Analysis of Genes Expressed in Rice Anthers during the Stage of Maximal Chilling Sensitivity

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Abstract: Rice enters its most chilling-sensitive stage 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 the anther is essential for fertile pollen formation. A cDNA for β-1,3-glucanase was isolated from rice anther and named Osg1. RT-PCR analysis revealed that Osg1 transcripts were present in leaves, roots, and anthers. 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 Arabidopsis AtGSL2, was specifically expressed in anthers, and was notably downregulated by chilling treatment. Rho-type small GTP-binding proteins are molecular switches that control callose synthase activity. Their gene expression profiles in rice anther were also analyzed. The gene expression profile during the microspore development process under chilling stress was revealed using cDNA microarray. Three novel genes whose expression levels were remarkably changed by chilling in rice anther were identified. A new cis element that includes a DNA transposon Castaway sequence was found in the 5' upstream region of two genes which were conspicuously down-regulated by chilling temperatures in rice anther. Analyses of these chilling-responsive genes are not only fundamental to the understanding of mechanisms that underlie chilling injury in rice pollen, but are also essential to the creation of a chilling-tolerant rice plant.

Key Words: Rice, Anther, Pollen, Chilling, Callose, Microarray, DNA transposon.

抄録：イネは小胞子遊離期に冷害感受性が最も高い。この時期に小胞子を覆う主にβ-1,3-グルカニンからなるカロース壁の合成・分解を精密に制御することが稔性花粉の形成に必須である。その分子機構を明らかにするため、イネ胞で発現するβ-1,3-グルカニーゼ遺伝子 Osg1 を単離し、その薬および葉、根での発現を確認した。イネゲノム中の全カロース合成酵素遺伝子の分子系樹と発現の解析を行い、小胞子形成に関与するシロイヌナズナ AtGSL2 のオルソログ（異種間での相同遺伝子）である OsGSL5 の発現が特異的で冷温により顕著に抑制されることを見出した。また、カロース合成酵素活性の調節因子である Rho 型小分子 GTP 結合タンパク遺伝子のイネ植物における発現を解析した。小胞子形成期イネ胞の冷温下での遺伝子発現変動を確認することも重要と考え、cDNA マイクロアレイ解析を行い、3 つの新規イネ胞冷温応答遺伝子を同定するとともに、冷温下のイネ胞で発現が抑制される遺伝子の上流領域に、DNA トランスポゾン Castaway を含む新規なシス因子（転写制御因子タンパク質（トランス因子）が結合して遺伝子の転写発現の調節を行う）を確認した。これら冷温感受性期に発現する遺伝子の解析は、花粉発育障害発生機構の解明、ひいては耐冷性イネ作成に向けた重要な足がかりとなるものである。

キーワード：イネ、胞、花粉、冷温、カロース壁、マイクロアレイ、DNA トランスポゾン、耐冷性

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References
I Introduction

Rice is the staple food of more than half of the world’s population, and about 1 billion households depend upon rice cultivation for employment and their major source of livelihood. Meanwhile, roughly 800 million people in developing nations suffer from hunger. Most of the world population that suffers from hunger and poverty lives in Asia and Africa.

The rice plant originated in the tropics, and is presently cultivated over a wide range, from 50°N (Aiwei, China) to 35°S (New South Wales, Australia), and from areas below sea level to over 2,600 m above sea level (Jumula Valley, Nepal). This wide range of cultivated area raises different kinds of climatic problems in different regions: low and high temperature, flooding, drought, strong wind, and so on. New research findings related to climatic problems are expected to help eliminate hunger and poverty among farmers and others in developing nations.

Compared with other cereal crops such as wheat and barley, rice is much more sensitive to low temperatures, probably as a result of its subtropical genesis. Male sterility induced by chilling temperature (12 to 18 °C) at the boot stage causes a serious loss of grain yield in rice and this chilling injury has been one of the most serious problems for agriculture in northern part of Japan. Using pot-grown rice under phytotron conditions, the most chilling-sensitive stage of rice was determined to be at the onset of microspore release (Hayase et al. 1969). Microscopic observation of developing rice anthers suggested that one possible reason for male sterility following chilling was the dysfunction of anther development. Observed abnormalities included the cessation of anther development, arrest of pollen development, anthers remaining within the flowers after anthesis, and partial or no dehiscence (Satake 1976). Cytological observation revealed a dilatation of tapetal layers and a vigorous augmentation of cytoplasmic organelles such as mitochondria, proplastids, Golgi bodies, and endoplasmic reticulum in chilled rice anthers (Nishiyama 1976). Chilling temperature treatment also affects the physiological status of anthers. Non-reducing sugar content was shown to increase rapidly, whereas acid phosphatase activity decreased in the chilled rice anthers (Nishiyama 1984). Possible involvement of phytohormones, such as gibberellic acid (GA) and auxin in chilling-induced male sterility, has been reported (Nishiyama 1975). However, it is still mostly unknown how chilling temperature induces the molecular phenomena that result in male sterility in rice plants.

Pollen development begins with the division of diploid archespores 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 (Eschrich and Currier 1964). 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 (Stieglitz and Stern 1973). In transgenic tobacco plants in which β-1,3-glucanase is induced prematurely, little fertile pollen is produced (Worrall et al. 1992). Therefore, the timing of β-1,3-glucanase activity is essential for the developing microspores. Beta-1,3-glucanases are hydrolytic enzymes commonly found in seed plant species. They have been referred to as pathogenesis-related (PR) proteins, belonging to the PR-2 family. They hydrolyze the β-1,3-linked glucans, major components of the cell walls of fungi, and synergistically act with chitinase to inhibit fungal growth in vitro (Kim and Hwang 1997). Besides pathogen attack, they have been shown to be induced by abiotic elicitors such as salicylic acid (Linthorst et al. 1990) and methyl jasmonate (Rickauer et al. 1997). Additionally, β-1,3-glucanases have been found to be involved in several physiological and developmental processes such as seed germination (Leubner-Metzger and Meins 2000) and pollen development (Bucciaglia and Smith 1994; Hird et al. 1993). However, little information has been available regarding β-1,3-glucanases that are expressed in the anthers of monocot plants. A novel β-
1,3-glucanase sequence was amplified from rice anther cDNA, and its molecular structure and the expression patterns were characterized. (Chapter II)

Barley’s tolerance to cool temperatures 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 (Koike et al. 2003). Plant callose is produced at specific stages of cell wall development in pollen and in response to pathogen attack and wounding. Twelve 

Gulcan Synthase-Like (GSL) genes have been identified in Arabidopsis (Verma and Hong 2001). Among these, AtGSL5 is developmentally expressed at the highest levels in flowers (Østergaard et al. 2002), and was shown to negatively regulate the salicylic acid (SA) pathway involved in disease resistance (Jacobs et al. 2003; Nishimura et al. 2003), while CalS5 (AtGSL2) is responsible for the synthesis of callose deposited at the primary cell wall of meioocytes, tetrads and microspores, and the expression of this gene is essential for exine formation in pollen wall (Dong et al. 2005). Two closely related and linked genes, AtGSL1 and AtGSL5, have been also shown to play essential but at least partially redundant roles in both sporophyte and pollen development (Enns et al. 2005). 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 complex (Qadota et al. 1996). In Arabidopsis, Rop1, a plant Rho-like protein, may act as a molecular switch that controls CalS1 (AtGSL6) activity at the forming cell plate through the interaction with UDP-glucose transferase (UGT) (Hong et al. 2001). 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 (Zheng and Yang 2000). In order to decipher the mechanisms of callose synthesis in rice pollen formation and its relationships with tolerance to cool temperatures, gene expression profiles of callose synthases and Rops that might be related to callose synthesis in rice anther were analyzed. A schematic diagram outlining callose synthesis and degradation in microspore development under normal or chilling temperature is shown in Fig. 1. (Chapter III)

The mechanisms of chilling tolerance in plant seedlings have long been intensively studied with a focus on membrane structure and function (Nishida and Murata 1996). Chilling tolerance was enhanced in transgenic tobacco into which a gene for glycerol-3-phosphate acyl transferases or chloroplastic fatty acid desaturases from Arabidopsis was introduced (Kodama et al. 1994; Murata et al. 1992). Another mechanism involves cellular defense against membrane lipid peroxidation caused by a chilling-induced increase in the generation of reactive oxygen species such as superoxide, hydrogen peroxide, and hydroxyl radicals (Prasad et al. 1994).

![Fig. 1 A schematic diagram outlining callose synthesis and degradation in microspore development under normal or chilling temperature](image-url)
In view of gene expression, cis-acting regulatory elements are important molecular switches of a dynamic network controlling various biological processes, including abiotic stress responses, hormone responses, and developmental processes. In particular, understanding the regulatory gene networks involved in stress response cascades depends on successful functional analyses of cis-acting elements. Expression profiling has led to the identification of various combinations of cis-acting elements in the promoter regions of stress-inducible genes involved in stress and hormone responses. Above all, major cis-acting elements that are a vital part of abscisic acid (ABA)-dependent and ABA-independent gene expression in osmotic and cold stress responses, such as the ABA-responsive element (ABRE) and the dehydration-responsive element/C-repeat (DRE/CRT), have been well described (Yamaguchi-Shinozaki and Shinozaki 2005).

To date, very little information is available regarding the molecular aspects of chilling damage in rice microspore development, and the anther genes which participate in this process have not been identified. Recently, the development of microarray technology has provided a potent tool for the nonexclusive analysis of gene expression (Schena et al. 1995). Several reports using microarray analysis in plants have been published (Akimoto-Tomiyama et al. 2003; Negishi et al. 2002; Petersen et al. 2000; Schaffer et al. 2001; Seki et al. 2001). A cDNA microarray containing 8,987 rice-expressed sequence tag (EST) clones was used to analyze the gene expression profile in rice anther at its early microspore stage under chilling. Three novel genes whose expression levels were conspicuously varied by chilling in rice anther have been identified. In particular, two genes have miniature inverted-repeat-transposable element (MITE) Castaway sequences at nearly the same position in the 5’ upstream region. Their molecular characteristics and expression patterns are discussed. (Chapter IV)

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II Molecular cloning and characterization of a novel β-1,3-glucanase gene from rice

1 Introduction

Beta-1, 3-glucanases are hydrolytic enzymes commonly found in seed plant species. They have been referred to as PR proteins, belonging to the PR-2 family. They hydrolyze the β-1,3-linked glucans, major components of the cell walls of fungi, and synergistically act with chitinase to inhibit fungal growth in vitro (Kim and Hwang 1997). Besides pathogen attack, they have been shown to be induced by abiotic elicitors such as SA (Linthorst et al. 1990) and methyl jasmonate (Rickauer et al. 1997). Additionally, β-1,3-glucanases have been found to be involved in several physiological and developmental processes such as seed germination (Leubner-Metzger and Meins 2000) and pollen development (Bucciaglia and Smith 1994; Hird et al. 1993).

Based on amino acid similarities, tobacco β-1,3-glucanases have been classified into three structural classes (Payne et al. 1990). Monocotyledonous β-glucanases together with nine reported rice glucanases (gns) have been classified into four subfamilies based on the structure and function of the genes, which are comprised of the β-1,3-gns subfamily A, the β-1,3,1,4-gns subfamily B, and β-gns subfamilies C and D with structures distinct from other subfamilies (Romero et al. 1998).

In this study, a novel β-1,3-glucanase sequence was amplified from rice anther cDNA, and its structure and expression pattern were characterized.

2 Materials and Methods

Rice plants (Oryza sativa L. cultivar (cv.) Hayayuki) were grown under greenhouse conditions. Anthers at the microspore stage, leaves, and roots were collected and immediately frozen in liquid nitrogen. Genomic DNA was extracted from the frozen leaves and roots using cetyltrimethylammonium bromide (CTAB) (Doyle and Doyle 1990). Total RNA was extracted from frozen samples by the CTAB method (Bekesiova et al. 1999). Poly(A)+ RNA was extracted from them using Oligotex-dT30 Super (Roche) according to the manufacturer’s instructions.

Double-stranded cDNA with EcoRI-NotI-BamHI adaptors (Takara Shuzo) was synthesized from 5 μg of poly(A)+ RNA using a cDNA Synthesis System Plus (Amersham) according to the manufacturer’s instructions. DNA fragments were cloned into pBluescript II SK+. DNA sequencing was done by the dideoxy chain-termination method using ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an automated DNA sequencer (Applied Biosystems; Model 373S). Sequence similarity searches were done in the Genbank, EMBL, Swiss Prot, and PIR Databases. Phylogenetic trees were analyzed using a multiple alignment program ClustalW version 1.7 (Thompson et al. 1994).

A cDNA library was screened using an RT-PCR fragment (g28) which had similarity with several β-1,3-glucanases,
and a positive lambda clone (G4A8) was obtained from about 500,000 recombinant plaques. This cDNA clone, 979 bp in length, lacked a 5'-end sequence. In order to isolate the complete rice anther glucanase gene, the G4A8 clone was used as a hybridization probe to screen a genomic DNA library constructed in λEMBL3 from MboI-partially digested fragments of DNA isolated from the rice (cv. Hayayuki) leaves. From the positive clone H2a4, a 2.3-kb SalI-Sall fragment was obtained that contained the full-length glucanase gene Osg1 (DDBJ Accession No. AB070742).

The cDNA clone (G4A8) was labeled with DIG-dUTP, the 5'-primer (5'-CATGGCATGAAGTCGTTCAGCTA-3') and 3'-primer (5'-CAACTATTCTGACGGTTGAGCCA-3') using a PCR DIG Probe Synthesis Kit (Roche), and used as a probe. Twenty μg each of genomic DNA was digested with either BamHI, EcoRI, or HindIII. The DNA fragments were electrophoresed in a 0.7% agarose gel, and the Southern blots were prepared with a nylon membrane (Hybond-N+; Amersham) according to the manufacturer’s instructions. Preliminary hybridization was done in hybridization buffer containing 0.5 M Na phosphate (pH 7.2), 1 mM EDTA, 7% SDS at 65°C for 3 hours. The filter was hybridized at 65°C for 16 hours in the same solution with 20 ng of labeled probes per 1 ml of hybridization solution, and washed three times in 40 mM Na phosphate (pH 7.2), 1% SDS at 65°C for 20 min. The hybridized probes were immunodetected with anti-DIG alkaline phosphatase conjugate and made visible with CSPD® and X-ray film as described in the instruction manual (Roche).

The first strand of the cDNA mixture was generated from 0.5 μg of total RNA and 2.5 μM random hexamer. The reverse transcription was done for 60 min at 37°C using a GeneAmp RNA PCR Kit (PE Applied Biosystems) according to the manufacturer’s instructions. The PCR reaction mixture (10 μl) contained 1.0 μl of 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 each of specific primers (Osg1r0: 5'-GTGATCCAGCCCTACTGCTG-3'; Act1f02: 5'-CGCAGTCCAAGGGGTACTC-3'; Act1r03: 5'-TCCTGGTCATAGTCCAGGGC-3'), 200 μM each dNTP mixture and 0.04 unit/μl of AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR products were separated by electrophoresis in 1.5% agarose gels, and transferred onto nylon membranes. Hybridization was performed using the method described above for the genomic Southern hybridization.

3 Results and Discussion

Fig. 2 shows a 2.3-kb contiguous sequence of the genomic clone H2a4, which includes 869 bp upstream of the coding region. Within the coding region, the genomic clone contains one small intron, the position of which is identical to that of the other cereal β,1,3-glucanase genes. Exon 1 encodes the majority of the signal peptide, while exon 2 encodes the last 3 aa of the signal peptide and all of the mature peptide. This N-terminal signal peptide directs the nascent polypeptide into the endoplasmic reticulum, and the mature protein is finally secreted into the apoplast. The mature peptide consists of 309 aa with a calculated molecular mass of 33765 and an isoelectric point of 5.42. A putative TATA box occurs at 729-735 bp (142 bp upstream of the ATG initiation codon).

Genomic DNA isolated from leaves was probed with a 0.65-kb fragment containing part of the coding region of the Osg1 gene. When the genomic DNA was digested with either BamHI, EcoRI, or HindIII, the probe hybridized to a single 10-kb, 9.1-kb, and 4.3-kb band, respectively (data not shown). Therefore, Osg1 existed as a single copy gene in the rice genome.

Screening the databases with the Osg1 protein sequence found some similarity of Osg1 to other plant β-glucanases. Conservation of residues potentially important in the structure and function of glucanases, such as the D (aspartate), E (glutamate), Y (tyrosine), and W (tryptophan) in Osg1 (Fig. 2) strongly suggests that Osg1 may function as either a β-
1,3-glucanase or a β-1,3,1,4-glucanase. Furthermore, the phylogenetic tree of the deduced Osg1 protein with several plant endo-β-1,3-glucanases (de Loose et al. 1988; Ori et al. 1990), a yeast endo-β-1,3-glucanase (Mrsa et al. 1993), and a monocot endo-β-1,3,1,4-glucanase (Simmons et al. 1992) (Fig. 3) shows that Osg1 belongs to the monocotyledon subfamily A, which is comprised of defense-related endo-β-1,3-glucanases and is the largest glucanase subfamily in monocotyledons. This subfamily was defined based on gene dendrogram analysis (Simmons 1994) and the substrate specificity of the encoded isozymes (Hrmova and Fincher 1993). These isozymes hydrolyze 1,3-β-glucan linkages, but differ greatly in their tolerance or requirements for 1,6-β-glucan branch linkages nearby on the polymer chain. Polyomers of 1,3;1,6-β-glucans are found only in fungi, but polymers of 1,3-β-glucans are found in both fungi and plants. Therefore, isozymes capable of hydrolyzing 1,3-β-glucans might function in plant development in addition to plant defense. So far, two isozymes, OsGns4 and OsGns5 of the rice subfamily A, have been characterized. OsGns4, which was first purified from rice bran, is basic and adapted for hydrolysis of 1,3-β-glucans and may be a defense-related isozyme (Akiyama et al. 1996). On the other hand, OsGns5, which was also purified from rice bran, is acidic and has much greater activity against linear-1,3-β-glucans than does the OsGns4 isozyme (Akiyama et al. 1997). Osg1 is most similar to OsGn5 in amino acid sequence.

**Fig. 2** Nucleotide and deduced amino acid sequence of the Osg1 gene. The sequence includes an 869-bp upstream region and one short intron which is depicted by small letters. A putative TATA box is underlined. Bold letters indicate residues conserved among several glucanases.

```plaintext
ACGAGTGCTGCGACGTCTTGGGAGGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGTGCTTGGGGAGAGAGCTCATGCTGCTAATGGGT
For RT-PCR expression analysis, two sets of fixed oligonucleotide primers were designed to cover an intron between them in order to distinguish spliced out cDNA (Osg1: 0.40 kbp; Act1: 0.50 kbp) from genomic DNA (Osg1: 0.78 kbp; Act1 (rice actin 1 gene): 0.75 kbp). As shown in Fig. 4, constitutive expression of Osg1 (Zhang et al. 1991) was confirmed by this RT-PCR experiment, except for anthers at anthesis, which have a high content of actin filaments in germinating pollens. Osg1 transcripts were present in leaves, roots, and anthers.
III Expression analysis of genes for callose synthases and Rho-type small GTP-binding proteins that are related to callose synthesis in rice anther

1 Introduction

In the northern part of Japan, rice crop production is occasionally severely damaged 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 (Hayase et al. 1969).

Pollen development begins with the division of diploid archespores 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 (Eschrich and Currier 1964). 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 (Stieglitz and Stern 1973). In transgenic tobacco plants in which β-1,3-glucanase is induced prematurely, little fertile pollen is produced (Worrall et al. 1992). 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 (Yamaguchi et al. 2002), and the role of this 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 (Yamaguchi et al. 2004). Particularly, two genes had miniature inverted-repeat transposable element (MITE) Castaway sequences at nearly the same position in the 5' upstream region.

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 (Yamaguchi et al. 2004). 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 (Koike et al. 2003). No information is available regarding the relationship between the thickness of callose envelopes and chilling tolerance. In order to analyze this relationship, the creation of transgenic rice plants was considered, in which the thickness of callose envelope is manipulated. Initially, it was planned that genes related to callose synthesis in rice anthers be identified, and be utilized. 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 (Verma and Hong 2001). Among these, AtGSL5 is developmentally expressed at highest levels in flowers (Ostergaard et al. 2002), and has been shown to negatively regulate the SA pathway involved in disease resistance (Jacobs et al. 2003; Nishimura et al. 2003). CalS5 (AtGSL2) is responsible for the synthesis of callose deposited at the primary cell wall of meioocytes, tetrads, and microspores, and the expression of this gene is essential for exine formation in the pollen wall (Dong et al. 2005). 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 (Enns et al. 2005). 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 complex (Qadota et al. 1996). 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 the interaction with UGT (UDP-glucose transferase) (Hong et al. 2001). A plant-specific family of Rho homologs, known as the Rop family, plays important roles in plant development, including pollen tube and root hair...
growth, cell wall synthesis, and cell proliferation in the meristem (Zheng and Yang 2000). In the present study, in order to decipher the mechanisms of callose synthesis in rice pollen formation and its relationship with cool tolerance, the gene expression profiles of callose synthases and Rops that might be related to callose synthesis in rice anther were analyzed.

2 Materials and Methods

1) 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 (Thompson et al. 1994). A phylogenetic tree was constructed using the NJ (Neighbor-Joining) algorithm and a dendrogram was drawn using the NJplot program (http://phil.univ-lyon1.fr/software/njplot.html). The transmembrane probability of OsGSL5 was predicted by means of the transmembrane hidden Markov model (TMHMM) program (http://www.cbs.dtu.dk/services/TMHMM/).

2) 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 (Satake and Hayase 1970). Chilling treatments were performed 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.

3) RNA preparation

Total RNA were extracted from frozen samples according to the method of Bekesiova et al. (Bekesiova et al. 1999). Total RNA was extracted from rice anthers, leaves, and roots.

4) 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 performed for 30 min at 42 °C using ReverTra Ace -α- (Toyobo) 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) 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, and visualized with the BioDoc-It System (UVP).
Table 1  OsGSLs and OsRops in rice genome and primers used in semiquantitative RT-PCR

<table>
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<th>GenBank accession no.</th>
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3 Results

1) 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. The gene names for AtGSLs and OsGSLs were largely designated by Somerville (see http://cell-wall.stanford.edu/gsl/index.shtml). Annotated rice genes for Rops are also shown in Table 1. Multiple alignments of the deduced amino acid sequences of GSL and Rop/Rho proteins from yeast, Arabidopsis, and rice were performed using ClustalW 1.83. The phylogenetic trees constructed using the NJ algorithm and drawn using the NJplot program are shown in Fig. 5 (GSL) and Fig. 6 (Rop/Rho proteins).
Fig. 5  Phylogenetic tree of glucan synthases.
Multiple alignment of deduced callose synthase (GSL, Glucan synthase-like) amino acid sequences were performed using ClustalW 1.83. Phylogenetic trees were constructed using the NJ algorithm, and drawn with the NJplot program. Abbreviations for species are Arabidopsis thaliana (At), Oryza sativa (Os), and Saccharomyces cerevisiae (Sc). Scale bar, 0.05 substitutions per site.

Fig. 6  Phylogenetic tree of Rop proteins.
Multiple alignment of deduced Rop amino acid sequences were performed using ClustalW 1.83, and phylogenetic trees were constructed using the NJ algorithm and drawn with the NJplot program. Scale bar, 0.05 substitutions per site.
2) Expression analysis of rice genes for Rho-type small GTP-binding protein and GSLs

In order to study the gene expression profile of Rops and GSLs during pollen development in rice, semiquantitative RT-PCR analyses were executed. For RT-PCR expression analysis, I used sets of paralog-specific oligonucleotide primers. Some of the primer sets were designed to put an intron between them in order to distinguish between the amplification from genomic DNA (e.g., \textit{OsGSL1}: 2.02 kbp; \textit{Act1}, rice actin 1 gene: 0.75 kbp) and that from spliced-out cDNA (e.g., \textit{OsGSL1}: 1.27 kbp; \textit{Act1}: 0.50 kbp). As shown in Fig. 7, constitutive expression of \textit{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, I could rule out the possibility of genomic DNA contamination in all of the RNA samples used. RT-PCR was conducted three times, and one series of data is shown in Fig. 7 and Fig. 8. As shown in Fig. 7, expressions of \textit{OsRops} overlapped in different rice organs, with some developmentally regulated differences. In particular, \textit{OsRop4} was specifically expressed in anthers at the microsporogenesis stage, and expression of \textit{OsRac1}, \textit{OsRac3}, \textit{OsRop5}, and \textit{OsRacD} in anthers at the microspore stage was also detected. As shown in Fig. 8, expression of \textit{OsGSL1}, \textit{OsGSL2}, \textit{OsGSL4}, \textit{OsGSL6}, \textit{OsGSL7}, \textit{OsGSL8}, \textit{OsGSL9}, and \textit{OsGSL10} was detected at all stages of anthers, leaves, and roots in a somewhat constitutive manner. \textit{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). \textit{OsGSL5} was significantly down-regulated in the anther by 12 °C treatment for 5 d (lane 2). At anthesis, expression of \textit{OsGSL5} decreased in anther (lane 4). Expression of \textit{OsGSL3} was not detected in six samples (lanes 1-6).

![Fig. 7 RT-PCR analysis of rice Rop genes.](image)

The first strands of the cDNA mixture were generated from total RNA. The PCR products were electrophoresed in agarose gel and visualized using ethidium bromide. The template cDNAs are the anthers at meiosis (lane 1), the anthers at microspore middle stage, just after chilling at 12 °C for 5 d (stages moved from the meiosis stage to the microspore middle stage during chilling, lane 2), the anthers at the microspore middle stage (lane 3), anthers at anthesis (lane 4), roots (lane 5), and leaves (lane 6).

![Fig. 8 Semiquantitative RT-PCR analysis of rice Glucan Synthase Like genes.](image)

The first strands of the cDNA mixture were generated from total RNA. The PCR products were electrophoresed in agarose gel and visualized using ethidium bromide. The template cDNAs are the anthers at meiosis (lane 1), the anthers at microspore middle stage, just after chilling at 12 °C for 5 d (stages moved from the meiosis stage to the microspore middle stage during chilling, lane 2), the anthers at the microspore middle stage (lane 3), anthers at anthesis (lane 4), roots (lane 5), and leaves (lane 6).
3) Sequence analysis of OsGSL5 and its derivative in mtDNA

As shown in Fig. 9, 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 kDa, 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 \( \alpha \) \( \beta \),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. In the present study, this 3.0 kb mitochondrial DNA was named OsGSLmt. As shown in Fig. 9, 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.

4 Discussion

Callose wall synthesis during microsporogenesis has been shown to be required for pollen fertility (Dong et al. 2005). In this study, the rice genomic sequence was annotated to determine the structure of 10 deduced rice callose synthases (OsGSLs). To my 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. 5). 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 roles in pollen development. In the present study, among 10 OsGSL genes in the rice genome, an anther-specific member was found. The results shown in Fig. 8 demonstrate 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 (Koike et al. 2003), but there is no information regarding the change in callose wall thickness due to chilling during early microsporogenesis. Since AtGSL2, an ortholog of OsGSL5, is required for exine formation during micromegatogenesis and pollen viability (Dong et al. 2005), OsGSL5 might play essential role in callose synthesis during microsporogenesis. Meanwhile, another 8 OsGSLs, 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 (Enns et al. 2005). Therefore, other OsGSLs, including OsGSL2, an ortholog of AtGSL1 and AtGSL5, might play key roles 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 (Dong et al. 2005). Thus it is possible that OsGSL5 is somehow downregulated by chilling temperatures during anther development and that microspore development is consequently 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 callose synthase (CalS) complex. The central loop of OsGSL5 contains the putative catalytic site, which characteristically lacks the D, D, D and QXXRW motifs implicated in the binding of UDP-glucose and the transfer of the glucosyl group in bacterial and plant cellulose synthases (Verma and Hong 2001). The catalytic subunit of CalS1 has been shown to interact tightly with UDP-glucose transferase (UGT1) containing the UDP-glucose binding signature (Hong et al. 2001). 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 (Notsu et al. 2002). Since OsGSLmt, which has been identified as orf241 by Notsu et al., is not transcribed in rice (Notsu et al. 2002), 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 further uncover 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 (Hong et al. 2001). 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 (Christensen et al. 2003). OsRacB was expressed highly in anther at the anthesis stage (Fig. 8), which is the same expression pattern as that of rop2 and rop9, maize orthologs of OsRacB (Christensen et al. 2003). 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. 8) and maize tassel at the meiosis stage (Christensen et al. 2003), overlapping Rops expression patterns were observed. Based on these observations, it was hypothesized that the rice Rops, such as OsRac1, OsRac3, OsRop4, OsRop5, and OsRacB belonging to multiple gene clusters, can cooperatively regulate the activity of callose synthesis at the microsporogenesis stage as members of the CalS complex. Recently, specific RNA silencing of each of the seven OsRac members was reported (Miki et al. 2005). Using this useful method, the functional importance of each OsRop and OsGSL member in callose synthesis during microsporogenesis and its chilling response will probably be clarified.

In summary, these data suggest possible roles of distinct members of OsGSLs and OsRops in pollen development and response to chilling temperatures. Further experimentation regarding 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 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.

IV cDNA microarray analysis of rice anther genes under chilling stress at the microsporogenesis stage revealed two genes with DNA transposon Castaway in the 5'-flanking region

1 Introduction

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 was determined to be at the onset of microspore release (Hayase et al. 1969). In effect, chilling treatment at this stage causes tapetum hypertrophy and disordered microspore development, and consequently gives rise to a high degree of male sterility (Nishiyama 1970).

Pollen development begins with the division of diploid archespores in the anther, giving rise to microsporocytes and tapetal cells. The tapetum forms a single layer of cells around the anther locule and provides nutrients and enzymes for microspore development. The microsporocytes produce a wall of callose composed primarily of β-1,3-glucan, and undergo meiosis (Eschrich and Currier 1964). 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 (Stiegitz and Stern 1973). In transgenic tobacco plants in which β-1,3-glucanase is induced prematurely, little fertile pollen is produced (Worrall et al. 1992). Therefore, the timing of β-1,3-glucanase activity is essential for the developing microspores. A β-1,3-glucanase gene was isolated from rice anther at the microspore stage (Yamaguchi et al. 2002), and the role of gene expression in chilling injury is now under investigation.

Mechanisms of chilling tolerance in plant seedlings have long been studied intensively with a focus on membrane structure and function (Nishida and Murata 1996). Chilling tolerance was enhanced in transgenic tobacco into which a gene for glycerol-3-phosphate acyl transferases or chloroplastic fatty acid desaturases from Arabidopsis was introduced (Kodama et al. 1994; Murata et al. 1992). Another mechanism involves cellular defense against membrane lipid peroxidation caused by a chill-induced increase in the generation of reactive oxygen species such as superoxide, hydrogen peroxide, and hydroxyl radicals (Prasad et al. 1994).

So far, very little information has been revealed regarding the molecular aspects of chilling damage in rice microspore development, and the anther genes which participate in this process have not been identified. Recently, the development of microarray technology has provided a potent tool for the nonexclusive analysis of gene expression (Schena et al. 1995). Several reports using microarray analysis in plants have been published (Akimoto-Tomiyama et al. 2003; Negishii et al. 2002; Petersen et al. 2000; Schaffer et al. 2001; Seki et al. 2001). In the present study, a cDNA microarray containing 8,987 rice EST clones was used to analyze the gene expression profile in rice anther at its early microspore stage under chilling. 160 ESTs were up- or down-regulated by chilling in rice anther. Three novel genes whose expression levels were conspicuously varied by chilling in rice anther were identified. In particular, two genes have MITE Castaway sequences at nearly the same position in the 5’ upstream region. Their molecular characteristics and expression patterns are discussed.
2 Materials and Methods

1) Plant materials and growth conditions

Rice plants (*Oryza sativa* L. cv. Hayayuki) were grown in 200 cm$^2$ Wagner pots filled with paddy field soil, to which 0.9 g each of nitrogen, phosphorus, and potassium, 0.3 g each of magnesium and calcium, and traces of iron and manganese were added. A growth chamber (12 h light (300 μE/m$^2$/s) at 26 °C; 12 h dark at 20 °C, relative humidity 75%) was used. Rice anthers at the early microspore stage, *i.e.*, the uninucleate microspore stage containing tetrads and microspores, were identified as previously described (Satake and Hayase 1970). 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 were collected and frozen immediately in liquid nitrogen.

2) RNA preparation

Total RNA was extracted from frozen samples according to the method of Bekesiova *et al.* (Bekesiova *et al.* 1999). Total RNA was extracted from rice anthers and 40 μg was used for each microarray analysis.

3) Microarray preparation

Microarray analysis was performed basically as previously described (Yazaki *et al.* 2000). The rice cDNA microarrays were prepared on aluminum-coated and DMSO-optimized glass slides. The sequences used in the construction were generated by PCR. The PCR products were purified by QIAquick 96-column (QIAGEN). DNA solutions were arrayed robotically using a Generation III ArraySpotter (Amersham Pharmacia).

4) Fluorescent labeling of probes

Isolated total RNA was reverse-transcribed with Cy5dCTP (Amersham Pharmacia). Reactions were incubated for 2.5 h at 42 °C with 80 μg of total RNA, oligo-(dT)25, random nonamer, control RNA, 1 × SuperScript II reaction buffer, 10 mM DTT, 2 mM dATP, 2 mM dTTP, 2 mM dGTP, 1 mM dCTP, 1 mM Cy5dCTP, and SuperScript II reverse transcriptase. The reactions were denatured at 94 °C for 3 min and the RNA was degraded by incubation with 4 μl of 2.5 N NaOH at 37 °C for 15 min. Following degradation, the mixture was neutralized with 20 μl of 2 M HEPES buffer. The labeled probes were purified using a QIAquick PCR Purification Kit (QIAGEN) and dried using a vacuum concentrator. The dried probes were resuspended in 9 μl of water and denatured at 95 °C for 4 min, and 6 μl of Oligo A80 (1 mg ml$^{-1}$) and 45 μl of ExpressHyb (Clontech) were added to the resuspended probe.

5) Hybridization on microarrays and analysis

Glass slides were incubated with a final volume of 30 μl of probe at 55 °C for 5 h in the dark. After hybridization, the glass slides were washed in 1 × SSC/0.2% SDS for 10 min at 55 °C in the dark, then in 0.1 × SSC/0.2% SDS for 10 min at 55 °C twice in the dark, and finally in 0.1 × SSC for 1 min at room temperature twice. After the final wash, the slides were briefly rinsed with distilled water and air-dried. The hybridized and washed microarrays were scanned using an Array Scanner Generation III (Amersham Pharmacia). ArrayGauge (FujiFilm) was used for image analysis.

6) 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) according to the manufacturer’s instructions. The PCR reaction mixture (10 μl) contained 0.1 μl of 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 MgCl$\_2$, 6 μM each of two primers (1904f: 5’-GTTCCATGTACGAAGCTCCC-3’ and 1904r: 5’-CACAAAGACGGGCGCATCAG-3’ for amplifying *SAMDC1*; 314f3: 5’-AGATATGGCACGTCGGCAGG-3’ and 314r3: 5’-CGTTGGCGCTGTCCTTCATG-3’ for amplifying *OPDARI*; *Sal* Tfl: 5’-GACGCTTGTGAAGTTGGCC-3’ and *Sal* Tr1: 5’-GGCCATGGTCCAGAAAATC-3’ for amplifying...
OsSalT; Act1f02: 5’-CGCAGTCCAAGAGGGGTATC-3’ and Act1r03: 5’-TCCTGGTCATAGTCCAGGC-3’ for amplifying rice actin 1; 4923f8: 5’-GAGAACACGCTCATCCACAG-3’ and 4923r: 5’-TGCTAGCAGCAGCTTGCTC-3’ for amplifying Radc1), 200 μM each of dNTP mixture, and 0.04 unit/μl of AmpliTaq Gold DNA polymerase (Applied Biosystems). Thirty-five cycles of PCR (94 °C for 0.5 min, 60 °C for 1 min, 72 °C for 2 min) were carried out. PCR products were separated by electrophoresis in 1.5% agarose gels, stained with ethidium bromide and visualized using the BioDoc-It System (UVP).

3 Results

1) Identification of chilling-regulated genes using the cDNA microarray

To analyze the gene expression profile during chilling stress in rice anther at the early microspore stage, a microarray containing 8,987 cDNAs of rice EST was used. As a total of 37,544 non-transposable-element-related protein-coding genes were identified (International Rice Genome Sequencing Project 2005), about one-fourth was contained in this array. Total RNA was isolated from the rice anthers at the tetrad stage and the middle microspore stage with or without chilling treatment during the tetrad stage, and fluorescence-labeled by reverse transcription before hybridization. The signal intensities from labeled targets derived from chilled and unchilled anthers were compared. On one slide, each cDNA clone was spotted in duplicate and the experiment was repeated twice. For each experiment, χ values were calculated by common logarithmic transformation of each signal intensity. Subsequently, Z scores were calculated to normalize the sample values to account for variations in RNA labeling, according to the formula $Z = (χ - μ) / δ$, where μ and δ are the mean and standard deviation of about 4500 χ values which are spotted on half of one slide, respectively. Finally, for each EST, two Z scores of duplicate spots on a glass slide were averaged. EST was considered to be chilling-inducible or chilling-repressible if the difference of averaged Z score (unchilled Z score - chilled Z score) was below -1.0 or above 1.0, respectively. Approximately, a difference of averaged Z scores of 1.0 corresponds to a two-fold difference in original signal intensity. As many as 160 ESTs were identified as being potentially responsive to chilling stress in rice anther at the early microspore stage, as they were found reproducibly in 2 experiments. Of these genes, the expression of 38 (24%) was up-regulated and that of 122 (76%) was down-regulated. These 160 ESTs were classified according to their putative functions based on EST descriptions and a protein database search by BLAST algorithm (annotations with similarity scores greater than 40 bits) (Table 2). About 36% of the chilling-responsive ESTs are of unknown function. Most of the chilling-responsive ESTs related to primary metabolism, signal transduction, defense, proteolysis including proteases, and secondary metabolism including 12-oxo-phytodienoic acid reductases (OPDARs) showed down-regulation. In contrast, genes related to translation, such as ribosomal protein, showed up-regulation in chilled anthers (Table 2).
### Table 2 Complete list of genes that are significantly up- or down-regulated by chilling stress in rice anther

<table>
<thead>
<tr>
<th>Clone</th>
<th>EST accession</th>
<th>Putative ID</th>
<th>difference of averaged Z scores</th>
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Continued on next page
Yamaguchi: Chilling-Responsive Gene Expression in Rice Anthers

2) Analysis of rice OPDARs and SAMDCs

Among the 8,978 rice ESTs on the microarray, 5 ESTs are annotated to encode OPDARs and 4 of them were downregulated by chilling stress in rice. There are at least 3 copies of OPDAR in the rice genome. The sequence of the most chilling-responsive clone (accession number C26097) was almost identical to the nucleotide sequence of an ORF in AP003525, which is derived from chromosome 6 at 32.7 cM. This gene was designated OPDAR1 (DDBJ accession No. AB122088). Two chilling-responsive clones (accession numbers C96685 and AU093231) also encoded OPDAR1.
One chilling-responsive EST clone (accession number AU057040) and one non-chilling-responsive EST clone (accession number D24670) encoded another copy of OPDAR on chromosome 6. These EST clones have up to 88% identities to OPDAR1, but the length of the responsive one is about 0.8 kb and that of the non-chilling-responsive one is about 0.5 kb. Among the 8,987 rice ESTs on the microarray, 5 ESTs were annotated to encode S-adenosylmethionine decarboxylases (SAMDCs) and 3 of them were up-regulated by chilling stress in rice anther. There are at least 3 copies of SAMDC in the rice genome. The sequence of the most chilling-responsive clone (accession number C28220) was almost identical to the nucleotide sequence of an ORF in AP005420 which is derived from chromosome 9. This gene was designated SAMDC1 (DDBJ accession No. AB122089). One chilling-responsive clone (accession number AU062686) also encoded SAMDC1. One chilling-responsive EST clone (accession number C26568) encoded another copy of SAMDC on chromosome 4. It has up to 89% identity to SAMDC1. Two non chilling-responsive EST clones (accession numbers AU100691 and AU056203) encoded yet another copy of SAMDC on chromosome 2, and has up to 85% identity to SAMDC1.

3) Sequence analysis of Radc1

Among the 8,987 rice ESTs on the microarray, 2 ESTs (accession numbers AU172968 and AU174285) with almost identical nucleotide sequences were remarkably down-regulated by chilling stress in rice anther. Their sequences were almost identical to the nucleotide sequence of an ORF in AC125471 which is derived from chromosome 3. This gene was designated Radc1 (rice anther down-regulated by chilling 1; DDBJ accession No. AB122090). The Radc1 amino acid sequence has an aspartic protease motif which shows 29/48% identity/similarity at the amino acid level with the tobacco chloroplast DNA-binding protein CND41, but lacks the N-terminal Lys-rich helix-turn-helix motif which is essential for DNA binding in CND41 (Fig. 10). In the upstream region of the Radc1 gene, Castaway, a kind of MITE sequence exists (Fig. 11). Castaway also exists in the upstream region of the high-salt- and drought-inducible OsSalT gene.

---

**Fig. 10** Domain organization of Radc1 and CND41.

The dotted box, the shaded box, and the black box represent transit peptide, Lys-rich helix-turn-helix motif, and active site aspartic acid residue, respectively.

**Fig. 11** Comparison of Radc1 and OsSalT genes.

The shaded box and the black box represent Castaway and Stowaway, respectively. The hatched boxes in Castaway represent ABA-responsive elements (TACGTGGC).
gene (Fig. 11) (Claes et al. 1990). OsSalT has another MITE sequence, Stowaway, in the upstream region (Fig. 11) (Bureau et al. 1996). Castaway found in Rade1 shows 84% sequence identity with Castaway found in OsSalT (Fig. 11).

4) Expression analysis of selected genes

To verify and confirm the microarray data, the expression of 5 ESTs, on which I focused for more detailed analysis, was examined by RT-PCR analysis. These 5 ESTs included up- and down-regulated genes and a non-responsive gene in the microarray experiment. For RT-PCR expression analysis, I used 4 sets of fixed oligonucleotide primers that are designed to put an intron between forward and reverse primers in order to distinguish between the amplification from genomic DNA (SAMDC1: 0.49 kbp; OPDAR1: 0.39 kbp; OsSalT: 0.35 kbp; Act1 (rice actin 1 gene): 0.75 kbp) and that from spliced-out cDNA (SAMDC1: 0.39 kbp; OPDAR1: 0.27 kbp; OsSalT: 0.25 kbp; Act1: 0.50 kbp). As shown in Fig. 12, constitutive expression of Act1 was confirmed by this RT-PCR experiment. As the 4 primer sets did not amplify the fragments which had sizes corresponding to non-spliced-out genomic DNA, the possibility of genomic DNA contamination in the RNA samples used was ruled out. A set of fixed oligonucleotide primers (4923fR and 4923r) was used to check the expression profile of the Rade1 gene by the above-mentioned RT-PCR method. All of the results were consistent with the microarray data shown in Table 2. Through the application of chilling stress in rice anther, the expression of OPDAR1, Rade1, and OsSalT was repressed and the expression of SAMDC1 was induced.

![Fig. 12 RT-PCR analysis of Act1, OPDAR1, SAMDC1, Rade1, and OsSalT genes.](image)

The first strands of the cDNA mixture were generated from total RNA from rice anthers. The PCR products were electrophoresed in agarose gel and visualized with ethidium bromide. The template cDNAs are the anthers at meiosis (lane 1), the anthers at microspore middle stage, just after chilling at 12 °C for 5 d (lane 2), and the anthers at the microspore middle stage (lane 3).

4 Discussion

In this study, microarray analysis was used to determine the gene expression profile in rice anther at the microspore release stage under chilling stress. Normalized log scores were obtained from two independent microarray experiments. As many as 160 ESTs were identified as chilling-inducible or -repressible in rice anther. These genes have a broad spectrum of potential functions based on their sequence similarities (Table 2). The scores of ESTs with similar sequences, such as OPDAR1, SAMDC1 and Rade1, showed similar changes in expression level (Table 2). These results indicate that microarray analysis is an efficient and reliable method for screening both up- and down-regulated genes under conditions of interest. However, ESTs homologous to a glucanase gene isolated from rice anther were not included in the list of 8,987 cDNAs on the microarray that was used. A potential problem of microarray research is that sequence similarity between closely related genes may lead to cross-hybridization. Also, in this experiment, orthologs of OPDAR1 or SAMDC1 which have up to 88–89% identity showed similar chilling-responsive expression patterns.
RT-PCR experiments with gene-specific primer might be used to confirm the chilling-responsive expression pattern of a particular gene (Fig. 12).

Aspartic proteinases (APs) (EC3.4.23) are one of the major classes of proteolytic enzymes showing acidic pH optima for enzymatic activity (Davies 1990). Typical plant APs have a high degree of similarity with those of animals and microbes, but plant AP proteins contain an extra plant-specific sequence (PSS) of approximately 100 residues, which shows high sequence and topological similarity to saposins, sphingolipid-activating proteins in mammalian cells. PSS has a putative membrane-binding region and may play a role in vacuolar transport of plant APs (Guruprasada et al. 1994; Mutlu and Gal 1999; Vaccaro et al. 1993). The tobacco chloroplast nucleoid DNA-binding protein CND41 and Radc1 protein lack the PSS sequence. As shown in Fig. 10, an aspartic protease motif of Radc1 shows significant similarity to CND41, but lacks the N-terminal Lys-rich helix-turn-helix motif that is essential for DNA binding in CND41 (Nakano et al. 1997). The results shown in Fig. 12 indicate that Radc1 is notably repressed by chilling in rice anther. Hence, Radc1 may play some regulatory role that is different not only from typical plant APs associated with cell death or plant defense, but from chloroplast DNA-binding protein. It is possible that Radc1 may degrade some proteins related to anther development and its response to chilling temperatures in rice plants.

Over 40% of the rice genomic sequence is repetitive DNA and most of this is related to transposable elements (Goff et al. 2002; Yu et al. 2002). The class 1 long-terminal repeat retrotransposons form the largest component of transposable elements, comprising 14% of the rice genome, but numerically, MITEs constitute the largest group, covering about 6% of the genome with over 100,000 elements classified into hundreds of families (Jiang and Wessler 2001; Tarchini et al. 2000). MITEs are non-autonomous class 2 elements, but Stowaway-like and Tourist-like MITEs can now be connected with two superfamilies of transposases: Tc1/mariner and PIF/harbinger respectively (Feschotte et al. 2002). Recently, an active MITE family, miniPing (mPing), was found in rice, and the correlation between mPing insertion in the slender glume allele and the slender mutation of glume was confirmed (Jiang et al. 2003; Kikuchi et al. 2003; Nakazaki et al. 2003). These mPing elements have undergone amplification more extensively in the temperate japonica than in the tropical japonica cultivar (Jiang et al. 2003). In wild rice, Oryza eichingeri, two MITEs, Castaway and Stowaway, in the 5’ upstream region of OsSalT, were shown to be not inserted (Bureau et al. 1996). ABA-responsive elements, which are known to regulate cold-inducible gene expression (Seki et al. 2001), were also conserved in the same position of Castaway, inserted in the 5’-flanking region of Radc1 and OsSalT. OsSalT mRNA accumulates very rapidly in the sheaths and roots of mature plants and seedlings upon treatment with ABA (Claes et al. 1990). Therefore, the similar down-regulated expression pattern of Radc1 and OsSalT under chilling in rice anther and the nearly identical position of the same Castaway insertion in the 5’-flanking region of the two genes is reminiscent of the possibility that the rice plant adapted to environmental extremes through transposon activation by stresses such as chilling and drought during the domestication of temperate japonicas. Since the Castaway-like sequence was not found in the 2 kbp 5’-flanking regions of 156 other chilling-responsive ESTs (data not shown), a further search for chilling-responsive Castaway elements in the rice genome and promoter-GUS assay under chilling conditions will be necessary in order to define the functions of Castaway in the chilling response in rice anther.

Jasmonate (JA) is involved in plant responses to several biotic and abiotic stresses and is a signal compound that regulates plant growth and development (Creelman and Mullet 1997). In Arabidopsis, the function of JA in anther development and pollen fertility is known to be essential. The JA-defective mutants in Arabidopsis are male-sterile because anther filaments do not elongate enough, and anther locules do not dehisce, and moreover, pollen grains on the mutant plants are inviable even though they develop to the trinucleate stage (Ishiguro et al. 2001; Sanders et al. 2000). JA has
been shown to increase the chilling tolerance of tomato fruit (Ding et al. 2002). OPDAR is the key enzyme in JA biosynthesis. To my knowledge, there is no information regarding the relationship between JA and anther development in rice and other cereal crops. The results shown in Fig. 12 indicate that \textit{OPDAR1} expression is remarkably repressed by chilling in rice anther, implying that \textit{OPDAR1} and JA play some role during anther development and its response to chilling temperatures in the rice plant.

Polyamines (PAs) are involved in many biological processes in plants (Kumar et al. 1997). The stress tolerance of plants is correlated with their capacity to enhance the biosynthesis of polyamines under stressful conditions (Bouchereau et al. 1999). A close correlation between the chilling tolerance of rice cultivars and putrescine accumulation in leaves under chilling stress has been found (Lee et al. 1995). In a chilling-tolerant cultivar of cucumber, synthesis of spermidine was increased in leaves during chilling treatment, while it was not in a chilling-sensitive cultivar (Shen et al. 2000). SAMDC is the key enzyme in PA biosynthesis that decarboxylates S-adenosylmethionine and supplies an aminopropyl moiety to putrescine and spermidine (Song et al. 2002). Low SAMDC activity is a major cause of poor performance of tomato pollen germination at high temperatures. To my knowledge, no prior study has elucidated the relationship between PA and microspore development in the rice plant. The results shown in Fig. 12 indicate that \textit{SAMDC1} expression is remarkably increased by chilling in rice anther, implying that \textit{SAMDC1} plays some role in microspore development and that it is tolerant to chilling temperatures in the rice plant.

In conclusion, 160 rice anther genes regulated by chilling were identified using high-throughput microarray analysis. The results suggest a network of gene functions and plant hormones involved in the chilling response at the early microspore stage of rice anther. Further experimentation including promoter-GUS assay or histochemical analysis of chilling-responsive genes will be necessary in order to define their functions in the chilling response of rice anther.

Fig. 13 Outline of this study
V Conclusion

In this study, genes expressed in rice anthers during the early microspore period, the stage of maximal chilling sensitivity, were analyzed in detail. This research not only provides a basis for an understanding of the mechanism by which chilling injury in rice anther arises, but is fundamental to improve markedly the chilling tolerance of rice during microspore development. An outline of this research is depicted in Fig. 13.

Beta-1,3-glucanases are referred to as PR proteins and they are also involved in several developmental processes. A cDNA for β-1,3-glucanase was isolated from rice anther, and was named *Oryza sativa glucanase 1 (Osg1)*. Phylogenetic analysis showed that Osg1 belongs to monocotyledonous endo-β-1,3-glucanase subfamily A. RT-PCR analysis revealed that Osg1 transcripts were present in leaves, roots, and anthers.

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. 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 possible roles of distinct members of OsGSLs and OsRops in pollen development and its response to chilling temperature are suggested.

The gene expression profile during the microspore development process under chilling stress was revealed using a microarray that included 8,987 rice cDNAs. As many as 160 cDNAs were up- or down-regulated by chilling during the microspore release stage. RT-PCR analysis of 5 genes confirmed the microarray results. Three novel genes whose expression levels were remarkably changed by chilling in rice anther were identified. A new cis element that includes a DNA transposon *Castaway* sequence was found in the 5' upstream region of two genes which were conspicuously down-regulated by chilling temperatures in rice anther.

References


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27.


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