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Analysis of gene-disruption mutants of a sucrose phosphate synthase gene in rice, *OsSPS1*, shows the importance of sucrose synthesis in pollen germination

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ABSTRACT

The molecular function of an isoform of sucrose phosphate synthase (SPS) in rice, *OsSPS1*, was investigated using gene-disruption mutant lines generated by retrotransposon insertion. The progeny of the heterozygote of disrupted *OsSPS1* (*SPS1*$^{+/-}$) segregated into *SPS1*$^{+/+}$, *SPS1*$^{+/-}$, and *SPS1*$^{-/-}$ at a ratio of 1:1:0. This distorted segregation ratio, together with the expression of *OsSPS1* in the developing pollen revealed by quantitative RT-PCR analysis and promoter–beta-glucuronidase (GUS) fusion assay, suggested that the disruption of *OsSPS1* results in sterile pollen. This hypothesis was reinforced by reciprocal crosses of *SPS1*$^{+/-}$ plants with wild-type plants in which the disrupted *OsSPS1* was not paternally transmitted to the progeny. While the pollen grains of *SPS*$^{+/-}$ plants normally accumulated starch during their development, pollen germination on the artificial media was reduced to half of that observed in the wild-type control. Overall, our data suggests that sucrose synthesis via *OsSPS1* is essential in pollen germination in rice.

1. Introduction

Sucrose is the major form of sugars translocated in higher plants and is synthesized by the cooperation of two enzymes, sucrose phosphate synthase (SPS, EC 2.3.1.14) and sucrose phosphate phosphatase (SPP, EC 3.1.3.24). SPS catalyzes the conversion of fructose-6-phosphate and UDP-glucose into sucrose-6-phosphate, and SPP subsequently hydrolyzes it to sucrose. Several studies have shown that SPS is the rate-limiting enzyme in sucrose synthesis [1,2]. In many plant species, SPSs are encoded by small gene families and the isoforms of SPS are phylogenetically classified into four distinct subfamilies, A–D [3]. In rice, five isogenes for SPS, *OsSPS1* (subfamily B), *2* and *6* (subfamily D), *8* (subfamily A) and *11* (subfamily C), exist and each gene has a different expression pattern and mode of regulation [3–5]. Among the five isogenes, *OsSPS1* is highly expressed in source tissues, particularly in leaf blades, and it plays a dominant role in sucrose synthesis [4]. In addition, some studies have associated *OsSPS1* with traits for rice growth and productivity such as plant height [6], plant seedling length [7], and spikelet number per panicle [8]. However, the role(s) of SPS and its isogenes in heterotrophic tissues has not been well investigated and remain unclear.

In our study of gene-disruption mutant lines of *OsSPS1*, we observed that there was no homozygote for gene disruption and that the segregation of heterozygotes to wild-type deviates from the expected 2:1 ratio, thus, implying a disorder of pollen function. Expression of *OsSPS1* in rice pollen has been reported by Chavez-Barcenas et al. [9], suggesting the role of *OsSPS1* in pollen. Castleden et al. [3] also found the expression of SPS genes in the anther of wheat and barley, especially for those belonging to B-subfamily, and pointed out any specific role of B-subfamily SPSs in anther/pollen.

In pollen, high level of starch accumulates during its development, and the sugars derived from the starch are utilized as an energy source to support pollen germination and also as an osmoticum (see Rounds et al. [10] for review). During pollen tube elongation, sugars are also required for the synthesis of cell wall materials in the rapidly growing pollen tube (see Hepler et al. [11]

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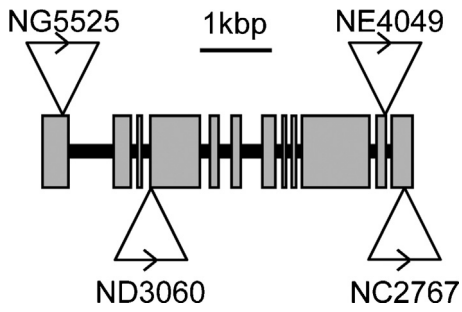

Fig. 1. Gene structure of *OsSPS1* and insertion position of *Tos17* in each line, in this study. Exons and introns are shown as gray boxes and black bars, respectively. Insertion position and direction of *Tos17* are indicated by triangles and arrowheads, respectively.

for review). Thus, starch and sugar metabolism in pollen can be of primary importance for the functionality of pollen. In fact, pollen sterility in rice induced by cytoplasmic male sterility [12], chilling stress [13], or heat-stress [14] is associated with altered patterns of carbohydrate metabolism and/or expression of related genes. Because of symplastic isolation from the sporophytic anther tissues, pollen requires a suite of transport systems to import sugar molecules during its development and germination, which is relatively well understood and extensively reviewed by Slewinski [15]. However, information on the role of carbohydrate metabolism and related genes in pollen function is limited. *Arabidopsis thaliana AtUSP* encoding UDP-sugar pyrophosphorylase [16], rice *CAP1* gene encoding arabinokinase-like protein [17], and rice *OsGT1* encoding glycosyltransferase [18] are essential for pollen wall formation, whereas the tomato *LeGWD* encoding glucan water dikinase [19] and rice *OsHXK*-encoding hexokinase [20] are essential for pollen germination and/or pollen tube elongation. In *A. thaliana*, genes for nucleotide sugar transporters, *AtUTr1* and *AtUTr2*, and for plastidic glucose 6-phosphate translocator, *AtGPT1*, are essential for pollen development, although their detailed mechanisms are unknown [21,22]. In addition, the inactivation of a rice gene for UDP-glucose pyrophosphorylase has been identified as the responsible gene for male sterility, *ms-h*, supposedly through impairment of pollen wall formation [23].

Here, we report further genetic analysis using the disruption mutants of *OsSPS1*. Based on the results, together with the expression pattern of *OsSPS1* in pollen development, possible roles of sucrose synthesis in pollen function is discussed.

2. Materials and methods

2.1. Plant materials and growth conditions

Four gene-disruption mutant lines of *OsSPS1* (Os01g0919400) by insertion of retrotransposon *Tos17* and their wild-type parental rice cultivar (*Oryza sativa* L., subsp. japonica cv. 'Nipponbare') were used. The four mutant lines, NC2767, ND3060, NE4049, and NG5525 were selected by BLAST searches against a dataset of *Tos17* flanking sequences in the rice genome (http://tos.nias.affrc.go.jp/, [24]), and were obtained from the National Institute of Agrobiological Science, Tsukuba, Japan. Each of the four mutant lines has an insertion of *Tos17* into different exons of the *OsSPS1* gene (Fig. 1).

Rice plants were grown in paddy fields at the Institute for Sustainable Agro-ecosystem Services, The University of Tokyo, Nishitokyo, Japan (35°44′N, 139°32′E). The planting density was 22.2 hills per square meter (hill spacing of 30 × 15 cm) with 1 seedling per hill. Compound fertilizer for paddy fields ($N:P_2O_5:K_2O = 12:16:18\%$) was applied at a rate of 50 g m^{-2} as basal dressing. For the measurement of pollen maturity and pollen germination, rice plants were grown in plastic pots (0.6 L volume) filled with nursery soil for rice seedlings in a growth chamber under 12 h light (30 °C) and 12 h dark (23 °C) photoperiod, ~800 μmol photons m^{-2} s^{-1}.

2.2. Genotyping mutant plants and pollen grains

The genotypes of each of the four mutant lines were determined by PCR. Genomic DNA was extracted from mature leaf blades of each plant using the method of Wang et al. [25], and used as template for the PCR with the primers listed in Supplementary Table S1.

2.3. Quantitative RT-PCR (qRT-PCR) analysis

From the panicles of a rice cultivar, Nipponbare, glumaceous flowers were taken and dissected on ice to collect anthers, from which total RNA was extracted using RNeasy Mini kit (QIAGEN Japan, Tokyo, Japan). First strand cDNA was synthesized from the total RNA samples using SuperScript III reverse-transcriptase (Life Technologies Japan, Tokyo, Japan), and then an aliquot of the resultant cDNA mixture corresponding to 10 ng of total RNA was subjected to qRT-PCR to detect the transcript levels of SPS isogenes using SYBR Premix Ex Taq II (Takara Bio, Shiga, Japan) and Smart Cycler II system (Cepheid, CA) following the manufacturers' instructions. The nucleotide sequences of the PCR primers were shown in Supplementary Table S1. The transcript level of each gene was standardized against that of a constitutively expressed polyubiquitin gene, *RUBIQ1* (Os06g0681400).

2.4. Promoter::beta-glucuronidase (GUS) analysis

The promoter region of *OsSPS1* (P_{SPS1}) covering from −2415 to +6 nucleotides from the translation start point was PCR-amplified from rice genomic DNA (cultivar Nipponbare) by a high-fidelity DNA polymerase (Prime Star GXL, Takara Bio, Shiga, Japan). Both *GUS* gene and NOS terminator were also amplified from pBI121 plasmid (GeneBank Accession No.: AF485783) in the same manner. The nucleotide sequences of the PCR primers used were shown in Supplementary Table S1. These elements were cloned into a destination vector, pIG-R4R3 [5] by use of Multiseite Gateway Technology (Life Technologies Japan). The resultant construct was transformed into rice by using the method of Yonekura et al. [5]. Glumaceous flowers from the P_{SPS1}::GUS transgenic lines were stained for GUS activity at 37 °C in the dark, overnight, in X-gluc solution containing 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]\cdot 3H_2O$, 0.3% (v/v) Triton X-100, 20% (v/v) methanol, and 2 mM 5-bromo-4-chloro-3-indolyl-glucuronide cyclohexylamine salt in 100 mM sodium phosphate buffer (pH 7.0). GUS staining was examined using a stereomicroscope (SZX10, Olympus, Tokyo, Japan).

2.5. Maturity of the pollen grains

Glumaceous flowers one or two days before anthesis were collected, immediately fixed in 50% (v/v) ethanol, and stored at room temperature until use. Anthers were excised from the glumaceous flowers, stained with an I_2/KI solution, and pollen grains densely stained by the I_2/KI solution were counted as mature pollen.

2.6. In vitro pollen germination

Pollen germination was examined on artificial media described by Kariya [26]. Pollen grains were germinated on 1% agar medium containing 20% (w/v) sucrose and 1 mM H_3BO_3. Glumaceous flowers just after anthesis were sampled and gently shaken above germination medium in 4.5-cm diameter Petri dishes to collect pollen grains. After incubation for 20 min at 20 °C, the pollen grains

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