

Identification and Characterization of the Duplicate Rice Sucrose Synthase Genes *OsSUS5* and *OsSUS7* Which Are Associated with the Plasma Membrane

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Systematic searches using the complete genome sequence of rice (*Oryza sativa*) identified *OsSUS7*, a new member of the rice sucrose synthase (*OsSUS*) gene family, which shows only nine single nucleotide substitutions in the *OsSUS5* coding sequence. Comparative genomic analysis revealed that the synteny between *OsSUS5* and *OsSUS7* is conserved, and that significant numbers of transposable elements are scattered at both loci. In particular, a 17.6-kb genomic region containing transposable elements was identified in the 5' upstream sequence of the *OsSUS7* gene. GFP fusion experiments indicated that *OsSUS5* and *OsSUS7* are largely associated with the plasma membrane and partly with the cytosol in maize mesophyll protoplasts. RT-PCR analysis and transient expression assays revealed that *OsSUS5* and *OsSUS7* exhibit similar expression patterns in rice tissues, with the highest expression evident in roots. These results suggest that two redundant genes, *OsSUS5* and *OsSUS7*, evolved via duplication of a chromosome region and through the transposition of transposable elements.

INTRODUCTION

Sucrose is the principal product of photosynthesis in the source tissues of higher plants and is also the major form by which carbon is transported from source leaves to various sink tissues via the phloem. Sucrose utilization in these tissues is initiated by its hydrolysis, catalyzed by two different classes of enzymes, invertase (INV) and sucrose synthase (SUS). Unlike INV, which catalyzes the irreversible hydrolysis of sucrose to glucose and fructose, SUS catalyzes the reversible conversion of sucrose and UDP to UDP-glucose and fructose (Sturm, 1999; Sturm and Tang, 1999). In higher plants, INV and SUS are both present as multiple isoforms. The INVs can be classified into three distinct types, cell-wall invertase (CIN), vacuolar invertase (VIN) and cytosolic neutral or alkaline invertase (NIN). SUSs exist as either free molecules in the cytosol or in association with various subcellular compartments, including the plasma membrane, actin filaments, mitochondria and vacuolar membrane (Duncan

and Huber, 2007; Etxeberria and Gonzalez, 2003; Subbiah et al., 2006; Winter et al., 1997; 1998).

The sucrose that is unloaded in sink tissues is hydrolyzed to monosaccharide sugars to serve as a storage material, carbohydrate backbone, and energy source in many higher plants. In this regard, SUS activities in sink tissues appeared to be essential for plant growth and development. Important roles of the SUS enzymes have now been demonstrated in the sink organs of many plant species, including potato tuber (Zrenner et al., 1995), tomato fruit (D'Aoust et al., 1999), cotton fiber (Ruan et al., 2003), maize endosperm (Chourey et al., 1998), watermelon seed (Kim et al., 2002) and the *Arabidopsis* silique and seed (Fallahi et al., 2008). In these previous reports, SUS activities correlated well with the sink strength of the storage tissues. For instance, the reduction of SUS activity in tomato fruit and potato tuber resulted in the reduction of the starch content and sucrose unloading capacity, coupled with a decrease in the fruit setting or tuber dry weight (D'Aoust et al., 1999; Zrenner et al., 1995).

SUS genes have been found in a number of plant species, including maize (Carlson et al., 2002), rice (Harada et al., 2005; Hirose et al., 2008; Huang et al., 1996; Wang et al., 1992), *Arabidopsis* (Barratt et al., 2001; 2009; Bieniawska et al., 2007), pea (Barratt et al., 2001), potato (Fu and Park, 1995), tomato (Chengappa et al., 1999), carrot (Sturm et al., 1999) and lotus (Horst et al., 2007). Through systematic searches of the complete genome sequence, it has been reported that the *Arabidopsis* and rice genomes both encode a small multigene family consisting of six *SUS* genes (Barratt et al., 2001; Bieniawska et al., 2007; Harada et al., 2005; Hirose et al., 2008). Phylogenetic analyses have further shown that *SUS* genes can be divided into three different groups, SUSI, SUSII, and SUSIII, each likely having distinct functions (Bieniawska et al., 2007). Of note, the SUSIII group has been found only in *Arabidopsis* and rice and its function remains unclear. Hence, further analysis of this third group of genes is necessary to more fully understand the function of the entire *SUS* gene family.

In our present study, we isolated a new member of the *OsSUS* gene family, *OsSUS7*, through analysis of the complete genome sequence of rice. *OsSUS7* is nearly identical to *Os-*

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SUS5 except for nine single nucleotide differences. We first investigated the evolutionary relationship between *OsSUS7* and the previously reported *SUS* genes including *OsSUS5*. We then compared the expression and subcellular localization of *OsSUS7* with that of *OsSUS5* and other *OsSUS* genes by RT-PCR analysis and transient expression assays using promoter-luciferase (LUC) fusion and GFP fusion constructs. Finally, we discuss the duplication of *OsSUS5* and *OsSUS7* genes and their function in rice.

MATERIALS AND METHODS

Plant materials

Rice seeds (*Oryza sativa* L. cultivar Jinmi) provided by the National Institute of Crop Science (Korea) were used in all experiments, except for the seed coat- and endosperm-specific cDNA libraries (Jun et al., 2004) which were constructed using the cultivar Dongjin. Plants were grown until maturity in a greenhouse at 30°C during the day and at 20°C at night in a light/dark photoperiod of 16/8 h. To examine the expression pattern of *OsSUS* genes, all rice samples were harvested as described by Cho et al. (2005; 2006), frozen in liquid nitrogen and kept at -80°C until needed.

Cloning of the *OsSUS5* and *OsSUS7* genes

Full-length cDNAs of the *OsSUS5* and *OsSUS7* genes were isolated by RT-PCR, using gene-specific primers designed to encompass the translation start and stop codons as listed in the rice sequence database (Supplementary Table 1). cDNAs synthesized using the total mRNAs isolated from the root were used in the PCR experiments. All cDNAs were cloned into the pENTR/D-TOPO vector (Invitrogen) and sequenced automatically. To avoid PCR errors, at least five independent cDNA clones for each gene were analyzed. These cDNA sequences have been submitted to the NCBI database and the accession numbers are HQ895723 and HQ895725 for *OsSUS5* and *OsSUS7*, respectively.

Comparative analysis of genomic structures

The genomic structure of each *OsSUS* gene was determined by aligning the cDNA and genomic sequences of BAC clones obtained from the NCBI database and the Rice Genome Annotation Project Database (<http://rice.plantbiology.msu.edu/>). The accession numbers (BAC clone numbers) of the clones used to analyze the genomic structure are as follows: *OsSUS1*, AC-084380 (OSJNBa0090P23); *OsSUS2*, AP004280 (P0648E08); *OsSUS3*, AP004988(B1056G08); *OsSUS4*, AC131175 (OSJNBa0030D15); *OsSUS5*, AL662942 (OsJNBa0033H08) and AL731596 (OsJNBa0024J22); *OsSUS6*, AP004082 (OJ1149_C12); and *OsSUS7*, AL663002 (OsJNBb0026I12).

Sequence comparisons and phylogenetic analysis

The deduced amino acid sequences of the translated *OsSUS* genes were aligned with those of previously reported genes using the Clustal W program (Thompson et al., 1994). Phylogenetic analyses were conducted using MEGA version 4 (Tamura et al., 2007) and the neighbor-joining method. The accession numbers of the sequences used to construct the phylogenetic tree are as follows: *AtSUS1-AtSUS6* from *Arabidopsis*, At5g20830, At5g49190, At4g02280, At3g43190, At5g37180, and At1g73370, respectively (Baud et al., 2004); *AgSUS* from *Alnus glutinosa*, X92378 (van Ghelue et al., 1996); *BoSUS1-BoSUS4* from *Bambusa oldhamii*, AF412036, AF412038, AF412037, and AF412039, respectively; *BvSBSS1* and *BvSBSS2* from sugar beet, AY457173 and EF660856,

respectively (Hesse and Willmitzer, 1996; Klotz and Haagen-son, 2008); *CISUS* from *Citrullus lanatus*, AB018561; *Cpss1* and *Cpss2* from *Craterostigma plantagineum*, AJ131999 and AJ132000, respectively (Kleines et al., 1999); *CrSUS* from *Chenopodium rubrum*, X82504 (Godt et al., 1995); *CitSUS1* and *CitSUS2* from citrus, AB022092 and AB022091 (Komatsu et al., 2002); *DcSUS1* and *DcSUS2* from carrot, X75332 and Y16091 (Sebkova et al., 1995; Sturm et al., 1999); *GhSUS* from *Gossypium hirsutum*, U73588; *GmSUS* from soybean, AF030231; *HvSUS1* and *HvSUS2* from barley, X65871 and X69931 (Sánchez de la Hoz et al., 1992); *LeSUS1* and *LeSUS2* from tomato, L19762 and AJ011319 (Chengappa et al., 1999; Wang et al., 1993); *MsSUS* from *Medicago sativa*, AF049487; *MtSUS1* from *Medicago truncatula*, AJ131943 (Hohnjec et al., 1999); *MySUS* from the tropical epiphytic CAM orchid *Mokara Yellow*, AF530568 (Li et al., 2004); *OgSUS* from the tropical epiphytic orchid *Oncidium goldiana*, AF530567; *OsSUS1-OsSUS7* from rice, LOC_Os03g28330, LOC_Os06g09450, LOC_Os07g42490, LOC_Os03g22120, LOC_Os04g24430, LOC_Os02g58480, and LOC_Os04g17650, respectively (Hirose et al., 2008; Huang et al., 1996; Wang et al., 1992); *PdSUS1* and *PdSUS2* from *Potamogeton distinctus*, AB193515 and AB193516; *PtSUS* from *Populus tremuloides*, AY341026; *PvSS* from bean, AF315375 (Silvente et al., 2003); *PsSUS1-PsSUS3* from pea, AJ012080, AJ001071, and AJ311496, respectively (Barratt et al., 2001; Craig et al., 1999); *SoSusy2* from *Saccharum officinarum*, AY118266; *StSUS2-StSUS4* from potato, AY205302, U24088, and U24087, respectively (Fu and Park, 1995; Salanoubat and Belliard, 1987); *TaSUS1* and *TaSUS2* from *Triticum aestivum*, AJ001117 and AJ000153; *TgSUS1* and *TgSUS2* from tulip, X96938 and X96939 (Balk and de Boer, 1999); *VfSUS* from fava bean, X69773; *Vrvss1* from mung bean, D10266 (Arai et al., 1992); and *ZmSH1*, *ZmSUS1* and *ZmSUS3* from maize, X02400, L22296 and AY124703, respectively (Werr et al., 1985).

Semi-quantitative RT-PCR analysis

Total RNA was prepared from various organs using Trizol reagent (Invitrogen), treated with DNaseI (Ambion) and then reverse-transcribed using an oligo-dT primer and a First-Strand cDNA Synthesis kit (Roche). Endosperm and seed coat cDNAs were prepared from seeds harvested 6-10 days after fertilization (DAF) in accordance with the method of Cho et al. (2005; 2006). To prevent genomic DNA contaminations, primers were designed to amplify a region encompassing at least one intron for each gene (Supplementary Table 2). The rice actin1 gene *OsAct1* was used as a PCR control (Cho et al., 2006). These experiments were repeated at least three times. To distinguish between the expression patterns of *OsSUS5* and *OsSUS7*, the RT-PCR products of both genes were further digested with the restriction enzyme *DraIII*, which specifically cuts the amplified product of *OsSUS5* only to yield 315- and 236-bp fragments, whilst the 551-bp *OsSUS7* amplicon remains intact.

Subcellular localization analysis

Full-length cDNAs of the *OsSUS* genes were amplified by PCR using proofreading DNA polymerase (SolGent, Korea) and the appropriate primer pairs (Supplementary Table 1). PCR products were inserted into the pENTR/D-TOPO vector (Invitrogen) and sequenced. Validated cDNAs were subcloned into p2FGW7 using LR clonase (Invitrogen) to produce N-terminal GFP fusion products (Karimi et al., 2002). The resulting GFP-*OsSUS* fusion constructs driven by the *CaMV35S* promoter were delivered into maize mesophyll protoplasts using a polyethylene glycol (PEG)-calcium mediated method followed by

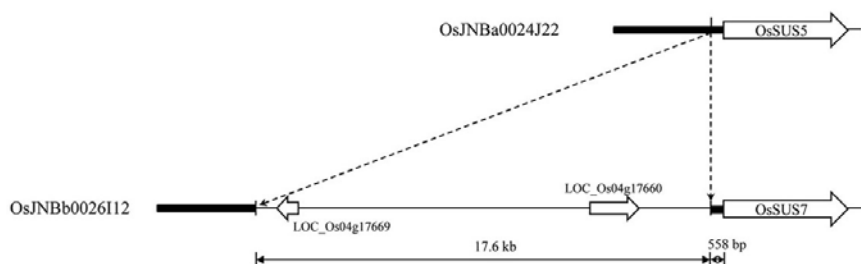


Fig. 1. Comparative analysis of the 5' upstream regions of *OsSUS5* and *OsSUS7*. An insertion region of 17.6-kb is present in the promoter of *OsSUS7*, but absent in the corresponding region of *OsSUS5*. The accession numbers of the BAC clones used to analyze 5' upstream regions of *OsSUS5* and *OsSUS7* are listed in "Materials and Methods".

12-24 h incubation to allow transient expression (Cho et al., 2009; Hwang and Sheen, 2001). Chlorophyll autofluorescence was used as a chloroplast marker. Expression of these fusion constructs was monitored using a confocal microscope (LSM 510 META, Carl Zeiss, Germany).

Transient expression assay

To construct reporter vectors, the native 2.19-kb *OsSUS5* promoter and 2.46-kb *OsSUS7* promoter, and the conserved 558-bp 5' regulatory region of *OsSUS5* and *OsSUS7*, were amplified by PCR using the appropriate primer pairs (Supplementary Table 1). The amplified PCR products were then inserted into the pENTR/D-TOPO vector (Invitrogen) and fused to the *LUC* gene of JJ2749 derived from pHGWL7 (Karimi et al., 2007) using LR clonase (Invitrogen). Three *OsSUS* promoter-*LUC* fusion constructs, *OsSUS5-LUC*, *OsSUS7-LUC* and *558-LUC*, were used in this study. The *ZmUBQ1-GUS* (β -glucuronidase) fusion construct (Cho et al., 2009) was used as an internal control. Maize mesophyll protoplasts ($1-2 \times 10^5$ cells/200 μ l) isolated from the second leaves of etiolated plants were cotransfected with reporter constructs and an internal control *ZmUBQ1-GUS* using a PEG-calcium mediated method (Cho et al., 2009; Hwang and Sheen, 2001). Transformed protoplasts were incubated for 6 h and then harvested. The harvested protoplasts were resuspended in lysis buffer and used for LUC and GUS assays. LUC and GUS assays were performed using previously described methods (Cho et al., 2009; Jefferson et al., 1987). The LUC and GUS fluorescence activity was measured using the VICTOR2 1420 multilabel counter (PerkinElmer Life Sciences). All transient expression experiments were repeated three times and produced similar results.

RESULTS

Identification and characterization of the *OsSUS7* genomic region

Systemic blast searches of the rice genome, using nucleotide and amino acid sequences of previously reported rice *SUS* genes (Harada et al., 2005; Hirose et al., 2008; Huang et al., 1996; Wang et al., 1992), identified seven genes encoding sucrose synthase enzymes. Thus, our analysis enabled the detection of a previously uncharacterized sucrose synthase gene, named *OsSUS7*. An in-depth examination further found that the genomic sequence of *OsSUS7* is nearly identical to that of *OsSUS5*, which are located near to each other on chromosome 4. Comparative analysis of both genomic regions revealed an insertion region of a 17.6 kb in the promoter of *OsSUS7*, which was absent in the corresponding region of *OsSUS5* (Fig. 1). Further analysis of the *OsSUS5* and *OsSUS7* genomic regions using BAC clones provided an interesting insight into the evolutionary history of both genes (Fig. 2). We found that several genes located at the periphery of *OsSUS5* and *OsSUS7* were nearly identical to each other. LOC_Os04g

17640 and LOC_Os04g24440, LOC_Os04g17700 and LOC_Os04g24410, LOC_Os04g17710 and LOC_Os04g24400, and LOC_Os04g17720 and LOC_Os04g24390, respectively, showed near identity indicating a conserved synteny between both loci. In addition, we found that a number of transposable elements are scattered among the *OsSUS5* and *OsSUS7* genes (Fig. 2). These data suggest that the *OsSUS7* chromosomal region might have been duplicated from the *OsSUS5* genomic region, which was followed by transposition events involving transposable elements. Alternatively, the *OsSUS5* genomic segment may have been duplicated from that of *OsSUS7* via an excision of the 17.6-kb insertion region.

To determine their coding regions, we cloned both the *OsSUS5* and *OsSUS7* cDNAs by RT-PCR. Alignment of the cDNA and deduced amino acid sequences of these two genes indicated that they harbored only nine single nucleotide substitutions within their coding regions, resulting in a change of six amino acids (Supplementary Table 3).

Phylogenetic relationship between *OsSUS7* and other *SUS* genes

Further alignment of the deduced amino acid sequences of all *OsSUS* cDNAs including *OsSUS7* confirmed the presence of two separate conserved domains in each case, a sucrose synthase domain and a glycosyl-transferase domain. These are common to *SUS* proteins (Baud et al., 2004; data not shown). To classify these plant *SUS* enzymes, we constructed a rooted phylogenetic tree using the neighbor-joining method (Fig. 3). Three major groups were thereby identified: *SUS* group I, *SUS* group II (also referred to as *SUSA*), and *SUS* group III. Group I was further subdivided into group I-1 consisting of dicot *SUS*s and group I-2 containing monocot *SUS*s. The rice *OsSUS1*, *OsSUS2*, and *OsSUS3* genes belong to the monocot group I-2 along with the maize sucrose synthases, *ZmSH1* and *ZmSUS1*, whereas the *Arabidopsis* genes *AtSUS1* and *AtSUS4* were classified into the dicot group I-1. This result suggests that *SUS* group I was subdivided into the dicot group I-1 and monocot group I-2 prior to the monocot-dicot divergence. The *SUS* group II gene, *OsSUS4*, could be classified with the maize *SUS*, *ZmSUS3*, and two *Arabidopsis* *SUS* genes, *AtSUS2* and *AtSUS3*. Interestingly, *OsSUS5*, *OsSUS6*, and *OsSUS7* belong to *SUS* group III, the members of which possess unique carboxy-terminal extensions relative to the other groups, as is the case for *AtSUS5* and *AtSUS6* (Baud et al., 2004).

Genomic structure of the rice *SUS* genes

The inferred genomic structures of seven *OsSUS* genes were determined by aligning the cDNA sequences and genomic sequences of the corresponding BAC/PAC clones obtained from the NCBI database. The *Arabidopsis* Information Resource (TAIR) (<http://arabidopsis.org/>) database was used to additionally determine the genomic structures of the six *Arabidopsis* *SUS* genes. The exon/intron structures of these *SUS*

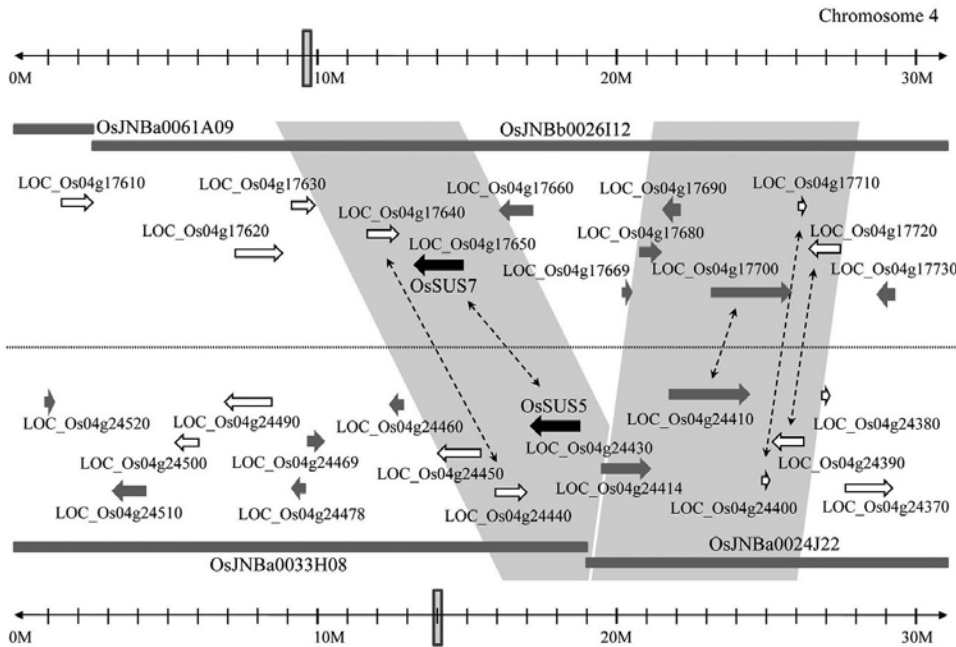


Fig. 2. Comparative analysis of the chromosomal regions encompassing *OsSUS5* and *OsSUS7* using BAC clones. Black arrows indicate *OsSUS5* and *OsSUS7* and open arrows indicate transposable elements. The accession numbers of the BAC clones used to identify the genomic structure of chromosomal region containing *OsSUS5* and *OsSUS7* are listed in “Materials and Methods”.

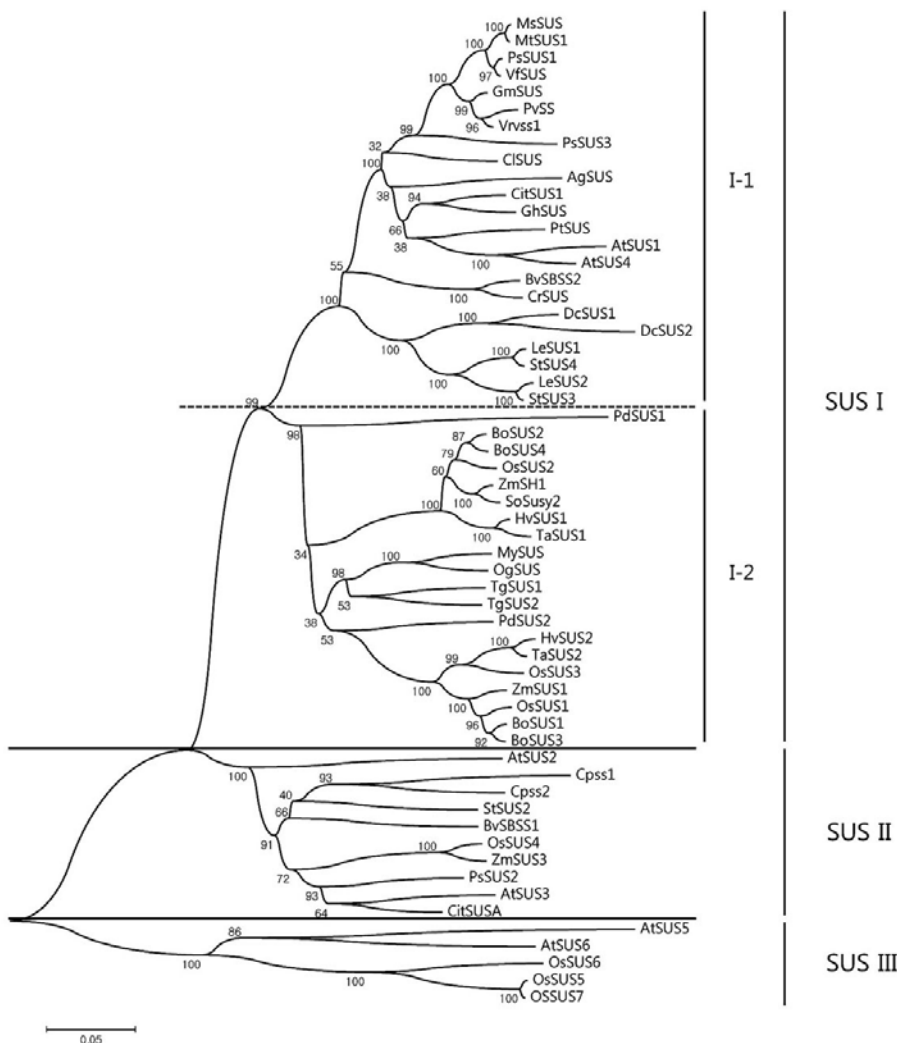


Fig. 3. Phylogenetic tree of the sucrose synthase (*SUS*) genes constructed using the neighbor-joining method with MEGA version 4 software. The scale bar corresponds to a distance of 5 changes per 100 amino acids. Bootstrap analysis was performed with 1000 replicates and numbers in the branches indicate bootstrap values as percentages. Accession numbers are listed in “Materials and Methods”.

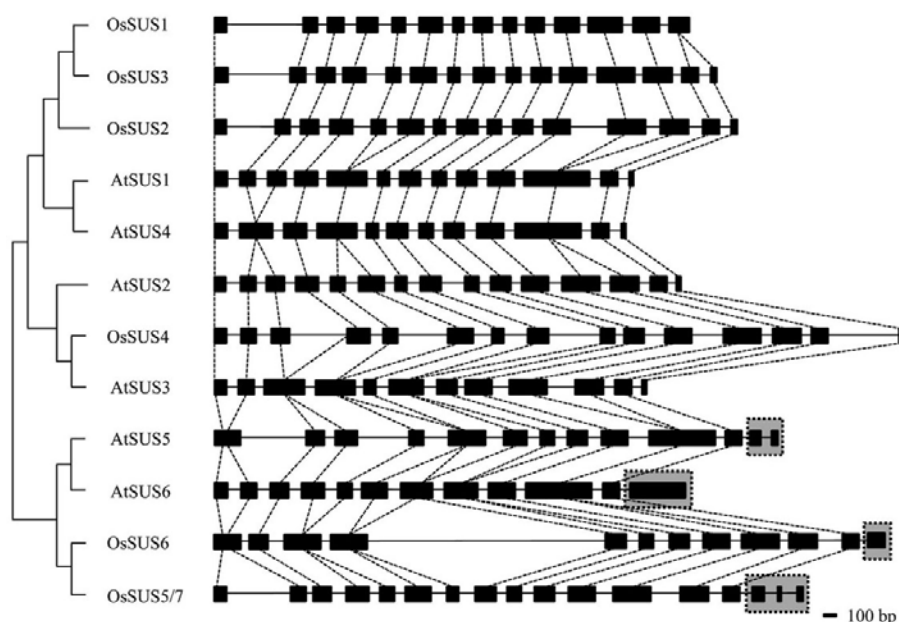


Fig. 4. Genomic structures of *Arabidopsis* *SUS* (*AtSUS*) genes and rice *SUS* (*OsSUS*) genes. Exons are indicated by black rectangles and introns by lines. The unique C-terminal regions of the group III genes are denoted by gray boxes with a dotted line.

genes were analyzed and compared using the coding region between the translation start and stop codons, except for the non-coding leader exon 1 found in some *SUS* genes (Fig. 4 and Supplementary Figs. 1 and 2). The results indicate that the exon sizes in the rice *SUS* genes are highly conserved except for the unique 3' extension regions of the group III genes. Among the four rice *SUS* genes belonging to the groups I-2 and II, *OsSUS1* is composed of 14 exons due to the presence of a 14th 187-bp unsplit exon, whereas the other three *OsSUS* genes comprised 15 exons. In the *SUS* group II gene *OsSUS4*, the number and sizes of the exons were quite similar to the three *OsSUS* genes belonging to the *SUS* group I-2. However, *OsSUS4* is the largest *SUS* gene in rice due to four relatively large introns of 400-600 bp. Three *OsSUS* genes belonging to group III, *OsSUS5*, *OsSUS6* and *OsSUS7*, possess unique 3' extension regions, similar to *AtSUS5* and *AtSUS6* (Fig. 4 and Supplementary Figs. 1 and 2).

Comparative expression analysis of *OsSUS5* and *OsSUS7*

The 17.6-kb fragment located 558-bp upstream of the translation start site of *OsSUS7* (Fig. 1) could inhibit gene expression. To test this possibility, we generated three promoter-LUC fusion constructs driven by the native 2.19-kb *OsSUS5* promoter and 2.46-kb *OsSUS7* promoter, and the conserved 558-bp 5' regulatory region of *OsSUS5* and *OsSUS7*, respectively (Fig. 5A). In transient expression analysis using maize mesophyll protoplasts, the relative expression levels of these three constructs were not significantly different, indicating that the 558-bp upstream region of *OsSUS7* is sufficient for its endogenous expression, and that the 17.6-kb insertion may not have a large influence on the expression of *OsSUS7*, at least in mesophyll protoplasts (Fig. 5).

Subcellular localization of the *OsSUS5* and *OsSUS7* proteins

The subcellular localization of *SUS* proteins was an important key to elucidating their intracellular function. To determine the subcellular localization of *OsSUS5* and *OsSUS7*, we generated GFP-*OsSUS* fusion constructs under the control of the *CaMV35S* promoter. In transient expression assays using maize meso-

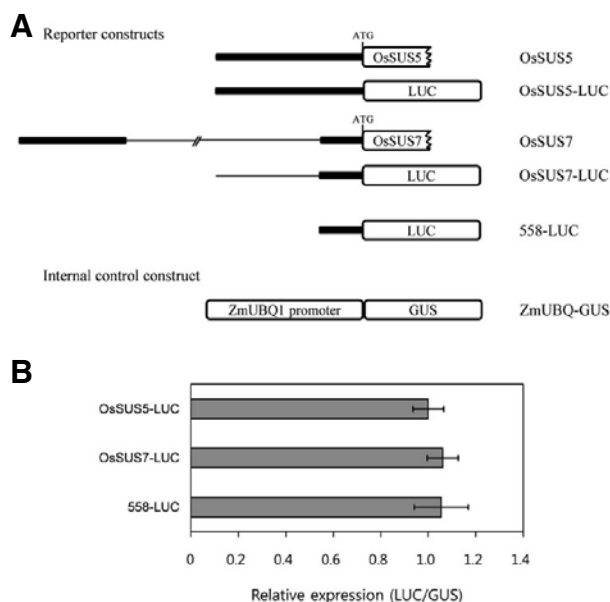


Fig. 5. Comparative expression analysis of the upstream regulatory regions of *OsSUS5* and *OsSUS7* using maize protoplasts. (A) Schematic diagram of the *OsSUS5* and *OsSUS7* genomic structure. Promoter regions are indicated by the thick line and the 17.6 kb region present in the promoter of *OsSUS7* is highlighted by the thin line. Vectors used in transient expression experiments; *OsSUS5-LUC*, *OsSUS7-LUC* and *558-LUC*. The *ZmUBQ1-GUS* construct was used as an internal control. (B) Transient expression assays of the three reporter constructs, *OsSUS5-LUC*, *OsSUS7-LUC* and *558-LUC*, in maize mesophyll protoplasts. The bar indicates standard errors.

phyll protoplasts, signals of GFP-*OsSUS5* and GFP-*OsSUS7* were found to be strongly associated with the plasma membrane and detectable in part in the cytosol (Figs. 6A and 6B). To confirm these plasma membrane-associated GFP signals,

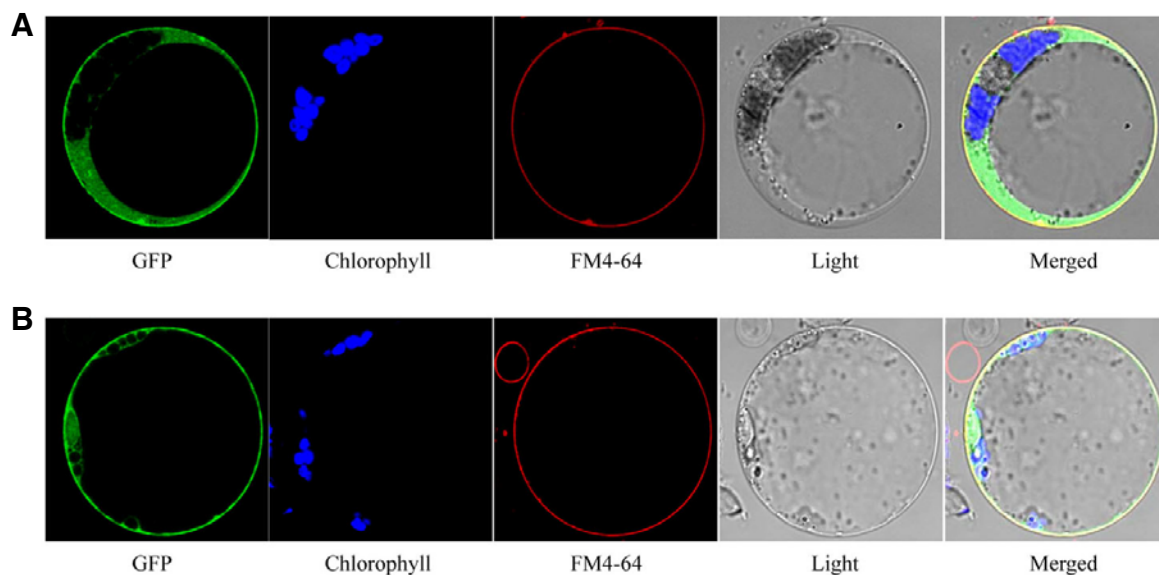


Fig. 6. Subcellular localization of GFP-OsSUS5 and GFP-OsSUS7 fusion proteins in transfected mesophyll protoplasts of maize. (A) GFP-OsSUS5, (B) GFP-OsSUS7. Chlorophyll autofluorescence and FM4-64 were used as chloroplast and plasma membrane markers, respectively. The GFP signals are indicated in green, and plasma membranes stained with FM4-64 are shown in red. A false color (blue) was used to monitor chlorophyll autofluorescence to distinguish it from GFP (green) and FM4-64 (red).

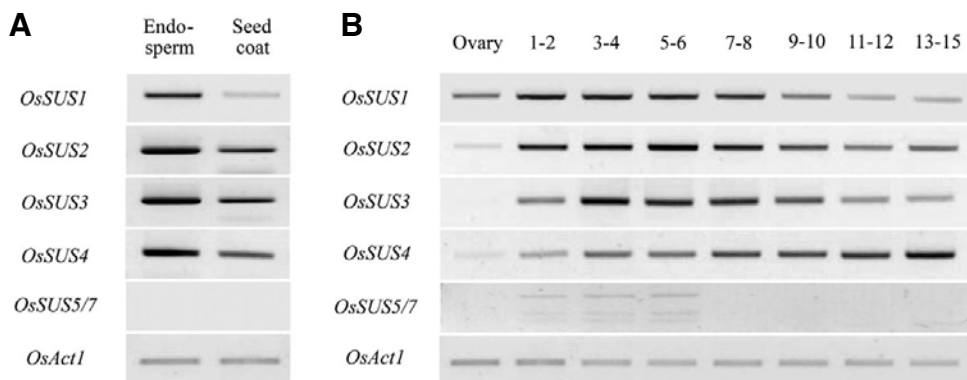


Fig. 7. Expression profiles of *OsSUS* genes during rice seed development. (A) Expression patterns of *OsSUS* genes in the endosperm and seed coat. (B) Changes in the gene expression levels of the *OsSUS* genes during rice seed development. Rice seeds were harvested over a time course up until 15 DAF. *OsAct1* is a PCR control. The two open arrowheads and single closed arrowhead indicate the

Dralll-digested *OsSUS5* and *Dralll*-resistant *OsSUS7* RT-PCR products, respectively.

protoplasts were stained with a lipophilic styryl dye, FM4-64, which primarily stains the plasma membrane (Cho et al., 2010; Ueda et al., 2001; Uemura et al., 2004). The GFP signals were indeed well merged with those of FM4-64 in the plasma membrane (Figs. 6A and 6B), indicating that *OsSUS5* and *OsSUS7* are predominantly associated with this structure.

Expression profile analysis of *OsSUS* genes during rice seed development

The spatiotemporal expression pattern of the *OsSUS* genes was examined by RT-PCR (Supplementary Fig. 3). Whilst only two genes, *OsSUS2* and *OsSUS4*, were found to be expressed at considerable levels in the leaf tissues, transcripts of many of *OsSUS* genes were more abundantly detectable in sink tissues such as roots, flowers, and immature seeds, suggesting that these SUS enzymes may have functions in these sink organs. Interestingly, we also found that *OsSUS5* and *OsSUS7* are highly expressed in roots with similar expression patterns in all tested rice tissues, which is consistent with the results of the

previous transient expression assay.

To elucidate the roles of *OsSUS* genes, particularly during rice seed development, we examined their expression in the endosperm and seed-coats of a series of seeds collected at different developmental stages (Cho et al., 2005). *OsSUS1* to *OsSUS4* are expressed abundantly in immature seeds and were found to be highly expressed in the endosperm rather than the seed coat prepared from 6 to 10 days after fertilization (DAF), whereas *OsSUS5/OsSUS7* transcripts were not detected in these samples (Fig. 7A). During different developmental stages in immature seeds, *OsSUS1* to *OsSUS4* were found to be expressed throughout the pre-storage phase (1-8 DAF) and up to the starch-filling phase (9-15 DAF). In contrast, *OsSUS5* and *OsSUS7* transcripts were detectable only in 1-6 DAF immature seeds (Fig. 7B), which is consistent with the finding that *OsSUS5* and *OsSUS7* are barely expressed in 6-10 DAF immature seeds (Fig. 7A). These expression analysis data suggest that the *OsSUS* gene products have a role in rice seed development.

DISCUSSION

***OsSUS5* and *OsSUS7* were formed by duplication and transposition events**

In the present study, we identified a new rice SUS member, *OsSUS7*, which is a duplicate of the *OsSUS5* gene. Our data indicate that *OsSUS5* and *OsSUS7* are almost identical at the nucleotide and amino acid sequence, genomic structure, expression pattern, and subcellular localization. We also found synteny between the *OsSUS5* and *OsSUS7* loci and identified a number of transposable elements widely scattered at the periphery of these loci. In addition, a 17.6-kb genomic region located 5' upstream region of *OsSUS7* consists of transposable elements. We thus concluded that *OsSUS5* and *OsSUS7* were formed via duplication, and by the transposition of transposable elements.

We previously reported three pairs of rice cell-wall invertases, *OsCIN2* and *OsCIN3*, *OsCIN5* and *OsCIN6*, and *OsCIN7* and *OsCIN8*, each located in pairs, and a number of transposable elements that could be observed at the periphery of these *OsCIN* genes (Cho et al., 2005). In addition, *OsCIN8*, which is quite similar to *OsCIN7*, harbors a transposable element similar to the maize transposon *MuDR* within its second intron which causes the inactivation of the *OsCIN8* gene (Cho et al., 2005). We therefore speculate that these *OsCIN* genes have also been formed via duplication and transposition events and that the surrounding regions of these genes are the preferred target loci of transposable elements.

Gene duplication and diversification events are important evolutionary processes. Duplication plays a central role in creating complex genetic systems by creating new loci such as the formation of resistance (R) gene cluster (Ronald, 1998). Transposition contributes to allelic diversity. Published sequence data have revealed that the rice genome contains more than 40% repetitive sequences related to transposable elements (Feng et al., 2002; Goff et al., 2002; Sasaki et al., 2002; Yu et al., 2002). Hence, retrotransposons are the most frequent transposable elements in rice and occupy approximately 15% of the rice genome (Jiang et al., 2004). Although most of the transposable elements are inactive in higher eukaryotes, some are active under normal conditions or can be activated in tissue cultures, such as *Tos17* or *Karma* which become active in rice culture cells in response to DNA hypomethylation (Hirochika et al., 1996; Komatsu et al., 2003; Kwon et al., 2009). It is therefore likely that duplication and the transposition of transposable elements has occurred in the *OsSUS5* and *OsSUS7* genes.

***OsSUS5* and *OsSUS7* are predominantly associated with the plasma membrane**

It is known that SUS proteins exist both freely in the cytosol and in association with various subcellular compartments including the plasma membrane (Duncan and Huber, 2007; Etxeberria and Gonzalez, 2003; Subbaiah et al., 2006; Winter et al., 1997; 1998). Interestingly, in our subcellular localization analysis of the *OsSUS* proteins, we found that *OsSUS5* and *OsSUS7*, belonging to SUS group III, are associated predominantly with the plasma membrane and exist in part in the cytosol in mesophyll cells. In this regard, the *in vitro* phosphorylation of the ZmSUS1 protein in maize, belonging to group I-2, was found to cause the release of SUS from the membrane fraction (Winter et al., 1997). In the developing tomato fruit, the subcellular localization of SUS isoforms has also been found to be developmentally controlled, the membrane form being specifically detected in actively growing fruits, depending on the phosphorylation of SUS proteins (Anguenot et al., 2006). It will thus be

interesting to determine whether the phosphorylation/dephosphorylation of *OsSUS5* and *OsSUS7* alters their subcellular localization. Notably, signals for the C-terminal GFP fusion constructs, *OsSUS5*-GFP and *OsSUS7*-GFP, were barely detectable in maize mesophyll protoplasts (data not shown). This may imply that the unique carboxy-terminal extensions of *OsSUS5* and *OsSUS7* are indispensable for the membrane association of these SUS proteins.

Generally, it is known that SUS activity correlates with the sink strength of various carbohydrate-storage or consuming organs (Hirose et al., 2008; Zrenner et al., 1995). In our current expression profile analysis, *OsSUS* transcripts, including those of *OsSUS5* and *OsSUS7* were abundantly detected in sink tissues such as roots, flowers, and immature seeds (Fig. 7 and Supplementary Fig. 3). Therefore, our results further support the contention that SUS enzymes play a major role in sink organs in the provision of carbon sources for sink metabolism. The SUS group III genes have recently been identified only in *Arabidopsis* and rice. The roles of these SUS isoforms are therefore still the subject of uncertainty and debate. Analysis of transgenic rice plants and loss-of function mutants for the novel group III genes, *OsSUS5* and *OsSUS7*, will be invaluable for our enhanced understanding of the *in vivo* function of these rice isoforms.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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