading frame without the stop codon was amplified by PCR. The amplified product was digested with *Xba*I and *Xho*I and was cloned into the region between the *CaMV35S* promoter and *sGFP* of the JJ461 binary vector (Cho et al., 2009). The primers used were 5′-GCTCTAGACTCTTCTGAACTAACCCAAAGAT-3′ and 5′-CCCTCGAGATCGGTGACCTCTCCTCCTTGAT-3′ (the underlined sequences indicate *Xba*I and *Xho*I sites, respectively). The resulting *CaMV35S*::*OsSUT2-GFP* (pJJ1747) construct was used to transform rice. Leaves or isolated protoplasts from the *CaMV35S*::*OsSUT2-GFP* transgenic rice plants were examined using a confocal microscope (LSM 510 META; Zeiss). Chlorophyll autofluorescence was used as a chloroplast marker. The plasma membrane was stained with a lipophilic styryl dye, FM4-64 (Molecular Probes).

## Construction of the *pOsSUT2*::*GUS* Fusion Vector

About 2 kb of the 5′ upstream region of the *OsSUT2* gene was amplified by PCR using genomic DNA as a template. The primers used were 5′-TACAATCCAATACTCCAGTAGAC-3′ and 5′-CTTCTTCTCGTGTTTGCCTCCG-3′. The amplified product was subcloned into a pGEM-T Easy vector (Promega) and confirmed by sequencing. The cloned fragment was then inserted in front of the promoterless *GUS* reporter gene of pBI101 (Jefferson, 1987). As a result, the promoter region of the *OsSUT2* gene was fused to a *GUS* gene linked to the terminator of the nopaline synthase gene (*Nos*). This construct was designated *pOsSUT2*::*GUS*.

## *Agrobacterium*-Mediated Rice Transformation

To produce transgenic rice (cv Hwayoung) plants expressing *CaMV35S*::*OsSUT2-GFP* and *pOsSUT2*::*GUS* constructs, *Agrobacterium tumefaciens* LBA4404 strains harboring each of these vectors were grown on *Agrobacterium* broth medium supplemented with 25 mg $L^{-1}$ kanamycin for 3 d at 28°C, and transgenic calli were obtained via the *Agrobacterium*-mediated cocultivation method as described previously (Jeon et al., 2000). Transgenic rice plants were regenerated from the transformed calli on selection medium containing 50 mg $L^{-1}$ hygromycin and 250 mg $L^{-1}$ cefotaxime.

## Histochemical GUS Analysis

Various tissues from *pOsSUT2*::*GUS* transgenic plants were collected and stained in GUS reaction solution containing 0.2 M sodium phosphate (pH 7.0), 10 mM EDTA, 20% methanol, 2% dimethyl sulfoxide, and 0.1% 5-bromo-4-chloro-3-indole-$\beta$-glucuronide, as described previously by Cho et al. (2010). Briefly, samples were incubated at 37°C overnight and then transferred to 70% ethanol to remove chlorophyll. After staining, samples were fixed in a solution containing 50% ethanol, 5% acetic acid, and 3.7% formaldehyde. The stained samples were observed with a dissecting microscope (SZX12; Olympus). For longitudinal and transverse sectioning, the stained samples were embedded in Paraplast Plus (Sigma), and 10-$\mu$m sections were prepared using a microtome (Microm HM360). The sections mounted on slides were observed with a compound microscope.

## Suc Transport Experiment in Yeast

An expression-optimized *OsSUT2* clone (GenBank accession no. HQ830493) was synthesized by Blue Heron Biotechnology and inserted into the Gateway entry vector pENTR221. The resulting amino acid sequence of this clone is identical to the sequence determined from cDNA isolated from rice. For expression in yeast, the entry clone was recombined in vitro with the Gateway destination vector pDR196/GW (provided by Dr. Wolf Frommer, Carnegie Institution). This construct, named $OsSUT2_{syn}$, as well as the native rice *OsSUT2* coding region, the potato (*Solanum tuberosum*) Suc transporter *StSUT1* (Schulze et al., 2000), and the empty vector were used to transform the yeast strain SEY6210 (*MAT$\alpha$ leu2-3,112 ura3-52 his3-$\Delta$200 lys2-801 trpl-$\Delta$901 suc2-$\Delta$9*; Brown and Bussey, 1993). The uptake of radiolabeled [$^{14}$C]Suc into yeast cells was determined as described previously (Reinders and Ward, 2001).

## Isolation of the *OsSUT2* Mutant

A T-DNA-tagged *OsSUT2* mutant, *ossut2*, was identified by searching the rice T-DNA Insertion Sequence Database (An et al., 2005a, 2005b; Jeong et al., 2006; http://www.postech.ac.kr/life/pfg/risd/index.html). A homozygous mutant (cv Hwayoung) was isolated by genomic DNA PCR analysis. The gene-specific primers used for the genotyping of *ossut2* were 5′-TGCTGTATCCTAAAATAACCTGTTGATGG-3′ and 5′-CATTTCATACACACACCTTAACACAGCAC-3′. The T-DNA-specific primer used was 5′-GACCAGAAAGCCCATAAACCTTGGAGGC-3′.

## Genetic Complementation Experiment

To complement the *ossut2* mutant, the full-length *OsSUT2* cDNA was amplified by PCR using the primers 5′-GGATCCCTCTTCTGAACTAACCCAAAGAT-3′ and 5′-GGTACCAACTCCTGCAACTTTTATTCACT-3′ (the underlined sequences indicate *Bam*HI and *Kpn*I sites, respectively). The PCR product was then subcloned into the pGEM-T Easy vector (Promega) and confirmed by sequencing. The insert digested with *Bam*HI and *Kpn*I was further subcloned between the maize (*Zea mays*) *Ubi1* promoter and the *Nos* terminator of the Ubi/NC4300 binary vector carrying the *Phosphomannose Isomerase* gene as a selectable marker (provided by Dr. Pamela Ronald, University of California, Davis). The resulting construct, *Ubi*::*OsSUT2* (pJJ2521), was used to transform the *ossut2* mutant as described previously (Lucca et al., 2001).

## RNA Isolation and PCR Analysis

For RT-PCR analysis of *ossut2* and transgenic lines, total RNA was prepared from harvested samples using Trizol reagent and reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). First-strand cDNAs were used in RT-PCR with gene-specific primers and control primers for the housekeeping genes *Act1* (McElroy et al., 1990) and *OsUBQ5* (Jain et al., 2006). The gene-specific primers used were as follows: for *OsSUT2*, 5′-CTATCATGATGGTGTGAGAATG-3′ and 5′-CTACCCAGTGACACAATAACCT-3′; for *Act1*, 5′-GGAACTGGTATGGTCAAGGC-3′ and 5′-AGTCTCATGGATACCCGCAG-3′; and for *OsUBQ5*, 5′-GACTACAACATCCAGAAGGAGTC-3′ and 5′-TCATCTAATAACCAGTTCGATTTC-3′. RT-PCR analyses were repeated at least three times, giving similar results using previously described methods (Cho et al., 2005).

For quantitative real-time PCR, endosperm and seed coat cDNA was prepared from seeds harvested 6 to 10 DAF according to the method described by Cho et al. (2005). The expression level of *OsUBQ5* was used for the normalization of real-time PCR results (Jain et al., 2006). All reactions were performed in triplicate using the HotStart-IT SYBR Green qPCR Master Mix (USB) and 7500 Real-Time PCR System (Applied Biosystems), according to the manufacturers' instructions. Changes in gene expression were analyzed by the comparative cycle threshold method and Sequence Detector Systems version 1.2 software (Applied Biosystems). The gene-specific primers used for quantitative real-time PCR were as follows: for *OsSUT2*, 5′-TGTGGCAAAGAATATGGATTAT-3′ and 5′-CTACCCAGTGACACAATAACCT-3′; and for *OsUBQ5*, 5′-GACTACAACATCCAGAAGGAGTC-3′ and 5′-TCATCTAATAACCAGTTCGATTTC-3′.

## Determination of Soluble Sugars and Starch

Approximately 100 mg of leaf blades was harvested at the end of day and night from 4-week-old plants grown in the greenhouse. Suc, Glc, Fru, and starch were measured using NAD(P)H-coupled enzymatic methods in the soluble and residual fractions of ethanol-water extracts (Lee et al., 2005). The measured metabolite contents were normalized to the leaf fresh weights.

## Sugar Export Assay

Sugar export assays were essentially carried out as described by Wingenter et al. (2010) with slight modifications suitable for rice. Briefly, mature leaf blades of 4-week-old plants grown in the greenhouse were collected by cutting the bottom of the leaf blades at the end of day, and the cut regions were soaked in a 15 mM EDTA solution (pH 7.25). To avoid blockage of the cut end regions, the end-most parts of each 1 mm were consecutively recut off four times at 1-min intervals under the buffer solution. The leaf blade ends were then immediately transferred into reaction tubes filled with 500 $\mu$L of the same buffer solution and incubated for 16 h under dark conditions. Subsequently, the solutions containing the exudates were collected to quantify total soluble sugars including Suc, and also Glc and Fru, since Suc is hydrolyzed into its monosaccharides.

Sugars were analyzed using a HPLC system (Agilent Technologies 1200 series) equipped with a temperature-controlled column chamber and autosampler and a refractive index detector (Agilent Technologies). The HPLC system and detectors were controlled, and results were calculated using the ChemStation software (Agilent Technologies). The carbohydrate column (5 $\mu$m, 4.6 $\times$ 250 mm; Agilent Technologies) was used for separation of the sugars in the exudates. Samples prepared were injected in a volume of 100 $\mu$L with a mobile phase in a 75% acetonitrile solution at a speed of 1.4 mL $min^{-1}$ after mixing with acetonitrile (1:1, v/v) and filtration through a 0.2-$\mu$m SmartPor syringe filter (Woongi Science). Peaks were identified by comparing the retention times with those of standard sugars. This experiment was carried out in triplicate.

## Measurement of Net Photosynthetic Activity

Net photosynthetic activity was measured on the most recent fully expanded leaves of 8-week-old rice plants using a portable gas-exchange system (Li-6400; Li-Cor) at sequentially changed photon flux density, leaf temperature of 25°C, and reference $CO_2$ concentration of 350 $\mu$mol $mol^{-1}$.

## Statistical Analysis

Student's *t* test was performed using Microsoft Excel to determine the significance of the differences in the morphological data and metabolite contents among wild-type, *ossut2*, and complemented rice lines.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers HQ540307 (*OsSUT2*) and HQ830493 ($OsSUT2_{syn}$).

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Phylogenetic tree of *SUT* genes constructed using the neighbor-joining method.

**Supplemental Figure S2.** Comparison of the deduced amino acid sequences for the rice tonoplast Suc transporter OsSUT2 and the previously identified tonoplast-localized SUTs, HvSUT2, LjSUT4, AtSUT4, and PtaSUT4.

**Supplemental Figure S3.** Subcellular localization of the OsSUT2-GFP fusion protein in leaf mesophyll protoplasts isolated from transgenic rice plants expressing *OsSUT2-GFP*.

**Supplemental Figure S4.** Expression levels of *OsSUT2* in endosperms and seed coats determined by quantitative real-time PCR.

**Supplemental Figure S5.** Net photosynthetic activity of the *ossut2* mutant.

## ACKNOWLEDGMENTS

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