Expression Analysis of Genes for Callose Synthases and Rho-Type Small GTP-Binding Proteins That Are Related to Callose Synthesis in Rice Anther

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The most chilling-sensitive stage of rice has been found to be at the onset of microspore release. The microsporocytes produce a wall of callose between the primary cell wall and the plasma membrane, and it has been shown that precise regulation of callose synthesis and degradation in anther is essential for fertile pollen formation. In this study, genes for 10 callose synthases in the rice genome were fully annotated and phylogenetically analyzed. Expression analysis of these genes showed that OsGSL5, an ortholog of microsporogenesis-related AtGSL2, was specifically expressed in anthers, and was notably downregulated by cooling treatment. Gene expression profiles of Rho-type small GTP-binding proteins in rice anther were also analyzed. The mechanisms of callose synthesis in rice pollen formation and its relationships with cool tolerance are discussed.

Key words: chilling stress; microsporogenesis; tetrad

In the northern part of Japan, rice crop production is occasionally damaged severely by low temperatures in summer. The most chilling-sensitive stage of rice has been found to be at the onset of microspore release. Chilling treatment at this stage actually causes tapetum hypertrophy and disordered microspore development, and consequently gives rise to a high degree of male sterility.

Pollen development begins with the division of diploid archespories in the anther-generating microsporocytes and tapetal cells. The tapetum forms a single layer of cells around the anther locule and supplies nutrients and enzymes for microspore development. The microsporocytes produce a wall of callose, composed primarily of β-1,3-glucan, between the primary cell wall and the plasma membrane. It is believed that the callose wall is formed temporarily to prevent cell cohesion and fusion. At the end of meiosis, tetrads of haploid microspores are freed into the locule by the action of a β-1,3-glucanase (callase), which is secreted by the tapetal cells. In transgenic tobacco plants in which β-1,3-glucanase is induced prematurely, little fertile pollen is produced. Therefore, the timing of β-1,3-glucanase activity is essential for the developing microspores. A β-1,3-glucanase cDNA was isolated from rice anther at the microspore stage, and the role of the gene expression in chilling injury is now under study.

A cDNA microarray technology was used to analyze the gene expression profile in rice anther at its early microspore stage under chilling, and three novel genes were found whose expression levels were conspicuously changed by chilling in rice anther. Particularly, two genes had miniature inverted-repeat transposable element (MITE) Castaway sequences at nearly the same position in the 5’ upstream region.

The cool tolerance of barley at the young microspore stage was much higher than that of rice, and microscopic observation revealed that callose envelopes that surround tetrad cells are thicker in barley than in rice. There is no information on the relationship between the thickness of callose envelopes and chilling tolerance. In order to analyze this relationship, we thought of making transgenic rice plants, in which the thickness of callose envelop is manipulated. Initially, we planned to identify genes related to callose synthesis in rice anthers, and utilize them. Plant callose is produced at specific stages of cell wall development in pollen and in response to pathogen attack and wounding. Twelve GSL (Gulcan Synthase-Like) genes have been identified in Arabidopsis. Among these, AtGSL5 is developmentally expressed at highest levels in flowers, and has been shown negatively to regulate the SA pathway involved in disease resistance. CalS5 (AtGSL2) is responsible for the synthesis of callose deposited at the primary cell wall of meiocytes, tetrads and microspores, and the expression of this gene is essential for exine formation in the pollen wall. Two closely related and linked genes, AtGSL1 and AtGSL5, have also been shown to play essential, but at least partially redundant roles in both sporophyte and pollen development. The activity of yeast β-1,3-glucan synthase requires the addition of GTP, and it has been demonstrated that the small GTP binding protein Rho1 is a subunit of the β-1,3-glucan synthase.
synthase complex. In Arabidopsis, Rop1, a plant Rho-like protein, might act as a molecular switch that controls CalS1 (AtGSL6) activity at the forming cell plate through interaction with UGT (UDP-glucose transferase). A plant-specific family of Rho homologs, known as the Rop family, has important roles in plant development, including pollen tube and root hair growth, cell wall synthesis, and cell proliferation in the meristem. In this study, in order to decipher the mechanisms of callose synthesis in rice pollen formation and its relationship with cool tolerance, we analyzed gene expression profiles of callose synthases and Rops that might be related to callose synthesis in rice anther.

Materials and Methods

Sequence and phylogenetic analysis. BLAST (http://www.ncbi.nlm.nih.gov/blast/) algorithms were used to search for sequence homology. Nucleotide and predicted amino acid sequences were analyzed using DNASIS Pro V2.6 for Windows (Hitachi Software Engineering, Yokohama, Japan). The ClustalW 1.83 program was used for multiple alignment of amino acid sequences. A phylogenetic tree was constructed using the NJ (Neighbor-Joining) algorithm and a dendrogram was drawn with Njplot program (http://pbil.univ-lyon1.fr/software/njplot.html). The transmembrane probability of OsGSL5 was predicted with transmembrane hidden Markov model (TMHMM) program (http://www.cbs.dtu.dk/services/TMHMM).

Analysis of rice genes for callose synthases and Rho-type small GTP-binding proteins. The rice genomic sequence was annotated to determine the structure of 10 deduced rice callose synthases (OsGSLs), as shown in Table 1. Gene names for AtGSLs and OsGSLs was largely designated by Somerville (see http://cellwall.stanford.edu/gsl/index.shtml). Annotated rice genes for Rops are also shown in Table 1. Multiple alignments of deduced amino acid sequences of GSL and Rop/Rho

| OsGSL1  | AP001389  | 6    | GSL01f1 | GCTCAAAATTGCTTCACACAAATACATC | 516  | 35 |
| OsGSL2  | AP003223  | 1    | GSL02f1 | GGTTAGGCTTTTCCATGCTGCTTTC    | 449  | 35 |
| OsGSL3  | AP003249  | 1    | GSL03f1 | CCAGTGAGACTGCAATGGAATAGCAG   | 526  | 35 |
| OsGSL4  | AP003445  | 1    | GSL04f1 | CATATAGTGGAGATGAAAGACAGAGG   | 629  | 35 |
| OsGSL5  | AP003454  | 6    | GSL05f1 | GAGCGAGGGTTGGATTTGAGCTGCTT   | 682  | 35 |
| OsGSL6  | AP004685  | 6    | GSL06f1 | ACTGGACAGAACACTAGCC           | 1,243| 35 |
| OsGSL7  | AP004082  | 2    | GSL07f1 | CAGGGAGGCCTGGAAAAAGGAGG       | 613  | 35 |
| OsGSL8  | AC118980  | 3    | GSL08f1 | CATGCCGCTGCTTGGTTGCC          | 468  | 35 |
| OsGSL9  | AP004347  | 1    | GSL09f1 | GACATTAGTGCAGTAACTCCTAGC      | 518  | 35 |
| OsGSL10 | AC104427  | 3    | GSL10f1 | CCACCATGAAATACGAGGGACAAAAGG   | 538  | 35 |
| OsGSLmt | BA000029  | mitochondria |  |  |  |  |
proteins from yeast, *Arabidopsis*, and rice were done with ClustalW 1.83. The phylogenetic trees constructed using the NJ (Neighbor-Joining) algorithm and drawn with the NJplot program are shown in Fig. 1 (GSL) and Fig. 2 (Rop/Rho proteins).

**Plant materials and growth conditions.** Rice plants (*Oryza sativa* L. cv. Hayayuki) were grown in a growth chamber, and anthers at the early microspore stage, the uninucleate microspore stage containing tetrads and microspores, were identified as previously described. Chilling treatments were done at 12°C for 120 h (5 d) from the early microspore stage (50 d after sowing; -10 to -7 cm of auricle distance). Anthers, leaves, and roots were collected and immediately frozen in liquid nitrogen.

**RNA preparation.** Total RNA were extracted from frozen samples according to the method of Bekesiova et al. Total RNA was extracted from rice anthers, leaves, and roots.
Semiquantitative RT-PCR analysis of genes. The first strands of the cDNA mixture were generated from 1.0 μg of total RNA and 2.5 μM Oligo d(T)16 primer. Reverse transcription was done for 30 min at 42 °C using ReverTra Ace-α- (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. The resulting cDNA solution was then diluted 10 fold with TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA). The PCR reaction mixture (10.0 μl) contained 1.0 μl of diluted reverse transcribed first strands of cDNA in 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.01 mg/ml gelatin, 1.5 mM MgCl2, 6 μM of each of two primers (shown in Table 1), 200 μM each of dNTP mixture, and 0.04 unit/μl of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) in a total volume of 10 μl. The temperature cycling parameters were as follows: 94 °C for 10 min; 30–35 cycles (Table 1) of 94 °C for 0.5 min, 60 °C for 1 min, 72 °C for 2 min; 72 °C for 10 min. To confirm the uniformity of cDNA synthesis, cDNAs for actin were also amplified. PCR products were separated by electrophoresis in 1.5% agarose gels, stained with ethidium bromide. The template cDNAs are the genomic DNA (e.g., OsGSL1: 2.02 kbp; Act1, rice actin 1 gene: 0.75 kbp) and that from spliced out cDNA (e.g., OsGSL1: 1.27 kbp; Act1: 0.50 kbp). As shown in Fig. 3, constitutive expression of Act1 was confirmed by this RT-PCR experiment. Since the fragments of sizes corresponding to non spliced out genomic DNA were not amplified by any primer sets, we could rule out the possibility of genomic DNA contamination in any of the RNA samples used. RT-PCR was conducted three times, and one series of data is shown in Fig. 3 and Fig. 4. As shown in Fig. 3, expressions of OsRops were overlapping in different rice organs, with some developmentally regulated differences. In particular, OsRop4 was specifically expressed in anthers at the microsporogenesis stage, and expression of OsRac1, OsRac3, OsRop5, and OsRacD in anther at the microspore stage was also detected. As shown in Fig. 4, expression of OsGSL1, OsGSL2, OsGSL4, OsGSL6, OsGSL7, OsGSL8, OsGSL9, and OsGSL10 was detected at all stages in anthers, leaves, and roots in a somewhat constitutive manner. OsGSL5 was specifically expressed in anthers, with moderately higher amounts at the young microspore stage (lane 1) and at the early middle stage (lane 3). OsGSL5 was significantly down-regulated in the anther by 12 °C treatment for 5 d (lane 2). At anthesis, expression of OsGSL5 decreased in anther
Expression of OsGSL3 was not detected in six samples (lanes 1–6).

Sequence analysis of OsGSL5 and its derivative in mtDNA

As shown in Fig. 5, the predicted OsGSL5 gene, which spans a region of chromosome 6 approximately 17 kb in length, comprises 41 exons with 40 introns and is transcribed into a 5.7 kb mRNA. The deduced peptide, with an approximate molecular mass of 218 kD, showed the highest sequence homology with Arabidopsis AtGSL2. Topology analysis of OsGSL5 revealed that, similarly to CalS1 and AtGSL2, it contains 16 predicted transmembrane helices with the N-terminal region and a large central loop, which contains a β-1,3-glucan synthase component, facing the cytoplasm. Thus the overall structure of this gene is very similar to other callose synthases.

Interestingly, a DNA sequence nearly identical to a part of OsGSL5 is found in the rice mitochondrial genome. We named this 3.0 kb mitochondrial DNA OsGSLmt. As shown in Fig. 5, OsGSLmt shows high sequence similarity (93% at the amino acid level, 74% at the nucleotide level) to the genome sequence of OsGSL5 (from the 29th intron to the 38th exon), which encodes the C-terminal half of the large central loop and four transmembrane helices.

Discussion

Callose wall synthesis during microsporogenesis has been shown to be required for pollen fertility. In this study, the rice genomic sequence was annotated to determine the structure of 10 deduced rice callose synthases (OsGSLs). To our knowledge, this is the first report of full annotations and phylogenetic analyses of all callose synthase genes in a monocot plant genome, and this information is fundamental to the understanding of diverse functions of callose synthases in monocot plants. Also, gene expression profiles of callose synthases and Rop proteins in rice anther were investigated.

By phylogenetic analysis of plant GSL sequences, at least four subgroups, each containing rice and Arabidopsis GSL members, were identified (Fig. 1). This suggests that the functional differentiation of plant GSLs occurred before monocot-dicot divergence. In Arabidopsis, 4 of the 12 AtGSL genes, AtGSL1, AtGSL2, AtGSL5, and AtGSL6, have been characterized previously, and AtGSL1, AtGSL2, and AtGSL5 have been shown to play essential role in pollen development. In this research, among 10 OsGSL genes in the rice genome, an anther-specific member was found. The results in Fig. 4 show that OsGSL5 was specifically expressed in anthers, with moderately higher amounts at the young microspore stage and at the early middle stage rather than the anthesis stage, and was notably downregulated by the cooling treatment. By microscopic observation, callose envelopes that surround tetrad cells have been shown to be thicker in barley than in rice, but there is no information regarding the change in callose wall thickness by chilling during early microsporogenesis. Since AtGSL2, an ortholog of OsGSL5, is required for exine formation during microgametogenesis and pollen viability, OsGSL5 might play essential role in callose synthesis during microsporogenesis. Meanwhile, other 8 OsGSL genes, except for OsGSL3, were also expressed in anthers, leaves, and roots. In Arabidopsis, two closely related and linked genes, AtGSL1 and AtGSL5, were expressed in all parts of the unwounded Arabidopsis plant. They are responsible for the formation of the callose wall that separates the microspore of the tetrad. Therefore, other OsGSLs, including OsGSL2, an ortholog of AtGSL1 and AtGSL5, might play a key role in callose synthesis during microsporogenesis. Chilling treatment of rice anther at the microspore release stage perturbs normal pollen development and causes male sterility. Mutations of the AtGSL2 gene resulted in severe sterility because of the degeneration of microspores. Thus it is possible that OsGSL5 is somehow downregulated by chilling temper-
ature during anther development and that consequently microspore development is disturbed.

OsGSL5 encodes a membrane protein of 1,913 amino acids. Similarly to other callose synthases, the transmembrane domains are clustered in two regions separated by a large hydrophilic domain (756 amino acids) that faces the cytoplasm and might play a role in the interaction with other components of the CalS complex. The central loop of OsGSL5 contains the putative catalytic site, which characteristically lacks the D, D, D and QXXRXW motifs implicated in the binding of UDP-glucose and the transfer of the glucosyl group in bacterial and plant CelSs. The catalytic subunit of CalS1 has been shown to interact tightly with UDP-glucose transferase (UGT1) containing the UDP-glucose binding signature. Therefore, OsGSL5 might be responsible for the synthesis of callose required for fertile pollen development in rice.

It is known that up to 13.4% of the mitochondrial genome was derived from nuclear genome in rice. Since OsGSLm1, which is identified as orf241 by Notsu et al., is not transcribed in rice, the transfer of OsGSL5 from nuclear genome to mitochondrial genome appears to confer no selective advantage, like most other transfer events. However, an OsGSL5 sequence flow from nucleus to mitochondrial genome might well uncover further the meanings and mechanisms of genetic fluidity and plasticity during flowering plant evolution.

In Arabidopsis, it has been suggested that CalS1 (AtGSL6) activity is regulated by Rop1 through interaction with UGT. The fully sequenced rice genome has seven Rops. Based on phylogenetic analyses, the monocot Rop family can be subdivided into at least three groups, and the orthology for specific rice and maize Rops have been predicted. OsRacB was expressed highly in anther at the anthesis stage (Fig. 4), which is the same expression pattern as that of rop2 and rop9, maize orthologs of OsRacB. This suggests a functional analogy of OsRacB together with maize rop2 and rop9 with the AtRop1-related group from dicots, which is required for pollen tube growth and polarity. Interestingly, in both rice anther at the microspore stage (Fig. 4) and maize tassel at the meiosis stage, overexpression of rice anther at the chilling stage revealed two genes responsible for the synthesis of callose required for fertile pollen development in rice.

In summary, our data suggest possible roles of distinct members of OsGSLs and OsRops in pollen development and response to chilling temperature. Further experimentation on OsGSL and OsRop genes, including genetic analysis of tagged lines or RNA-mediated gene-specific suppression lines, and/or promoter-GUS assay or histochemical analysis, will be necessary in order to define their functions in rice anther under the chilling response. In order to improve the chilling-tolerance of rice anther at the microspore stage, it should also be useful to execute biochemical analyses regarding the interactions between GSL, UGT, and Rop proteins in callose synthesis, and their involvement in the chilling-tolerance of rice anther.

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References


