

Full Length Research Paper

Isolation of a novel MYB transcription factor *OsMyb1R* from rice and analysis of the response of this gene to abiotic stresses

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To understand mechanisms underlying stress responses and discover new stress-tolerance genes in rice (*Oryza sativa* L.), we analyzed a global genome expression profiling of the *indica* cultivar Pei'ai 64S subjected to cold, drought, or heat stresses. Expression profiles were obtained for leaf and panicle tissues at seedling, booting and heading stages from plants under no stress, or cold, drought or heat stresses using the GeneChip Rice Genome Array (Affymetrix) representing 51 279 transcripts from *japonica* and *indica* rice. A large number of genes highly up-regulated or down-regulated were identified under the stresses. One of these genes, *OsMyb1R*, was highly induced in leaf and panicle at the heading and flowering stages, in response to all stresses. The expression profile of *OsMyb1R* obtained by the microarray analysis was confirmed by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of the gene. The two sets of data matched very well, suggesting that *OsMyb1R* is a multiple stresses responsive gene in rice. In order to study its function in stress tolerance, we cloned the cDNA of the gene through amplification by RT-PCR. Sequence analysis showed that the cDNA encodes a protein of 489 amino acid residues with molecular weight (M.W.) \approx 56 kD and $pI \approx$ 6. Bioinformatics analysis revealed that the protein contains a conservative domain of the MYB family gene. Analysis of the putative promoter region for candidate *cis*-regulatory elements identified 5 matches to *cis*-elements related to stress responses. Based on the earlier mentioned analysis and results obtained, we propose that *OsMyb1R* is a novel candidate gene involved in stress tolerance in rice.

Key words: *Oryza sativa* L., stress, microarray, real-time PCR, MYB, bioinformatics.

INTRODUCTION

There is a very serious impact on the yield and quality when the process of rice cultivation is under cold, heat, drought and other abiotic stress. Therefore, to understand the mechanism by which rice perceives environmental signals and transmit the signals to cellular machinery to activate adaptive responses and isolate stress-related genes for developing stress-tolerant rice which is significant for ensuring stable and high yield, raising the utilization efficiency of low-yield fields, expanding rice planting area, we investigated the gene expression levels of rice in different stresses. Based on previous study, stress tolerance of plant is a quantitative trait controlled

cold and other stresses, the expression patterns will be by multiple genes. When plants are subjected to drought, greatly changed (Hannah et al., 2005) of many genes involved in lots of metabolic and physiological processes of adaptation, including the regulation of secondary metabolism (Borevitz et al., 2000; Jin et al., 2000; Nesi et al., 2001; Baudry et al., 2004) control of cell morphogenesis (Lee and Schiefelbein, 1999, 2001; Higginson et al., 2003), regulation of meristem formation, floral and seed development (Penfield et al., 2001; Gregor et al., 2002; Shin et al., 2002; Steiner et al., 2003) and the control of the cell cycle (Ito et al., 2001; Araki et al., 2004). Some were also involved in various defense and stress responses (Vaillau et al., 2002; Abe et al., 2003; Denekamp and Smeekens, 2003; Nagaoka and Takano, 2003) and in light and hormone signaling pathways

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(Gocal et al., 2001; Seo et al., 2003; Newmana et al., 2004).

Transcription factors are important regulators of gene expression that are composed of at least four discrete domains, DNA binding domain, nuclear localization signal (NLS), transcription activation domain, and oligomerization site, which operate together to regulate many physiological and biochemical processes by modulating the rate of transcription initiation of target gene (Du et al., 2009e). MYB proteins constitute a huge family with the largest number of members in plants: 190 have been deduced in *Arabidopsis*, 156 in rice, and more than 200 estimated in maize (Borevitz et al., 2000; Goff et al., 2002; Dias et al., 2003). The large size of the MYB family in plants indicates their importance in the control of plant-specific processes. MYB transcriptional regulators contain conserved DNA-binding domains that are usually composed of one, two, or three imperfect 51 or 52 residue repeats (R1, R2, and R3). Each repeat encodes three α -helices, with the second and third helices forming a helix-turn-helix (HTH) structure when bound to DNA (Gabielsen et al., 1991; Ogata et al., 1994). In plants, MYB family members are classified into three subfamilies including R1-MYB, R2R3-MYB, and R1R2R3-MYB. In the past decades, R2R3-MYB genes have been extensively studied and members of the MYB family have been found to be involved into diverse physiological and biochemical processes. R1-MYB proteins, such as LHY, CCA1, and CCP1 in *Arabidopsis* and IBP1 in Maize, containing only one conserved domain, play a significant role in maintaining the integrity of chromosome structure and regulating gene transcription (Lugert and Werr, 1994; Yu et al., 2000; Mizoguchi et al., 2002). Some members of the MYB family have been found to be involved in control of cell cycle and regulation of cell differentiation, such as MYB3R1 and MYB3R4 in *Arabidopsis* (Dai et al., 2007; Haga et al., 2007). However, in plants, the overwhelming majority of MYB proteins are R2R3-MYB containing two conserved MYB domains.

A large number of MYB proteins have been found in plants, most of them are R2R3-MYB, and however, only a few of R1-MYB have been functionally verified in terms of the largest number of associations for MYB transcription factors in plants. The trials introduced here aimed to isolate the cDNA sequence of a new R1-MYB gene, *OsMyb1R* in rice, whose expression patterns in expression profiles were confirmed by quantitative real-time RT-PCR analysis. According to the data from both microarray and qRT-PCR, and sequence analysis, *OsMyb1R* is assumed as a putative MYB transcription factor and may play an important role in rice response to diverse abiotic stresses.

MATERIALS AND METHODS

Plant materials and stress treatments

Seeds of cultivated rice, Pei'ai 64S (*Oryza sativa* L.), were

sterilized with 0.1% HgCl₂ for 10 min, water washing 3 times, seeds soaking was done for 3 days under 25°C, changing the water once a day, regeneration for 2 to 3 days under 37°C, broadcast in batches in net basin of Institute of Subtropical Agriculture, Chinese Academy of Sciences. At five-leaf stage, part of them are took as the test material of the seedling stage; other parts, as test material of booting and flowering stage, are transplanted to the other pots, 5 plants each pot, and was put in the greenhouse under natural conditions, regular water and fertilizer management, pest and disease control. For drought treatment, pots were put in dry shed, the basin water was tossed away and collected materials after 16 h; the leaves began to curl, meanwhile, the control group was also put in the dry shed, but with water in pots. For heat treatment, materials were put in climate incubator, PGC15.5 (produced by Percival, USA) for 2 h under 45°C, while the control group was put into PGC15.5 under 45°C. Both treatment and control group are under the dark conditions. For cold treatment, put seedling state materials into PGC15.5 for 12 h under 4°C, booting and heading stage materials for 16 h under 12°C. The control group was placed into another PGC15.5, and both control and treatment group was under dark conditions.

Isolation of total RNA and cDNA cloning

Total RNA was extracted using TRIzol (Invitrogen) from materials collected from second to last leaf of both treatment and control group when plants had 4 to 5 leaves and from the middle of the young panicle or flower spike. RNA quality and concentration were checked by agarose gel electrophoresis and spectrophotometer analysis, and the RNA samples were stored at -70°C until use. Full-length *OsMyb1R* was amplified using PCR incorporating a unique *Hind* III restriction site immediately upstream from the translational start codon (*OsMyb1R*-F: 5'-AAGCTTATGGAGATGGCCTGTTTGC-3') and a unique *Eco*R I restriction site immediately downstream from the termination codon (*OsMyb1R*-R: 5'-GATCCGCCTC-TTCTTCCCGCCTTA-3'). All the PCR products were purified using Gel Extraction Mini Kit (Biomed, China) and the amplified fragment was cloned into vector, pMD18-T (TaKaRa, Dalian, China), sequenced by Invitrogen.

Microarray data analysis and real-time quantitative PCR analysis

The process was according to protocols previously described by Xu, Jiang and Dong (Xu et al., 2008; Jiang et al., 2011; Dong et al., 2011). Briefly, according to Affymetrix expression microarray experiments manual provided by GeneTech Biotechnology Limited Company, Shanghai, the following steps are: (1) Total RNA extraction and purification; (2) cDNA synthesis and purification; (3) cRNA synthesis and cRNA transcription purification in vivo; (4) cRNA fragmentation, preparation of hybridization solution; (5) chip hybridization; (6) elution chip; (7) scan chips; (8) data analysis. Expression levels of *OsMyb1R* in the microarray were verified further by real-time qRT-PCR. Total RNA was extracted with Trizol reagent. Prior to qRT-PCR, RNA was diluted to 20 ng/ul. All primers were designed through software Primer Expression 3.0. For quantitative real-time PCR, SYBR Green RT-PCR One Step Kit (QIAGEN, Cat. No. 204243) and fluorescent real-time quantitative PCR instrument was used to detect the PCR products, and used 18S as beta-actin. According to the sequence information obtained from the Gramene (<http://www.gramene.org/>), we designed the primer pairs used in the real-time PCR. *OsMyb1R*-F: 5'-GAGCAGCATGCAACAGTTTCTC-3', *OsMyb1R*-R: 5'-GGCTCCCGTTGTAGCTCAAC-3'; 18S-F: 5'-CGTCCCTGCCCTTTGTACAC-3', 18S-R: 5'-CGAACACTTCACCGGATCATT-3'. The relative expression levels

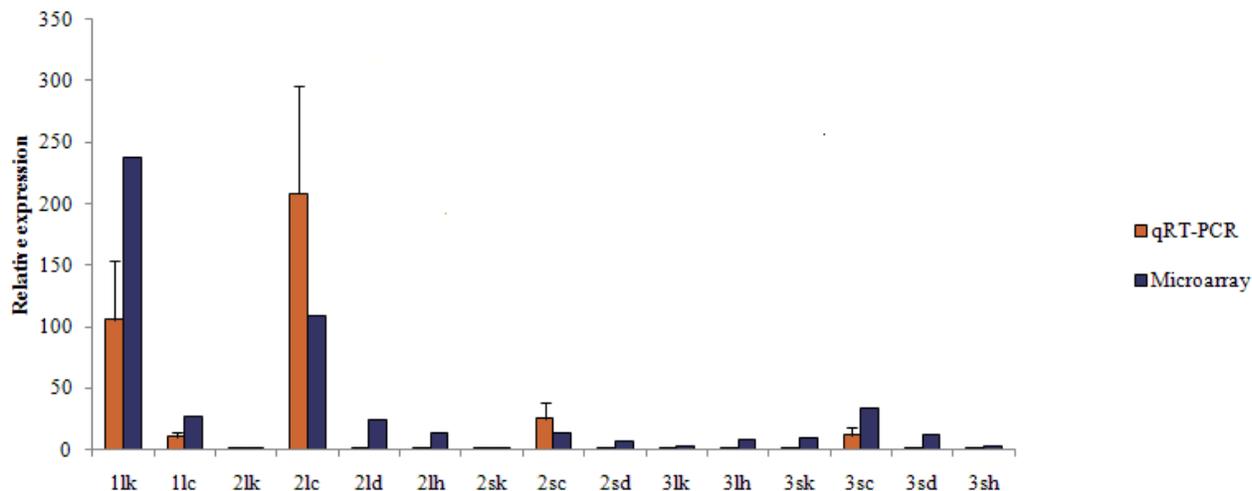


Figure 1. Relative expression of *OsMyb1R* in leaves and panicles of *indica* rice cultivar Pei'ai 64S under the various stresses and in normal growth conditions, at different developmental stages. 1: Seedling stage; 2: Booting stage; 3: Heading and flowering stage; L: Leaf; S: Spike; K: Control; C: Cold; H: Heat; D: Drought. (The error bars for the qRT-PCR represent standard error).

of *OsMyb1R* were normalized to the 18S gene using the method described by Ma and Chen (Ma et al. 2009; Chen et al. 2011).

Sequence analysis

The analysis and comparison of the deduced amino acid sequence with published sequences were performed with BLASTp (Standard Protein-FASTA) (Altschul et al., 1990) on the NCBI server (<http://www.ncbi.nlm.nih.gov/>). Promoter analysis of 1500 bp, upstream of *OsMyb1R* gene was performed with PlantCARE on the web (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). *OsMyb1R* gene was aligned with other MYB proteins from different species using DNAMAN.

RESULTS

Microarray and qRT-PCR analysis of *OsMyb1R* subjected to different abiotic stress

Expression profiles were obtained for leaf and panicle tissues at seedling, booting and heading stages from plants under no stress, or cold, drought or heat stresses using the GeneChip Rice Genome Array (Affymetrix) representing 51 279 transcripts. A large number of genes highly up regulated or down regulated were identified under stress. One of these genes, *OsMyb1R*, coding for a putative MYB trans-acting protein showed significant changes at expression level at different developmental phases subjected to different abiotic stress. In order to verify the reliability of microarray data, the expression patterns of *OsMyb1R* was further studied by using qRT-PCR to analyze the materials which were used to microarray analysis (Figure 1). The differential level and dividing point of *OsMyb1R* relative expression level was the same as microarray results. Under cold treatment, the

expression patterns of *OsMyb1R* changed greatly at different developmental phases, tissues and organs. The *OsMyb1R* was down-regulated greatly at seedling stage under 4°C cold treatment, while up-regulated at the booting and flowering stage under 12°C cold treatment. Under both drought and heat treatment, *OsMyb1R* was up-regulated significantly compared to control at different developmental stages, tissues and organs.

Cloning and characterization of the rice *OsMyb1R* gene

Sequence analysis revealed that *OsMyb1R* consists of 3.04 kb nucleotides corresponding to Nipponbare LOC_Os02g46030. Six exons and five introns were identified in *OsMyb1R*. We analyzed the possible promoter region, 1500 bp upstream from the start codon of *OsMyb1R*, which may contain different kinds of promoters by using online software, PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). Different cis-elements relating to response to different abiotic stress in rice are observed in this region, including ABRE (ABA Binding Responsive Element), ARE (Anaerobic Responsive Element), CE3 (involved in ABA and VP1 responsiveness), HSE (involved in heat stress responsiveness), LTR (low-temperature responsiveness), and MBS (MYB binding site involved in drought-inducibility) (Figure 2). The 1467 bp open reading frame (ORF) encodes a putative protein, OsMybR1 which contains 489 amino acids and has a calculated molecular weight of 51 kDa with a $pI = 6$. A nuclear localization signal (NLS) sequence was found immediately downstream of the R1 domain and an acidic Ser/Thr rich area was observed in the downstream region of the NLS

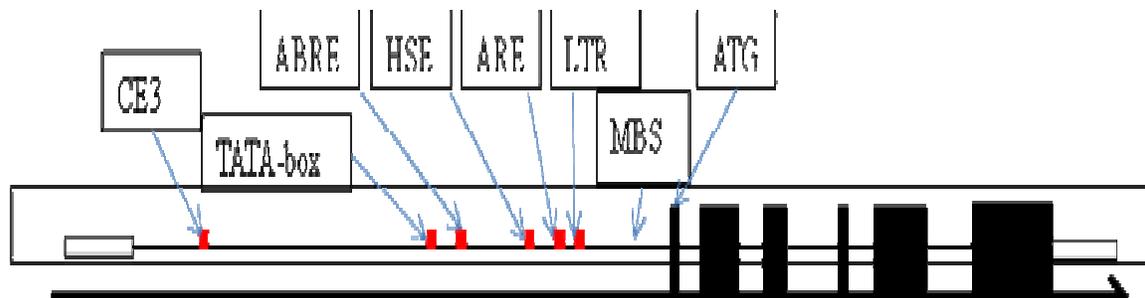


Figure 2. Structure of *OsMybR1* gene and candidate cis-elements in the putative promoter region. CE3, LTR, HSE, ARE, MBS, and ABRE are cis-elements involved in ABA and VP1, low temperature, heat stress, anaerobic, drought, and ABA responses respectively. The exons are boxed with black.

fragment (Figure 3).

OsMybR1, the deduced protein, contains a highly conserved single DNA binding domain that is very similar to the DNA binding domains of other mammalian, *Drosophila*, and plant MYBs (Figure 4). Compared with other MYB proteins that contain a 1R in their DNA binding domains, the 1R residues of the *OsMybR1* is most closely related to *AtEPR1* and *ZmMYBE1*, is most distant from *AtMYBL2*, and *PcMYB1*. *StMYB1* (*MybSt1*) transactivates the 35S RNA gene of the Cauliflower mosaic virus (*CaMV35S*) promoter and is expressed in various organs of potato (Baranowskij et al., 1994). *AtMYBL2* is expressed in *Arabidopsis* leaf, but its function has not been characterized (Kirik and Umlein, 1996). *PcMYB1* interacts *in vivo* with a light-regulatory promoter unit and is expressed in parsley cell cultures and seedlings (Feldbrugge et al., 1997). *AtMYBCCA1* binds to the promoter of an *Arabidopsis* light-harvesting chlorophyll *a/b* binding protein gene, *Lhcb*, and mediates the phytochrome regulation of *Lhcb* (Wang et al., 1997). *OsMYBS1*, *OsMYBS2* and *OsMYBS3* mediate sugar and hormone regulation of α -*Amylase* gene expression (Lu et al., 2002). *AtLHY* controls circadian rhythms and the photoperiodic control of flowering (Schaffer et al., 1998). *AtEPR1* mediate the correct oscillatory behavior of target genes (Kuno et al., 2003). *ZmMYBE1* has an important role in developmental regulation in maize (Guan J et al., 2009).

Comparison of amino acid residues among the 1R regions of *OsMybR1* and other MYBs in different species shows that the 1R regions contain conserved Trp (W), Glu (E), Gly (G), and Arg (R) at the same positions. The Trp residue, which plays a critical role in stabilizing the DNA binding domain of animal MYBs (Ogata et al., 1992) is conserved at the first and second positions, but not at the third position, among most of the 1R regions. Phylogenetic tree was obtained using the DNAMAN program based on R1-domain sequences of R1 proteins.

DISCUSSION

In the present study, an *OsMybR1* gene that encodes

MYB protein with single DNA binding domain was isolated and characterized. The structurally conserved R1 MYB domain suggests its important role in rice as compared with other species (Jin and Martin, 1999) and the existence of the acidic Ser/Thr-rich area reveals its potential function in transcriptional regulation. It has been proposed that a single original MYB repeat was replicated to give rise to two- and three-repeat MYBs (Lipsick, 1996). Because in c-Myb, both R2 and R3 are necessary for DNA binding and the putative base-contacting residues in the third helix of 1R is different from that of R2R3, it is likely that the 1R MYB binds DNA in a different manner (Lu et al., 2002). Proteins containing only one MYB DNA-binding domain play vital roles in chromosomal structural maintenance and light cycle response in plants. In maize, *IBP1* with a single MYB domain has been revealed (Lugert and Werr, 1994), which encodes a telomeric DNA binding protein (Ogata et al., 1992) and affects developing cells from the root or shoot apex and the gibberellins hormone balance (Klinge et al., 1997). In *Arabidopsis*, several R1-MYB proteins, such as *EPR1* (Kuno et al., 2003), *LHY* and *CCA1* (Mizoguchi et al., 2002) have been isolated and confirmed as circadian oscillators involved in developmental modulations in plants. Alignment of the MYB domains of eleven plant 1R MYBs shows that some of these domains contain the highly conserved SHAQK (Y/F)F motif in the third predicted α -helix (Figure 4). This family of MYBs containing 1R may recognize a DNA sequence motif different from those recognized by other MYBs, for the reason that the third α -helix of MYBs has been shown to be involved in the sequence specificity of DNA binding (Gabrielsen et al., 1991).

In this trial, at seedling stage, the *OsMybR1* gene showed significant decline under 4°C for 16 h as compared to the control, however, it showed great increase under 12°C for 16 h as compared to the control at booting stage. Two main reasons may account for these changes. First, different temperature used to treat the materials may result in the significant change of *OsMybR1* at seedling and booting stage. The second is that different developmental phases may have great

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1  ATGGCCTGTTTGCAGGGAACGCCATGGCAACCGACGAAAACGGTGCCGACGATCGGCCGGCGGAGAGCACCCTGGATCATCTCAGG
1  M A C L Q G N A M A T D E N G A D D R A G G E S T V D H L R
91  AGCCATATGAACACTACGGCGACATGGATTTGTGAGGGAAGAGCACGTGCCAAAGGCGGAAAGCCGTACACGATCACGAAGCAGCGCGAG
31  S H M N Y G D M D L S G E E H V P K A R K P Y T I T K Q R E
181  AAGTGGACGGACGAGGAGCAGGCTGTTCTTGAAGCCCTGCAGCTGCACGCCGCGCATGGCGCCGTATAACAAGAGCACATAGGTACC
61  K W T D E E H R L F L E A L Q L H G R A W R R I Q E H I G T
271  AAGACTGCCGTGCAAAATCCGTAGCCACGCGCAGAAGTCTTCTCTAAGGTCGTCAGAGAATCTTCGGGGAGTAACACCGGCTCGGGCGGC
91  K T A V Q I R S H A Q K F F S K V V R E S S G S N T G S G G
361  GCGTCCCGCGGGCGGCGGCCATCCAGATCCCTCCGCGCGGCGGAAGAGGAAGCCGCGCACCCGTACCCGCGCAAGGTGGAC
121  A S A A A A A A A I Q I P P P R P K R K P A H P Y P R K V D
451  GCGCGGCCAAGAAGCAGTCCCGCGCTCAGGACGTGGAGAAGCCCGTGTGGATGACAGTCCCTGTCGAGCAGGAGGGCTCGCCG
151  G A A K K H V P A L R Q L E K P P L W M Q S L S E Q E G S P
541  ACGTCGGTGTGACGGCGCGCAGATAGCACCGAGGCCCTGGGCGTGGTCTCGAATAACTCGAGCGGCAGCGGGTTCGTTGGCTCCG
181  T S V L T A A Q I G T E A L G G G F S N N S S G S G S L A P
631  TCAGCCCGGTTACGGATGAGCATGTCGACGGTGGCGGCTCGCCGCGTCTGCGTGGACAGAGAGCAGGGTGCCTCTACCGAGCATC
211  S A A G T D E H V D G G G S P A S S V D R E D G C L S P S I
721  CCGACTGCTGAGTTGGCTATGCAGGCGCAAATACTAAGATGTCAATTGCAACCACGGATGCCAAAGAAGCATCCTCAGAAGCATCAGTC
241  P T A E L A M Q A P N T K M S I A T T D A K E A S S E A S V
811  TTCAGGCTATTCGAAAGAGCGTAGTGGTTAAGGATTCAGACCAGTGCACCTGCTTAATGGCAGTAACATTGCAACGAGTGGTTCAGTT
271  F R L F G K S V V V K D S D Q L H L L N G S N I A T S G S V
901  GAGAGAGCAACCAGAAACATACTAGTACCTTCTTTGCTGCTCCCCAGAAGGGAGCTCGTCAATCCATGGCCGAGCAGCATGCAACAG
301  E R A T R N I L V P S F A A A P E G S S S N P W P S S M Q Q
991  TTTCTACTTCTCTCGATCGGATGGTTTCGCCGCGCAACCTGTCATGCCATGGTTCAGCTACAACGGGAGCCTTCCATGCGCGCTG
331  F L Y F L P R S D G F A A Q P V M P W F S Y N G S L P C A L
1081  TTCTACCCGGCGGGCGGCGGCTGCGAACCAGCAGTGCACCGTATTGAGAGGGCGTAGAGTTCAGAGTCTCGCAGAGGGAAGGA
361  F Y P A A A A A A N Q Q C H R D S E G V E F R V S Q R E G
1171  TCGCTGACGGGCTCGAACACGGCTCCAGCGTCTGCTGGATCGTCGGCGCGGTACCGCGGCGGCGGCGGCTCAGAATTCGGAC
391  S L T G S N T A S S V V L G S S A A V P A A A A A A Q N S D
1261  GTCGCGGAGTCCCGTGGCAAGGAACAGCAGGAGGCGGCGCTCGCCACGGCTGACCAAGTGCAGAGCTCGGCGTCCGTACCCTG
421  V A E S R G Q G N S R E A A A S P R L T K C E S S A S V T L
1351  CTCGAGAGGGCTTCATGCCGTACAAGAGGTGCGCGCGGAGAGCGAGTCTGTCGATCGGAGGCCCGGAGGAGGAGGCGCTCGCC
451  L Q R G F M P Y K R C A A E S E L L R S E A A G G E E A V A
1441  GACGGTGAAGCTGACAAGGCTGTGCTTGA
481  D G E L T R L C L ☆

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Figure 3. The deduced ORF of *OsMybR1*. The putative translation initiation site (ATG) and stop codon (TAG) are highlighted with yellow. The R1 domain is single underlined and the SNL is double underlined.

impacts on expression of *OsMybR1* gene. Therefore, the expression pattern of *OsMybR1* may be affected by both temperature and developmental phase. In both microarray and qRT-PCR experiments, *OsMybR1* showed great changes at expression level under different abiotic stress at different developmental stages, which implies that there are more important roles for *OsMybR1* at both the seedling and booting stage in response to abiotic stress. Therefore, we take *OsMybR1* as an

important candidate gene for abiotic stress tolerance.

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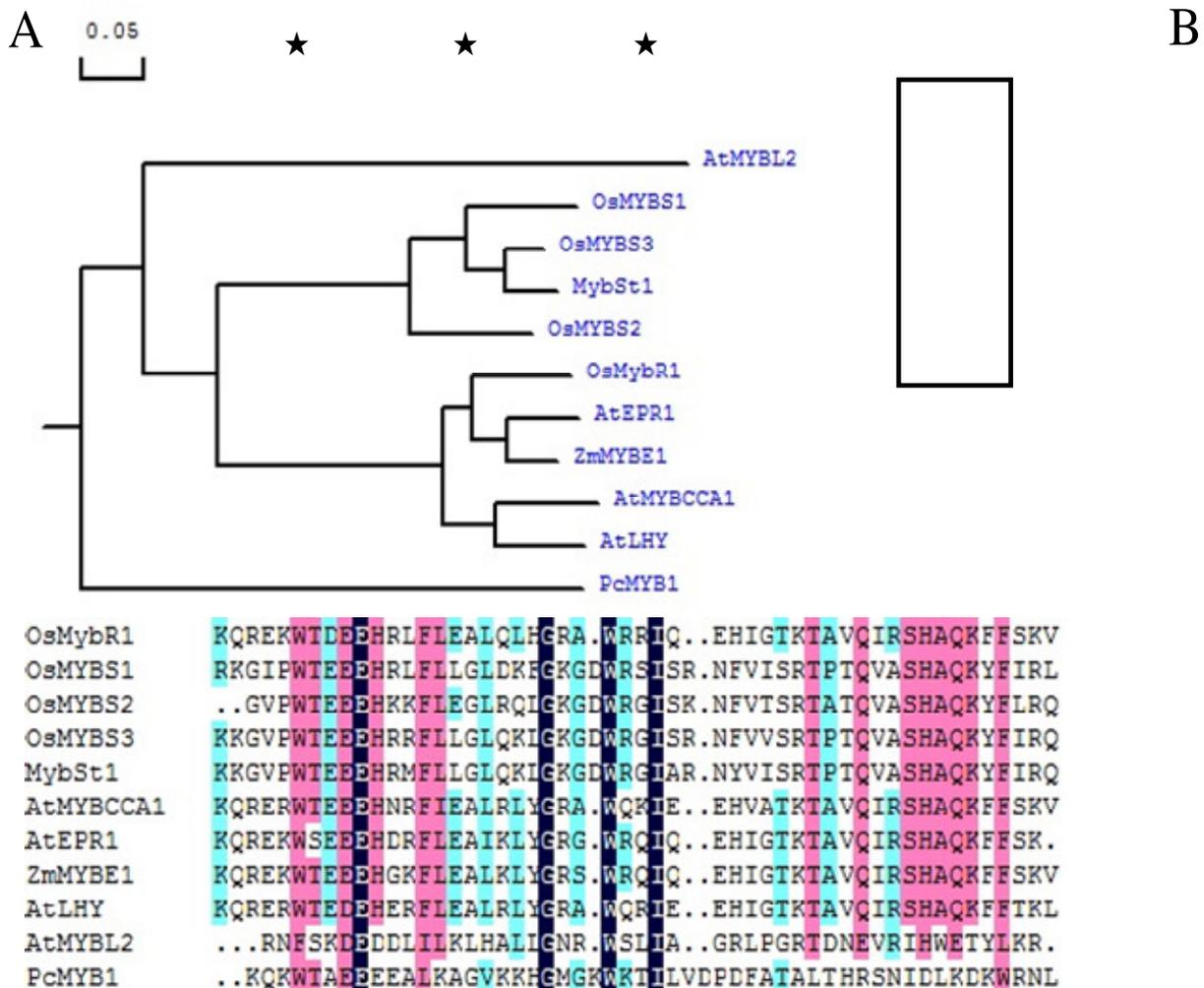


Figure 4. Highly conserved amino acid residues are present in 1R domains of the plant MYBs and phylogenetic tree. A: Highly conserved amino acid residues are present in 1R domains of the plant MYBs. The amino acid sequences of the 1R domains were derived from the DNA binding domains of the following sources: AtMYBL2, AtMYBCCA1, AtEPR and AtLHY from Arabidopsis (Kirik and Bäuml, 1996; Wang et al., 1997; Kuno et al., 2003; Schaffer et al., 1998); STMYB1 from potato (Baranowskij et al., 1994); and PcMYB1 from parsley (Feldbrügge et al., 1997); Three OsMYBSs from rice (Lu et al., 2002). ZmMYBE1 is from maize (Jia et al., 2009). The three regularly spaced Trp residues present in each repeat of the MYBs are labeled with asterisks; The amino acid sequences are aligned, and gaps (dots) have been introduced to maximize the alignment. The highly conserved SHAQK(Y/F)F motifs in 1R are boxed. B: The phylogenetic relationship of OsMybR1 and other ten R1-MYB proteins based on R1 domain sequences using the DNAMAN program.

analysis.

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