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

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

Tomoya YAMAGUCHI *1)

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：イネ葯は小胞子解離期に冷温感受性が最も高い。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，イネ葯で発現するβ-1,3-グルカナーゼ遺伝子 *Osg1* を単離し，その葯および葉，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統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* の発現が葯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 型低分子 GTP 結合タンパク遺伝子のイネ葯における発現を解析した。小胞子形成期イネ葯の冷温下での遺伝子発現変動を俯瞰することも重要と考え cDNA マイクロアレイ解析を行い，3つの新規イネ葯冷温応答遺伝子を同定するとともに，冷温下のイネ葯で発現が抑制される遺伝子の 5'上流域に，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出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，葯，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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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 reticula 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 envelops 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 meiocytes, 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).

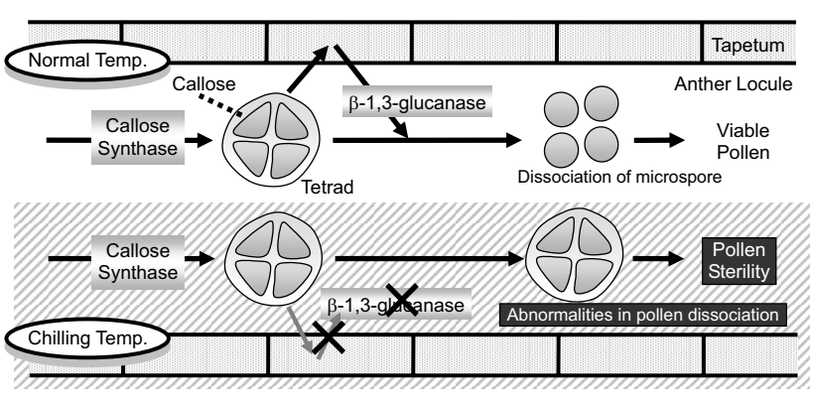


Fig. 1 A schematic diagram outlining callose synthesis and degradation in microspore development under normal or chilling temperature

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)

I wish to express my sincere gratitude to Professor Dr. Jiro Sekiya, Kyoto University, for his courteous guidance and continuous encouragement throughout my research. I wish to express my sincere appreciation to Dr. Setsuo Koike, National Agricultural Research Center for Tohoku Region (NARCT), for his invaluable suggestions and discussion throughout my research. I greatly appreciate Dr. Masaharu Yajima and Dr. Hiromitsu Kanno, NARCT, for their useful suggestions regarding my research and for their critical reading of the manuscripts. I wish to also thank my collaborators in NARCT, Ms. Takami Hayashi, Mr. Katsuhiro Nakayama, presently at Iwate University, Ms. Junko Sakurai, and all the other members of NARCT for their substantial collaboration and kind suggestion concerning my research. I also thank Mr. Akira Tanifuji, Mr. Eisaku Kumagai, Mr. Akio Yoshida, Mr. Kazuhiro Hayasaka, Ms. Keiko Iwabuchi, and Ms. Hiromi Suzaki, NARCT, for their technical assistance. I wish to express my deep gratitude to Professor Dr. Takayuki Kohchi, Professor Dr. Kazufumi Yazaki, Associate Professor Dr. Hideya Fukuzawa and Dr. Katsuyuki T. Yamato, Kyoto University, for their excellent guidance, valuable advice, and critical reading of the manuscripts. Special thanks must be given to Dr. Kanji Ohyama, presently at Ishikawa Prefectural University and Dr. Yasuyuki Yamada, for their interest in and very thoughtful criticism of my research and for their continuous encouragement. I wish to thank Dr. Yoshiyuki Tanaka, Dr. Atsunori Fukuda, and Dr. Atsuko Nakamura, of National Institute of Agrobiological Sciences (NIAS), for their kind support in the construction of the cDNA and genomic library and hybridization analysis. I wish to thank Dr. Shoshi Kikuchi, Dr. Naoki Kishimoto, and Dr. Junshi Yazaki, NIAS, and their colleagues for their gracious support of the microarray analysis. I wish to thank Dr. Masato Wada, National Institute of Fruit Tree Science, for his valuable suggestions and kind help regarding the molecular biological analysis. This research is already published as original paper (Yamaguchi *et al*. 2002, Yamaguchi *et al*. 2006, Yamaguchi *et al*. 2004), and is a revision of the dissertation for the doctoral degree at Kyoto University.

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 *Eco*RI-*Not*I-*Bam*HI 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. The resulting products were ligated into the λgt10 vector and then packaged using a Gigapack Gold (Stratagene).

Complete degenerate oligonucleotide primers (Glu1: 5'-GGIGTITGYTAYGG-3'; Glu4: 5'-GGCCAICCISWYTC-3'; Y, S, and W are mixtures of (C, T), (C, G), and (A, T), respectively; I is inosine) were designed based on the sequence of conserved pentapeptides (GVCYG and ESGWP). The PCR reaction mixture (25 μl) contained 0.5 μl of reverse-transcribed first strands of cDNA mixture in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01 mg/ml gelatin, 1.5 mM $MgCl_2$, 4 or 8 μM each of the degenerate primers, 200 μM each dNTP mixture and 0.04 unit/μl of AmpliTaq DNA polymerase (Applied Biosystems). Thirty-two cycles of PCR (5 cycles of 94˚C for 0.5 min, 46˚C for 0.5 min, 72˚C for 1.5 min, 27 cycles of 94˚C for 0.5 min, 52˚C for 0.5 min, 72˚C for 1.5 min) were performed. The PCR products were separated by electrophoresis in 1.5% agarose gels and stained by SYBR Green I (BMA).

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 *Mbo*I-partially digested fragments of DNA isolated from the rice (cv. Hayayuki) leaves. From the positive clone H2a4, a 2.3-kb *Sal*I-*Sal*I 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 *Bam*HI, *Eco*RI, or *Hin*dIII. 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 $MgCl_2$, 6 μM each of specific primers (*Osg1*-ff3: 5'-GTATGGGACAAAGGATCTCC-3'; *Osg1*-r0: 5'-GTGATCCAGCCCTACTGCTG-3'; Act1f02: 5'-CGCAGTCCAAGAGGGGTATC-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 *Bam*HI, *Eco*RI, or *Hin*dIII, 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. Polymers 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;1,6-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 OsGns5 in amino acid sequence.

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GTCGACGAATAATGCCTTCCTGTCAGTATTCAATCTCGACATGGAGTACGTGAAGATTCA   60
TATCGCAAAATCGAGAATCACCAGAGTCGTGCAGCTTGGGCGGTGGAAGAGGTCAAGGAA  120
GCACCACATCGAGCTGGTGGGAGACAACGACCTACTCAAAAAAGTTTTGGCGTGCAAGAA  180
CCCTGAGGTACCTCAGCCTCAGGGGCATCTCCCTCATCGAGTCCATACCCGAGGCCATCG  240
GCACGCTTGCCGAACTCCTCGTGCTCGACCTCAGGGCGTGCCACAACTTGGAGAAGTTAC  300
CGGGGTCTATCGGCTCGTTGCTGAAGCTAGAGTACCTGGACTTGTCCGAATGCTTCTTAC  360
TTGAAGAAATGCCCAAAGAGATCGGCGAGCTGTCCAAGCTGCAGGTCCTCAAGGGTTTTC  420
TCGTTGGCAGTTCAAGAAAGAAGAGCAGCCCATGCCGTCTCGCCGACCTGGCCACAAAGG  480
CGCAGAATCTACGGAAGCTAAACATTACCACTGGACGGCAATCTTTGGTGTGTGATGAAG  540
ATGAGCTCTGCCAATTGGCGAAATGCCAGCATCTTGAGTCCTTGACAATAACTTGGATAG  600
GAGAGGCATCTACCGAGCCATATCTTCCTCTTCCTTCCAGCCTCACAAAGCTTGATCTCC  660
GGCGTGCCCCTACCGCAAGCCTTCTTAATATCATTCATCCATCGACGTCGGTGAGCCTGA  720
AACGGCTGTATATTAGGGGGAGGGAAGCTCCGTACACTGGGGCAAGACGGCGGCTGGAACG  780
TTGAGACACTGCGTGCTAGATGTCTCAATGATTTGGAGTGTGAGTGGAGTGAGCTCCATG  840
GTTTGTTCCGAGAACTCCGTTTTGTGGAGATGTGGAGGTGTGCCCGCCTTAGTTTCTGGC  900
                          M  W  R  C  A  R  L  S  F  W     10
CTTGCGATGGGCGGGGCGTATGGGACAAAGGATCTCCTTCTCTGGgtaagtgtcttacat  960
P  C  D  G  R  G  V  W  D  K  G  S  P  S  L                  25
ctccatgcatattactacctctgtttcaaaataagttcatttttcatccatcccacatac 1020
caatacaaactcaaaaatactataataccatattttatcaaatcccaatgcaactattca 1080
tctcactttatctactcctaatacagttattccttgttttcacaaactaagatacaatga 1140
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gcagCTCGCCGATCCATTGGAGTCTGCTACGGAGTGAAAGGCAACAATCTGCCACCATGG 1380
    A  R  R  S  I  G  V  C  Y  G  V  K  G  N  N  L  P  P  W    44
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 H  E  V  V  Q  L  Y  A  S  N  N  I  P  A  M  R  I  F  Y  P    64
CACCACGATGTTCTTGAGGCTCTGAGAGGCACAGGCATCGGTATCAGCCTCGACGTTGAG 1500
 H  H  D  V  L  E  A  L  R  G  T  G  I  G  I  S  L  D  V  E    84
GGCCAATTCCTCCCATCCTTCGCCTCTGAACCCTCCGTTGCGGCTGCCTGGGTGAAGACC 1560
 G  Q  F  L  P  S  F  A  S  E  P  S  V  A  A  A  W  V  K  T   104
AATGTGCAAGCCTTTTACCCTGCAGTCTCATTCAAGTTCATCACAGTCGGCAACCAGGTT 1620
 N  V  Q  A  F  Y  P  A  V  S  F  K  F  I  T  V  G  N  Q  V   124
GCCCTGAGAGAGATGAGATACATCCTCCCAGCGATGCAGAATATCTATGCAGCGTTGTCA 1680
 A  L  R  E  M  R  Y  I  L  P  A  M  Q  N  I  Y  A  A  L  S   144
GCAGTAGGGCTGGATCACATCAAGGTGTCAACGTCGGTGAGAAGGGACGTGCTGGGTTTG 1740
 A  V  G  L  D  H  I  K  V  S  T  S  V  R  R  D  V  L  G  L   164
TCCTATCCTCCCTCTGCTGGTGCTTTCTCCTCAGCGATGGAACAGTACATGGCACCTATT 1800
 S  Y  P  P  S  A  G  A  F  S  S  A  M  E  Q  Y  M  A  P  I   184
GTGCAGTTCTTGGCCAAGATTGGGGCTCCACTTCTGGCCAGTGTGTTCCCTTACTTTACT 1860
 V  Q  F  L  A  K  I  G  A  P  L  L  A  S  V  F  P  Y  F  T   204
TACGTGCACAATCAGGAAGGCATTGATATCGATTACGCCCTTTTCACCTCCCCGGGAACT 1920
 Y  V  H  N  Q  E  G  I  D  I  D  Y  A  L  F  T  S  P  G  T   224
GTGGTGCAGGACGGTGAACATAGCTATCAGAACCTCTTCGATGCCATTGTGGATGCTCTG 1980
 V  V  Q  D  G  E  H  S  Y  Q  N  L  F  D  A  I  V  D  A  L   244
TATTCGGCGATGGAGAAGGTGGGTGGCTCAACCGTCAGAATAGTTGTCTCAGATAGCGGC 2040
 Y  S  A  M  E  K  V  G  G  S  T  V  R  I  V  V  S  D  S  G   264
TGGCCATCGGCTGGGGCTCCTGCAGCGACCAAAGATAATGCTAGGGCCTACGTCCAGAAC 2100
 W  P  S  A  G  A  P  A  A  T  K  D  N  A  R  A  Y  V  Q  N   284
CTGATTAACCATGTGAGCAAAGGGACACCCAAGAGGCCCGTACCTATAGAGACATACATT 2160
 L  I  N  H  V  S  K  G  T  P  K  R  P  V  P  I  E  T  Y  I   304
TTTGCCATGTTCAATGAGAACGAGAAAACCGGTGACGAGATAGAGAGGAACTTTGGGCTC 2220
 F  A  M  F  N  E  N  E  K  T  G  D  E  I  E  R  N  F  G  L   324
TTTGAACCTGACAAGAGCCCTGTGTACCCAATCACCTTCTCTTAACCGAAAGGATAGTAC 2280
 F  E  P  D  K  S  P  V  Y  P  I  T  F  S  *                 338
AAGGAGGCAGCTGCTACAAAAACTCAACCACTTTTCACTGATCCGTTGACCTGCAGGTCG 2340
AC                                                            2342
```

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.

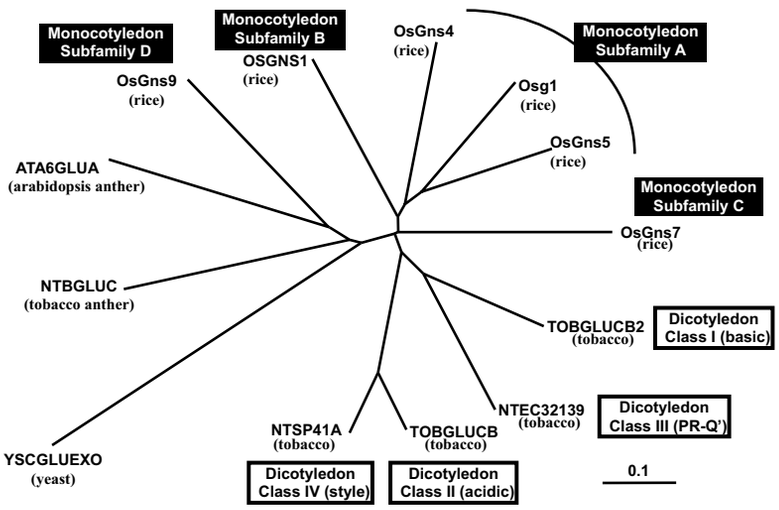


Fig. 3 Phylogenetic trees of *Osg1* and known glucanases.
The sequences of known glucanases were adapted from (Linthorst *et al*. 1990); (Bucciaglia and Smith 1994; Hird *et al*. 1993; Payne *et al*. 1990); (Romero *et al*. 1998). Scale bar, 0.1 substitutions per site.

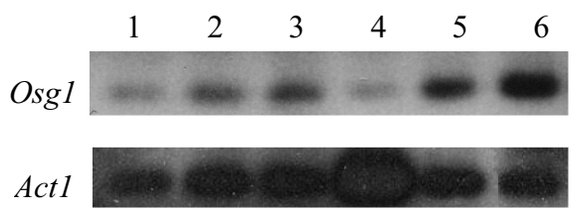


Fig. 4 Quantitative RT-PCR analysis of the *Osg1* and *Act1* genes.
Template cDNAs were prepared from anthers at the young microspore stage (lane 1), anthers after cooling at 12 ℃ for 5 days (stages moved from the young microspore stage to the early middle stage during chilling) (lane 2), anthers at the early middle stage (lane 3), anthers at anthesis (lane 4), roots (lane 5), and leaves (lane 6). PCR conditions were 24 cycles of (94 ℃ for 30 sec, 60 ℃ for 1 min, 72 ℃ for 1 min) for *Osg1* and 19 cycles of (94 ℃ for 30 sec, 56 ℃ for 1 min, 72 ℃ for 1 min) for *Act1*.

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.

The cool tolerance of barley at the young microspore stage was much higher than that of rice, and microscopic observation revealed that callose envelops 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 envelops and chilling tolerance. In order to analyze this relationship, the creation of transgenic rice plants was considered, in which the thickness of callose envelop 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 (Østergaard *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 meiocytes, 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://pbil.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 $MgCl_2$, 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 *OsGSL*s and *OsRop*s in rice genome and primers used in semiquantitative RT-PCR

	GenBank accession no.	Chromosome location	Specific primers used for RT-PCR		RT-PCR product size (bp)	Number of PCR cycles
OsGSL1	AP001389	6	GSL01f1	gctcaaaattgcttcacacaatacatc	516	35
			GSL01r1	ggttaggcttttcatgcctcctg		
OsGSL2	AP003223	1	GSL02f1	gtggctcttgtgacagttgcttac	449	35
			GSL02r1	ccacatatcatacctcaaactccatg		
OsGSL3	AP003249	1	GSL03f1	ccagttgaagatcgcaaatgaaagcag	526	35
	AP003268		GSL03r1	ggcttattgtactgtacctcaaactgc		
OsGSL4	AP003445	1	GSL04f1	cacatagtgcatgggaacagaagc	629	35
			GSL04r1	catgatcagtccctctttgagcc		
OsGSL5	AP003454	6	GSL05f1	ggagcaagtggattagcagccg	682	35
			GSL05r1	gtgcgggctcagctctgcttc		
OsGSL6	AP004685	6	GSL06f1	actggaccaagcactagcc	1,243	35
			GSL06r1	agccctcgccgctcagtc		
OsGSL7	AP004082	2	GSL07f1	ctgggaagcgtggaattgttctcg	613	35
			GSL07r1	gaatggcagacaggctactactcc		
OsGSL8	AC118980	3	GSL08f1	catgggctgtgcttggtggcc	468	35
			GSL08r1	gcagctacagtcatactcccgc		
OsGSL9	AP003447	1	GSL09f1	catagcacgccgcagcaaaagc	518	35
			GSL09r1	ccattcatccattcattcattcctc		
OsGSL10	AC104427	3	GSL10f1	ccacttaaatatcacaggcgacaaaag	538	35
	AC144426		GSL10r1	gggtagagaagatcagtccttg		
OsGSLmt	BA000029	mitochondria				
OsRac1	AB029508	1	Rac1-F1	ttgctgatcatccagcttctt	229	35
			Rac1-R1	ccgcaaaagtaccgcctta		
OsRac2	AB029509	5	Rac2-F1	tgttcttgttgggaccaagtt	310	35
			Rac2-R1	caggtcctaaacaacacatgc		
OsRac3	AB029510	2	Rac3-F1	ggatctccgtgaagacaagc	320	30
			Rac3-R1	cacccagaacattgcatcat		
OsRop4	AF380335	6	Rop4-F2	gtgcttgccttctccctagtg	459	35
			Rop4-R2	acacaagacatcattttgtctc		
OsRop5	AF376055	2	Rop5-F2	tttccctcatcagcaaagcg	434	35
			Rop5-R2	gcatctaatctgcaacacac		
OsRacB	AY579208	2	RacB-F1	gcattatgcacctggtgtgc	280	30
			RacB-R1	tcacaaaatggagcacgccc		
OsRacD	AF329814	2	RacD-F1	gcattatgctcctggtgtgc	277	35
			RacD-R1	caagatggcacatcctttctg		
Act1	AB047313	3	RAcf02	cgcagtccaagaggggtatc	505	35
			RAcr03	tcctggtcatagtccagggc		

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 *AtGSL*s and *OsGSL*s 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).

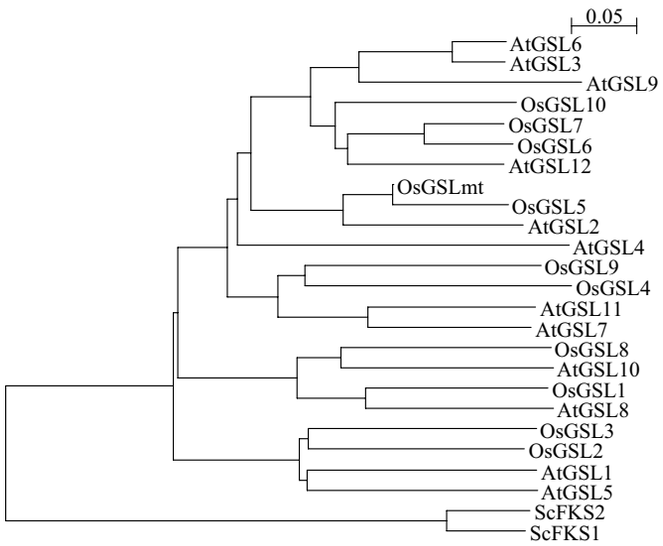


Fig. 5 Phylogenetic tree of glucan synthases.

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.

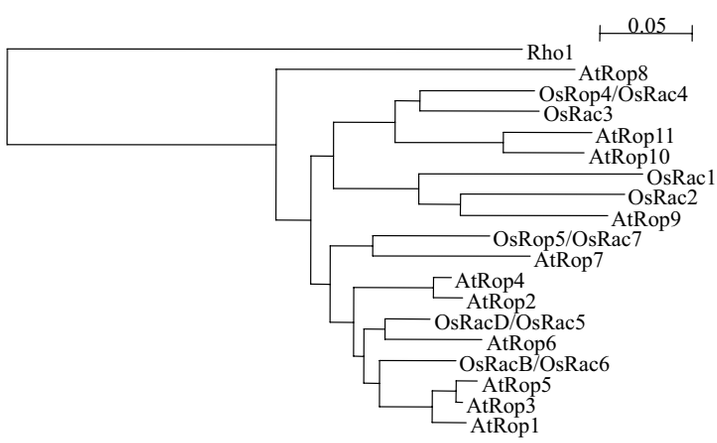


Fig. 6 Phylogenetic tree of Rop proteins.

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 *Rop*s and *GSL*s 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., *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. 7, 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, 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 *OsRop*s overlapped 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 anthers at the microspore stage was also detected. As shown in Fig. 8, expression of *OsGSL1*, *OsGSL2, OsGSL4, OsGSL6, OsGSL7, OsGSL8, OsGSL9*, and *OsGSL10* was detected at all stages of 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 (lane 4). Expression of *OsGSL3* was not detected in six samples (lanes 1-6).

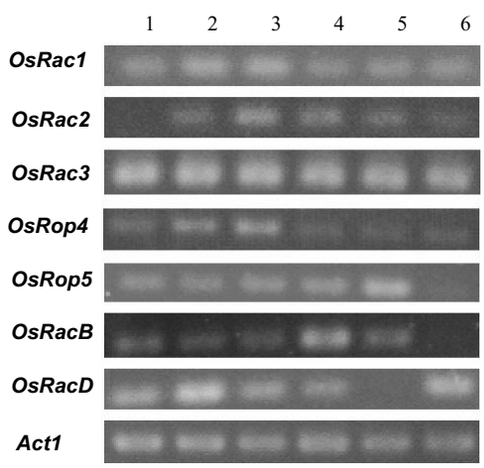


Fig. 7 RT-PCR analysis of rice Rop genes.
The first strands of the cDNA mixture were generated from total RNA. The PCR products were electrophoresed in agarose gel and visualized using ethidium bromide. The template cDNAs are the anthers at meiosis (lane 1), the anthers at microspore middle stage, just after chilling at 12 ℃ 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).

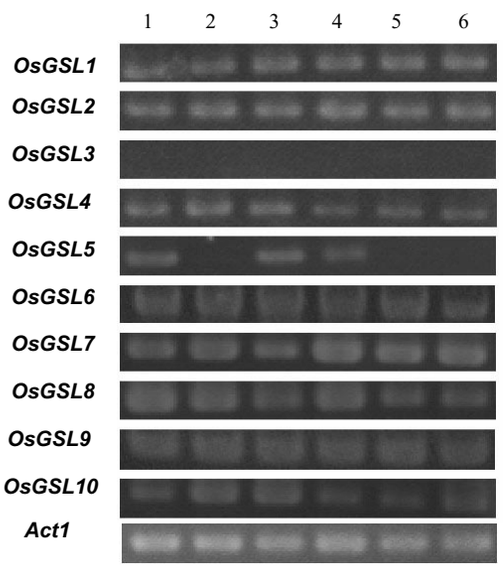


Fig. 8 Semiquantitative RT-PCR analysis of rice Glucan Synthase Like genes.
The first strands of the cDNA mixture were generated from total RNA. The PCR products were electrophoresed in agarose gel and visualized using ethidium bromide. The template cDNAs are the anthers at meiosis (lane 1), the anthers at microspore middle stage, just after chilling at 12 ℃ 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 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. 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.

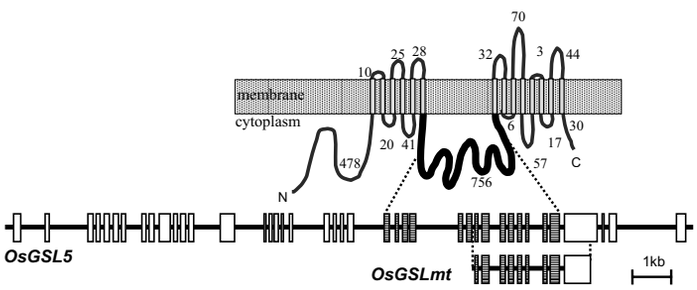


Fig. 9 Gene structure of *OsGSL5* and OsGSLmt and predicted organization of *OsGSL5* protein in a plasma membrane.

The long rectangle and the vertical grey bars indicate the membrane and the transmembrane regions, respectively. Numbers represent the sizes of loops in amino acids, and the N-terminus (N) and the C-terminus (C) are indicated. Exons are indicated by open and striped boxes.

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 microgametogenesis and pollen viability (Dong *et al*. 2005), *OsGSL5* might play essential role in callose synthesis during microsporogenesis. Meanwhile, another 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 (Enns *et al*. 2005). Therefore, other *OsGSL*s, 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 *Rop*s. 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 *Rop*s expression patterns were observed. Based on these observations, it was hypothesized that the rice *Rop*s, such as *OsRac1*, *OsRac3*, *OsRop4*, *OsRop5*, and *OsRacD* 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 Os*Rop*s 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 (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 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; Negishi *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, 0.3g each of magnesium and calcium, and traces of iron and manganese were added. A growth chamber (12 h light (300 $\mu E/m^2/s$) 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 *OPDAR1*; *Sal*Tf1: 5'-GACGCTGGTGAAGATTGGCC-3' and *Sal*Tr1: 5'-GGCCATGGGTTCCAGAAATC-3' for amplifying

*OsSal*T; *Act1*f02: 5'-CGCAGTCCAAGAGGGGTATC-3' and *Act1*r03: 5'-TCCTGGTCATAGTCCAGGGC-3' for amplifying rice actin 1; 4923f8: 5'-GAGAACACGCTCATCCACAG-3' and 4923r: 5'-TGCTAGCAGCAGCTTG-GCTC-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 $Z = (\chi - \mu)/\delta$, 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

Clone	EST accession		Putative ID	difference of averaged Z scores
primary metabolism				
7438	AU056550		3-ketoacyl-CoA thiolase-like protein	1.9
3092	AU064119	AU101254	acyl-CoA oxidase	1.1
7555	AU076256	AU076257	adenosine monophosphate binding protein 3	1.0
3787	AU064238	AU172746	adenosylhomocysteinase	1.4
163	AU166291	AU166292	alpha-amylase isozyme 3D precursor	1.3
826	AU068387	AU166535	alpha-amylase isozyme 3D precursor	1.2
3730	C73655	AU108182	β-ketoacyl synthase	1.4
8098	D48949	AU097625	bifunctional nuclease	1.1
6219	D24338	AU173217	β-ketoacyl reductase	1.0
7281	AU056257	AU056258	carbonic anhydrase	2.3
5419	AU095385	AU095386	cysteine synthase	1.6
5474	AU164935	AU164936	cysteine synthase	1.9
469	C93441	AU166412	hexokinase 1	1.6
2519	C71989	AU101080	invertase	1.5
2559	AU101108	AU101109	lipase	1.0
1425	AU108817	C97061	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	1.0
8085	D48732	AU032835	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	2.1
8589	D40492	AU174256	oxalate oxidase	1.2
2791	C72731	AU165528	plant short chain alcohol dehydrogenase	1.0
8304	D39365	AU163158	sucrose synthase	1.9
8428	AU174192		sucrose synthase	1.7
5214	AU101521	AU101522	3-hydroxy-3-methylglutaryl-CoA reductase	-1.0
31	C25787	AU102080	glutamine synthetase shoot isozyme	-1.1
414	C26203	AU100677	glycine decarboxylase complex H-protein	-1.1
3444	C99346	AU101318	*Homo sapiens* h-bcs1 (BCS1) mitochondrial protein	-1.5
5695	AU174855	AU174854	nonspecific lipid-transfer protein 2 precursor (LTP 2)	-1.2
signal transduction				
4154	C74259	AU091362	CaM-like protein	2.0
7219	AU056086	AU056087	CBL-interacting protein kinase 1 (CIPK1)	1.0
6586	D25079		finger protein WZF1	1.1
4082	C74114	AU091318	NAC-like protein	1.2
2459	C71770	C98483	RING finger-like protein	1.2
4278	AU162272	AU029581	WD domains, G-beta repeats	1.0
3298	C19151	AU091905	homeobox 1 protein OSH1	-1.2
4105	C74163	AU101400	RING-box protein	-1.2
transcription				
7594	AU057316	AU057317	*Arabidopsis thaliana* scarecrow-like 1 (SCL1)	1.1
4389	C91785	AU172851	Dof zinc finger protein	1.1
7463	AU162697	AU056522	OsNAC6 protein	1.1
641	AU166454	AU166455	the largest subunit of RNA polymerase II	1.1
defense				
2693	C72401	AU172487	*Arabidopsis thaliana* ERD15 protein	1.0
121	D22210	C96640	chitinase	1.0
8614	D40768	AU174271	low temperature and salt responsive protein LTI6B	1.1
7113	AU055773	AU055774	metallothionein-like protein	1.3
7645	AU057501	AU057502	metallothionein-like protein	1.3
2142	AU063562	AU172350	*OsSal*T	2.1
8849	AU066156	AU161751	*OsSal*T	2.4
995	AU068611	AU166664	*P. vulgaris* PVPR3 protein	1.3
7493	AU075857	AU075858	PR protein	1.5
8556	D40265	AU163222	peroxidase FLXPER4 (PER4)	1.0
7179	AU162644	AU056017	ascorbate peroxidase	1.4
315	C25998	AU092225	ascorbate peroxidase	-1.0

Continued on next page

Table 2 (Continued)

Clone	EST accession		Putative ID	difference of averaged Z scores
translation				
7640	AU070286	AU173957	chloroplast 30S ribosomal protein S7	2.1
8135	AU070441	AU174085	chloroplast 30S ribosomal protein S8	1.2
2302	AU175087	AU175088	protein translation factor SUI1	1.2
7320	AU077773	AU077774	protein translation factor SUI1	1.2
9031	AU161922	AU161923	protein translation factor SUI1	1.0
124	D22238	C96682	elongation factor 1-gamma	-1.0
159	AU102118	C96770	elongation factor 1-gamma	-1.1
5341	AU031252		40S ribosomal protein S15	-1.1
312	AU062501	AU092216	60S acidic ribosomal protein P0	-1.0
668	AU062621	AU108592	60S acidic ribosomal protein P0	-1.0
5464	AU164919	AU031524	60S ribosomal protein L18	-1.1
5501	AU095410	AU031615	60S ribosomal protein L2 (L8)	-1.1
5905	AU164554		60S ribosomal protein L7	-1.0
104	D22132		ribosomal protein L18a	-1.2
3427	C19518	C99258	ribosomal protein S4 type I (rps4)	-2.1
cell structure				
7891	D47590	AU082750	HMG protein	1.1
5402	AU095352	AU031408	pistil extensin like protein	-1.0
125	AU166286		villin 3	-1.0
transport				
6457	D24715	AU173362	*E. coli* cation transport protein ChaC	1.0
7621	AU173952	AU057343	vacuolar sorting receptor	1.1
603	C26456	AU164984	beta prime COP coatomer protein	-1.1
133	C96646	AU166285	cation-chloride co-transporter	-1.0
proteolysis				
651	C26503	AU091279	aspartic proteinase precursor	1.4
8125	D49033	AU101829	AtAPG8c mRNA for autophagy 8c	1.1
4332	C91690	AU029682	cysteine proteinase 1 precursor	1.0
7147	AU055940	AU055941	oryzacystatin-I	1.4
2744	C98695		polyubiquitin	1.0
4923	AU172968	AU166209	Radc1	2.6
8640	AU174285	AU174286	Radc1	3.6
4949	AU065677	AU030354	serine proteinase	1.1
528	C26358	AU100706	SEC61 alpha subunit	-1.4
secondary metabolism				
7405	AU056351		dihydrofolate reductase	1.3
132	C96685	C96686	OPDAR	1.2
314	C26019	AU092237	OPDAR	1.2
339	C26097	AU093231	OPDAR	1.4
7329	AU057040	AU057041	OPDAR	1.0
9071	AU175176	AU176556	shikimate kinase precursor	1.0
689	C26568	AU108589	SAMDC	-1.0
883	AU062686	AU166559	SAMDC	-0.8
1904	C28220	C97560	SAMDC	-1.0
53	AU067858	AU067859	xanthine dehydrogenase	-1.0
storage protein				
8808	AU174343	AU174344	OsSBP for selenium binding protein	1.1
8232	D49189	AU082276	maize Fer1	1.2
energy production				
7813	AU075867	AU075868	ATP synthase C chain	1.6
4506	AU064654	AU094876	ATP synthase delta chain	1.2
5918	D23879	AU031680	cytochrome b5 reductase (NFR)	-1.6
other				
6872	AU070733		cytochrome P450	1.5
7846	AU173996		Cyt-P450 monooxygenase	1.0
6249	AU173240	AU173241	isopenicillin N epimerase	1.1
2365	AU174453		lectin precursor (agglutinin)	1.0
3764	C73711	C99490	male sterility protein 2	1.7
2371	AU174447		Acyl-CoA-binding protein	-1.2

Clone	EST accession		difference of averaged Z scores
unknown			
348	AU062521	AU166365	1.1
479	C26301	AU166411	-1.1
1218	C26865	AU166775	1.3
1559	AU069076		1.4
1690	C27876	AU100890	1.6
3003	AU078191		1.5
3026	C73175	AU172643	1.0
3068	C98974		1.0
3273	AU172688		-1.2
3297	C19175	AU094360	-1.9
3325	C19310		-1.7
3434	AU162217	C99361	-1.1
3653	C20268		1.2
3673	C20375		1.0
3693	C20472		1.0
3726	C73631	C99434	2.0
3780	AU078130	C99517	1.0
4012	AU164283		1.0
4019	AU064364		1.0
4174	AU058082		-1.3
4217	AU101426	AU172815	4.0
4271	AU162267	AU029519	1.0
4339	C91704	AU029692	1.0
4345	C74445	AU172829	1.2
5694	AU174850		-1.2
5766	AU174902	AU174903	1.4
6204	D24309	AU173209	-1.6
6255	D24399	AU031803	-1.2
6666	AU181075		1.5
6714	AU032158	AU175073	1.1
6921	AU077754	AU077755	1.1
7003	AU078041	AU078042	1.3
7022	AU071077	AU162620	1.4
7059	AU071233		1.9
7323	AU057050		1.5
7369	AU057246	AU057247	2.1
7381	AU057197	AU057198	1.4
7386	AU057208		1.4
7388	AU173946	AU057231	1.3
7586	AU057277		1.1
7611	AU162748	AU057349	1.4
7627	AU057395	AU057396	1.1
7639	AU057483	AU057484	1.3
7748	AU057862		1.0
7757	AU057824	AU057825	1.1
8101	D48962	AU163113	1.5
8133	D49049		1.2
8153	AU101838	AU101839	1.5
8199	AU162105	AU162106	1.8
8244	D49239	AU101865	1.3
8258	D49335	AU101883	1.4
8727	D41415	AU033134	1.0
8814	AU161719	AU161720	1.7
8909	C25216	AU174362	1.0
8934	AU181057		1.1
9000	AU161904		1.2
9005	AU102062	AU102063	1.6
9054	AU097686		1.0

2) Analysis of rice *OPDARs* and *SAMDCs*

Among the 8,987 rice ESTs on the microarray, 5 ESTs are annotated to encode OPDARs and 4 of them were down-regulated by chilling stress in rice anther. 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 *OsSal*T

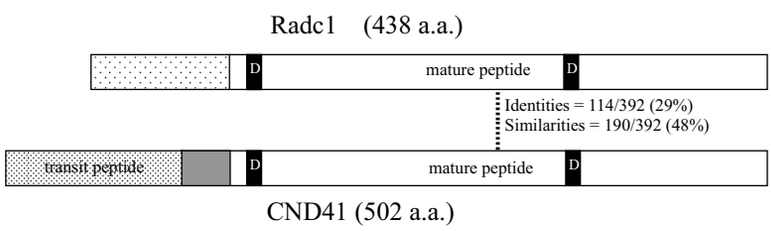


Fig. 10 Domain organization of *Radc1* and CND41.
The dotted box, the shaded box, and the black box represent transit peptide, Lys-rich helix-turn-helix motif, and active site aspartic acid residue, respectively.

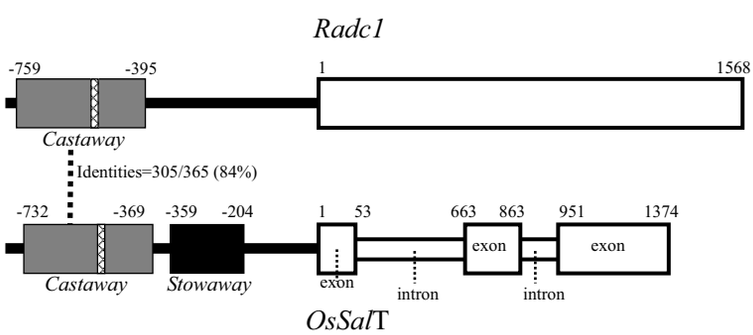


Fig. 11 Comparison of *Radc1* and *OsSal*T genes.
The shaded box and the black box represent *Castaway* and *Stowaway*, respectively. The hatched boxes in *Castaway* represent ABA-responsive elements (TACGTGGC).

gene (Fig. 11) (Claes *et al*. 1990). *OsSal*T has another MITE sequence, *Stowaway*, in the upstream region (Fig. 11) (Bureau *et al*. 1996). *Castaway* found in *Radc1* shows 84% sequence identity with *Castaway* found in *OsSal*T (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; *OsSal*T: 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; *OsSal*T: 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 (4923f8 and 4923r) was used to check the expression profile of the *Radc1* 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*, *Radc1*, and *OsSal*T was repressed and the expression of *SAMDC1* was induced.

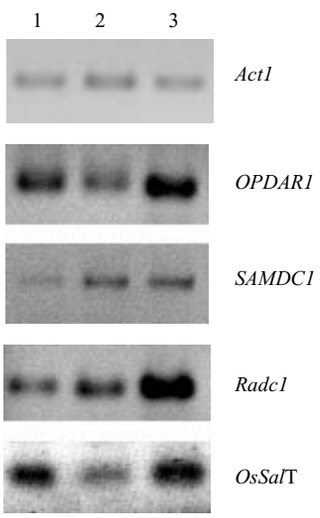


Fig. 12 RT-PCR analysis of *Act1*, *OPDAR1*, *SAMDC1*, *Radc1*, and *OsSal*T genes.
The first strands of the cDNA mixture were generated from total RNA from rice anthers. The PCR products were electrophoresed in agarose gel and visualized with ethidium bromide. The template cDNAs are the anthers at meiosis (lane 1), the anthers at microspore middle stage, just after chilling at 12 ℃ 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 *Radc1*, 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, *miniature Ping (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 *OsSal*T, 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 *OsSal*T. *OsSal*T 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 *OsSal*T 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 *japonica*s. 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 *OPDAR1* expression is remarkably repressed by chilling in rice anther, implying that *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 *SAMDC1* expression is remarkably increased by chilling in rice anther, implying that *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.

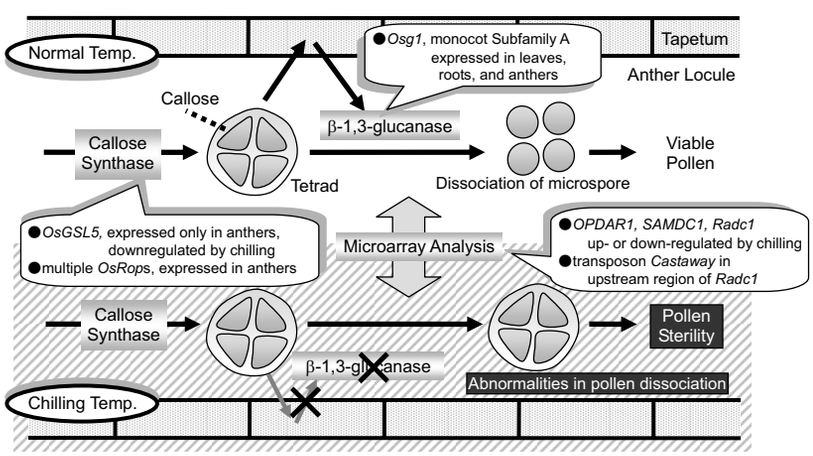


Fig. 13 Outline of this study

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.

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