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The *OsMyb4* gene family: stress response and transcriptional auto-regulation mechanisms

E. BALDONI*, A. GENGA, A. MEDICI**, I. CORAGGIO, and F. LOCATELLI

CNR, Institute of Agricultural Biology and Biotechnology, via Bassini 15, I-20133 Milan, Italy

Abstract

The rice *OsMyb4* gene, which encodes a Myb transcription factor (TF), improves the stress tolerance/resistance when expressed in both monocotyledonous and dicotyledonous transgenic plants. In this study, a phylogenetic analysis showed the existence of putative OsMyb4 homologues in monocot and dicot species. In particular, the analysis revealed that *OsMyb4* belongs to a small rice gene subfamily conserved among monocots. The expression analyses of the *OsMyb4*-like genes in rice, wheat, and *Arabidopsis* indicated that these genes are involved in the response to dehydration, cold, and wounding. Moreover, the *in silico* analysis of the 5' upstream regions of the *Osmyb4*-like genes highlighted that the positions of some *cis*-elements involved in the stress response were conserved among the putative promoters, especially between *OsMyb4* and its putative paralog *Os02g41510*. Finally, our transient expression assays in tobacco protoplasts demonstrated that OsMyb4 is able to repress the activity of both its own promoter and the *Os02g41510* promoter by acting on the same binding site. A compensatory mechanism of auto-regulation is consistent with the well-known complexity of the OsMyb4-activated pathway, and this mechanism could regulate the transcription of other genes belonging to the family.

Additional key words: *Arabidopsis thaliana*, Myb transcription factor, *Nicotiana tabacum*, *Oryza sativa*, *Triticum aestivum*.

Introduction

Plants respond to abiotic and biotic stresses through the coordinated expression of many genes that are modulated by the action of several transcription factors (TFs). The overexpression of these TFs in transgenic plants may result in an enhanced stress tolerance (Dubouzet *et al.* 2003, Fujita *et al.* 2006, Ding *et al.* 2009, Agarwal and Jha 2010, Lourenço *et al.* 2011, Mao *et al.* 2011, He *et al.* 2012, Movahedi *et al.* 2012, Todaka *et al.* 2012, Yang *et al.* 2012).

Among the TF-encoding genes, the rice *OsMyb4* gene (accession No. Y11414, LOC_Os04g43680), which was previously isolated in our laboratory (Pandolfi *et al.* 1997), is involved in the response to abiotic and biotic stresses. We demonstrated the ability of OsMyb4 to positively regulate the transcription of genes involved in the cold response, *i.e.*, genes encoding the *Phaseolus vulgaris* phenylalanine ammonia lyase and the *Solanum commersonii* Δ^9-desaturase *via* the direct interaction with their promoters (Vannini *et al.* 2004). A comparative microarray analysis of wild-type and *OsMyb4*-expressing *Arabidopsis* plants revealed that OsMyb4 affects the expression of genes involved in both the abiotic and biotic stress responses (Vannini *et al.* 2006). Moreover, the *OsMyb4* gene is able to improve the stress tolerance/resistance when ectopically expressed in both monocots and dicots *via* the activation of some metabolic pathways, such as the chorismate and phenylpropanoid pathways (Vannini *et al.* 2004, 2006, 2007, Mattana *et al.* 2005, Pasquali *et al.* 2008, Laura *et al.* 2010, Soltész *et al.* 2012, Docimo *et al.* 2013). Recently, Park *et al.* (2010) reported that OsMyb4 induces stress tolerance in transgenic rice plants through the direct or indirect activation/repression of several genes with putative roles in stress defence, confirming in the homologous system the results that were previously obtained in heterologous species. Altogether, the results from the transgenic plants

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Abbreviations: ABA - abscisic acid; BS - bluescript; CaMV - cauliflower mosaic virus; GUS - β-glucuronidase; MBS - Myb binding site; qRT-PCR - quantitative reverse transcription-polymerase chain reaction; RH - relative humidity; TF - transcription factor.

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** Present address: Integrative Biology Institute for Plants, UMR5004, Biochemistry and Plant Molecular Physiology, CNRS/INRA/Supagro/UM2, 2 Place Viala 34060 Montpellier Cedex 1, France

* Corresponding author; fax: (+39) 0223699411, e-mail: baldoni@ibba.cnr.it.

indicate that OsMyb4 is a crucial component of the stress-signalling network and strongly suggest that it is conserved among species.

In this study, we used a phylogenetic analysis to identify putative OsMyb4 homologues in both monocot and dicot species. Subsequently, expression analyses of the *OsMyb4* gene and its cognates in rice, wheat, and *Arabidopsis* were performed to evaluate their responses to environmental stimuli. Moreover, *in silico* analyses of the 5' upstream regions of the *OsMyb4*-like genes were carried out to identify putative *cis*-elements involved in the stress response. Finally, we tested the ability of OsMyb4 to regulate the transcription driven by its own promoter or by the promoter of its putative paralog *Os02g41510*.

Materials and methods

A *BLASTp* search in the *Phytozome* and *NCBI* databases (www.phytozome.net; www.ncbi.nlm.nih.gov) using the complete amino acid sequence of OsMyb4 was performed on selected species to identify the putative homologous proteins. The Myb factors showing the maximum score and the maximum query coverage (5/6 proteins for each species) were selected to construct the phylogenetic tree. The sequence alignments were generated with *ClustalW2* (http://www.ebi.ac.uk/Tools/msa/clustalw2/; Larkin *et al.* 2007). To reveal the relationship between OsMyb4 and its homologues, a neighbour-joining tree was constructed with the package *PHYLIP v. 3.69* (Felsenstein 1989) and visualised with the *TreeView* software (Page 1996).

For the gene expression analysis under stress conditions, seeds from each species were sterilised and placed onto Murashige and Skoog (1962; MS) agar medium. Rice (*Oryza sativa* L. ssp. *japonica* cv. Arborio) was sterilised with 70 % (v/v) ethanol for 2 min followed by 5 % (m/v) Ca(ClO)$_2$ for 30 min. Wheat (*Triticum aestivum* L. cv. Chinese Spring) was sterilised with 5 % (m/v) NaClO for 30 min. *Arabidopsis thaliana* L. (cv. Wassilewskija) was vernalised at 4 °C in the dark for 3 d and sterilised with 4 % NaClO for 7 min. The seedlings were grown *in vitro* in a growth room under irradiance of 300 µmol m^{-2} s^{-1} and 60 % relative humidity (RH) with the following species-specific conditions: rice at 28/23 °C and a 14-h photoperiod for 3 weeks; wheat at 22/20 °C and a 12-h photoperiod for 2 weeks; and *Arabidopsis* at 24/22 °C and a 14-h photoperiod for 2 weeks. For the cold treatment, the seedlings were placed at 4 °C in the light for 24 h (long-term) or 4 h (short-term). For the desiccation treatment, the seedlings were placed in a closed Petri dish at room temperature (22 - 24 °C, 40 % RH) for 45 min. For the wounding treatment, the leaves were scratched with a scalpel. To evaluate the effect of the exposure to volatile compounds generated by wounded plants, the unwounded plants were maintained in the same tray as the scratched plants (equal numbers of wounded and unwounded plants). Both the wounded and unwounded plants were sampled 30 min after scratching. For each treatment, leaf pools of 7 - 8 seedlings were collected, frozen in liquid nitrogen, and stored at -80 °C until analysis. The leaves from untreated plants were pooled and collected at the same time of day as the treated seedlings.

Total RNA was extracted using the *TRIzol*® reagent (*Invitrogen*, Carlsbad, CA, USA) and subsequently treated with DNase I (*Roche Applied Science*, Indianapolis, IN, USA). Next, cDNA was synthesised from 0.5 µg of total DNase I-treated RNA using the *SuperScript III* first-strand synthesis *SuperMix* for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) according to the manufacturer's instructions (*Invitrogen*). The qRT-PCR was performed using 0.02 cm^3 triplicate reactions on a *7300* real time PCR system (*Applied Biosystems*, Foster City, CA, USA) according to Caruso *et al.* (2012). The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) using "not treated 1" samples (collected at 10 a.m.) as the calibrator for all experimental conditions. The following gene-specific forward (F) and reverse (R) primers were used: *OsMyb4* (LOC_Os04g43680): *F* 5'-AACCTTACTCTGCAGCCTCGG-3', *R* 5'-GTGCTTGCTCCTTCCCTGCT-3'; *Os02g41510* (LOC_Os02g41510): *F* 5'-ACCACGGACAGTTTCACCTC-3', *R* 5'-CTCATCTCCATCCCGGAGT-3'; *OsMyb8* (LOC_Os10g33810): *F* 5'-AACGATGGGAAATTCAGGTG-3', *R* 5'-TGAAGCCAGCTGATAACGAA-3'; *TaMyb2/2A* (AY615199): *F* 5'-GGAGACTTGGCTCCTGATTG-3', *R* 5'-GCAGCGCACTCTTCTCTGTT-3'; *TaMyb2B*: *F* 5'-GCTTCTGGTCGGAGACGCTG-3', *R* 5'-GCCGCCCTCCATGAACTCT-3' (Mao *et al.* 2011); *TaMyb7/2D* (AB252147): *F* 5'-GACGCGTTCGGCAAGC-3', *R* 5'-CGTCGTTGTTGTCGTCGT-3'; *TaMyb32* (JF951915): *F* 5'-GACAGCTTCTGGTCGGAGAC-3', *R* 5'-CATCTCATCGTTGCTCGAAG-3'; *TaMyb33* (JN584645): *F* 5'-GGCTCCTCGGCTAGTTTCTT-3', *R* 5'-ACCCACCGAATGATCAGAAC-3'; *TaMyb73* (JN969051): *F* 5'-GACAGCTTCTGGTCGGAGAC-3', *R* 5'-CGACGACGGCGATAAACTAT-3'; *AtMyb13* (AT1G06180): *F* 5'-ACCTCTCCGCAACAACAATC-3', *R* 5'-CGCGGAGTCTTTGTTGCTAT-3'; *AtMyb14* (AT2G31180): *F* 5'-AGAGGATTGGGAAGGATTGA-3', *R* 5'-AACTCGGGTATGTCGGAAAA-3'; and *AtMyb15* (AT3G23250): *F* 5'-ACGACGGCTATAGCAACGAG-3', *R* 5'-CGATATCCGCACCAAAAGTT-3'. The following genes were used as endogenous references: the rice *UBQ5* gene (AK061988; Jain *et al.* 2006), the *Arabidopsis UBQ5* gene (AT3G62250; Lolas *et al.* 2010), and the wheat *RNase L inhibitor-like protein* gene (Ta2776; Paolacci *et al.* 2009). The primers reported by the authors were used. The results of the qRT-PCR expression analysis of rice and *Arabidopsis* genes were compared to those available on the *Bio-Array Resource for Plant Biology* (*BAR*, http://bar.utoronto.ca/welcome.htm) using the *Arabidopsis* and rice *eFP Browser*.

Using *PlantPan* (http://plantpan.mbc.nctu.edu.tw; Chang *et al.* 2008), the 5’ upstream regions of the rice *myb* genes (1 000 bp from the start codon) were analysed for putative Myb binding sites (MBS) and for sites involved in the response to abscisic acid (ABA) and abiotic or biotic stresses, as reported in the *PLACE* database (http://www.dna.affrc.go.jp/PLACE/). The *cis*-elements most represented in the rice putative promoters were searched for in the 5’ flanking regions (when available on the *Phytozome* and *NCBI* databases) of the *OsMyb4*-like genes belonging to other species.

To construct pMyb4GUS, the 935 bp sequence upstream of the *OsMyb4* gene was PCR-amplified using the following primers: *pMyb4-F* 5’-GGTCCGTCAACCTCTCAACCA-3’ and *pMyb4-EcoRI-R* 5’-TTTGAATTCAACTGCTCTGCTCC-3’. Using an internal *Hind*III restriction site at -853 bp from the ATG, the 825 bp fragment (*Hind*III-*Eco*RI) was cloned into the Bluescript (BS) vector. Subsequently, the *Hind*III-*Bam*HI putative promoter fragment was cloned into the pCaMVGUS vector (Vannini *et al.* 2004) to yield pMyb4GUS. To construct pMyb41510GUS, the 944 bp sequence upstream of the *Os02g41510* gene was PCR-cloned into the pUCGUS vector (Giovinazzo *et al.* 1992) using the following primers: *pMyb41510-PstI-F* 5’-AACTGCAGCAGTGTTGTGCTAG-3’ and *pMyb41510-BamHI-R* 5’-CGGGATCCTCCTCTCGTTCGA-3’. The pMyb4GUS and pMyb41510GUS plasmids were mutagenised with the *QuikChange*$^{\text{TM}}$ site-directed mutagenesis kit (*Stratagene*, La Jolla, CA, USA) to yield pMyb4mutGUS and pMyb41510mutGUS following the manufacturer’s instructions. Namely, the site ELRECOREPCRP1/MYB2AT (ATTGACC) was mutated using the following primers: 5’-CTTTCCACGAAGTTGCCAAAA**GGT**ACCCATTGGAGCACGTTTTTGC-3’ and 5’-GCAAAAACGTGCTCCAATGGGT**ACC**TTTTGGCAACTTCGTGGAAAG-3’ for the *OsMyb4* putative promoter, and 5’-GCTGCGACGAAGCTGCCCCAA**GGT**ACCACGTTCGCTGTAGCCGCG-3’ and 5’-CGCGGCTACAGCGAACGTGGT**ACC**TTGGGGCAGCTTCGTCGCAGC-3’ for the *Os02g41510* putative promoter (the mutated bases are indicated *in bold*). The *Kpn*I site (GGTAC-C), which was created in the mutants, was used for colony selection.

Protoplast isolation and transformation, protein determination and β-glucuronidase (GUS) activity assays were performed as described in Locatelli *et al.* (2003). The results are the means of 4 independent experiments.

Results

An *in silico* analysis was performed to identify putative OsMyb4-like proteins in six dicot (apple, *Arabidopsis*, grapevine, *Medicago truncatula*, poplar, and tobacco) and four monocot (*Brachypodium distachyon*, maize, sorghum, and wheat) species in addition to rice. The resulting phylogenetic tree (Fig. 1) revealed that in rice, OsMyb4 belongs to a small subfamily that contains three members: OsMyb4, Os02g41510, and OsMyb8 (OsMYB55, OsMYB30, and OsMYB110, respectively, according to the *GRASSIUS* nomenclature). In monocots, three subgroups corresponding to the three rice Myb factors were present whereas in dicots, the subfamily of

Table 1. Relative expression of rice, wheat, and *Arabidopsis myb* genes under stress treatments. The samples for “Control 1”, “Desiccation” (45 min), “Cold 24 h”, “Wounding”(30 min), and “Volatile“ (compounds generated by the wounded plants) were collected at 10 a.m. The samples for “Control 2” and “Cold 4 h” were collected at 2 p.m. Means ± SD of three biological replicates. Comparisons of differences between means were performed with Student's *t*-tests (ns - not significant, * - $P \leq 0.05$, ** - $P \leq 0.01$, *** - $P \leq 0.001$). For statistical analysis, “Control 2”, “Desiccation”, “Cold 24 h”, “Wounding”, and “Volatile” were compared to “Control 1”, whereas “Cold 4 h” was compared to “Control 2”.

		Control 1	Control 2	*P*	Desiccation	*P*	Cold 24 h	*P*	Cold 4 h	*P*	Wounding	*P*	Volatile	*P*
Rice	*OsMyb4*	1.01±0.13	1.28±0.32	***	23.27±1.93	***	47.70±8.12	**	27.56±6.47	***	24.39±2.57	***	16.78±4.53	***
	Os02g41510	1.03±0.25	1.85±0.28	*	17.36±0.66	***	39.02±8.05	***	27.65±9.72	**	21.97±3.32	***	19.80±5.98	**
	OsMyb8	1.01±0.15	1.67±0.09	**	8.38±2.24	**	38.67±1.36	***	12.74±4.65	*	7.32±0.60	***	6.32±0.10	***
Wheat	*TaMyb2/2A*	1.00±0.04	1.66±0.02	***	1.80±0.15	***	0.67±0.24	*	0.95±0.11	***	4.95±1.10	**	2.22±0.68	*
	TaMyb7/2D	1.00±0.05	1.42±0.11	**	3.83±0.51	***	0.74±0.14	*	1.46±0.13	ns	6.80±1.67	**	2.45±0.70	*
	TaMyb2B	1.01±0.11	0.89±0.03	ns	6.12±0.76	***	0.51±0.02	**	1.87±0.07	***	11.32±3.58	**	2.20±0.68	*
	TaMyb32	1.09±0.44	3.89±0.60	**	16.91±0.88	***	0.65±0.16	ns	7.20±0.57	**	12.61±3.59	**	2.21±0.08	*
	TaMyb33	1.14±0.78	2.07±0.04	*	12.37±4.22	**	0.51±0.22	ns	5.45±0.40	***	10.78±3.00	*	2.22±0.06	*
	TaMyb73	1.05±0.32	2.71±0.44	**	8.48±0.60	***	0.52±0.14	ns	6.80±0.27	***	11.15±2.28	**	1.81±0.08	*
Arabidopsis	*AtMyb13*	1.10±0.66	1.25±0.30	ns	3.60±2.18	ns	4.29±0.20	***	3.12±1.02	**	1.44±0.76	ns	1.26±0.50	ns
	AtMyb14	1.07±0.37	0.99±0.24	ns	2.77±0.82	*	1.54±1.16	ns	4.35±1.25	**	7.13±0.32	***	0.77±0.04	ns
	AtMyb15	1.00±0.07	1.03±0.34	ns	11.20±2.07	***	0.53±0.22	*	0.98±0.40	ns	33.76±1.87	***	1.07±0.22	ns

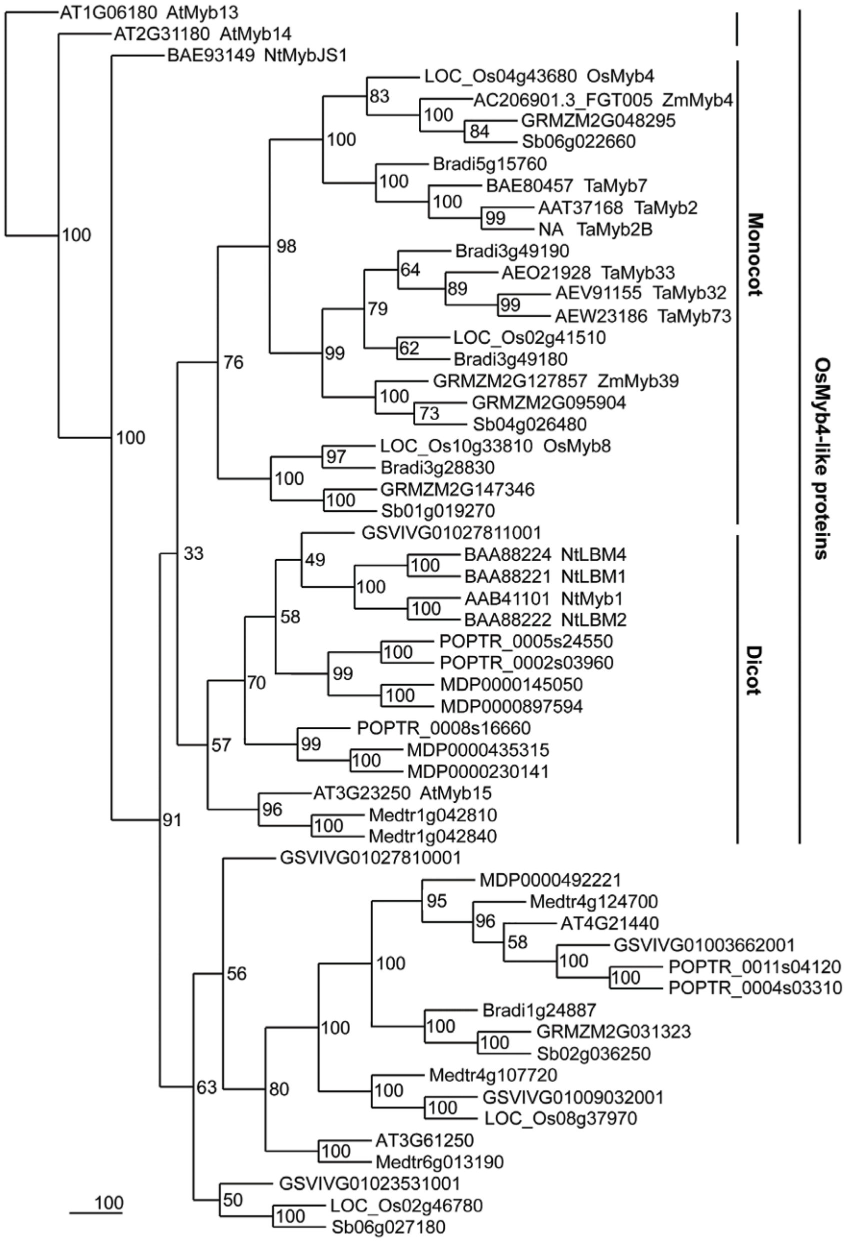

Fig. 1. Phylogenetic tree of OsMyb4 and Myb proteins in selected plant species. The phylogenetic tree was constructed based on peptide sequences using a neighbour-joining method with the *PHYLIP 3.69* package. The protein accession numbers and bootstrap values are shown. At - *Arabidopsis thaliana*, Bradi - *Brachypodium distachyon*, MDP - *Malus domestica*, Medtr - *Medicago truncatula*, Nt - *Nicotiana tabacum*, Os - *Oryza sativa*, POPTR - *Populus trichocarpa*, Sb - *Sorghum bicolor*, Ta - *Triticum aestivum*, GSVIV - *Vitis vinifera*, GRZM (Zm) - *Zea mays*, NA - not annotated.

putative OsMyb4-like proteins did not show a clear organisation in the three subgroups. Some closely related Myb factors, from both monocot and dicot species, clustered independently to the OsMyb4-like proteins. A multiple sequence alignment of the monocot OsMyb4-like proteins showed the expected high similarity of the Myb domain and the presence of two more conserved amino acid motifs in the COOH region; further conserved amino acid regions characterised the three subgroups (Fig. S1, *see on-line*). A multiple alignment of both monocot and dicot proteins showed a lower similarity of the two conserved motifs (data not shown).

Expression analyses of rice, wheat, and *Arabidopsis OsMyb4*-like genes were performed on the seedlings subjected to desiccation, cold, wounding, and exposure to volatile compounds generated by the wounded plants (Table 1). In rice, the expression of the three genes was induced in all experimental conditions with stress-specific expression patterns. The transcription of *OsMyb4* and *Os02g41510* was strongly induced by all treatments (17- to 48-fold change) whereas *OsMyb8* expression was strongly induced by the long-term cold treatment (39-fold change) and to a lesser extent by the other stress conditions (6- to 13-fold). A comparison with the stress-driven expression data available on the rice *eFP* browser confirmed the response to the drought and cold treatments of all three rice genes. In wheat, the expression of the three *TaMyb2* genes (*TaMyb2/2A*, *TaMyb7/2D*, and *TaMyb2B*) was induced by desiccation, wounding, and to a lesser extent by volatile compounds. Under the long-term cold treatment, the expression of the three *TaMyb2* genes was slightly repressed. Under the short-term cold treatment, *TaMyb2B* transcription was slightly induced whereas *TaMyb2/2A* transcription was slightly repressed. *TaMyb32*, *TaMyb33*, and *TaMyb73* transcription increased in response to the desiccation, short-term cold treatment, wounding, and to a lesser extent to volatile compounds. In *Arabidopsis*, the *myb* genes showed specific expression patterns under these stresses. *AtMyb13* transcription was slightly induced under long- and short-term cold stress whereas *AtMyb14* transcription significantly increased under desiccation, short-term cold treatment, and wounding. *AtMyb15* expression was strongly induced under desiccation and wounding treatments (11- and 34-fold change, respectively) and repressed under long-term cold stress. Unlike the rice and wheat genes, the *Arabidopsis* genes did not show any transcriptional changes depending on the day-time or in response to the exposure to volatile compounds generated by the wounded plants. The data available on the *Arabidopsis eFP* browser indicate that *AtMyb13* is down-regulated both in the shoots and roots after 12 and 24 h of cold treatment; *AtMyb14* is down-regulated in the shoots after 3 and 6 h of cold treatment, whereas it is up-regulated in the roots after 30 min and 12 h of the treatment. *AtMyb14* expression is induced by desiccation and wounding both in the shoots and roots, with some differences in the treatment time and induction level. For

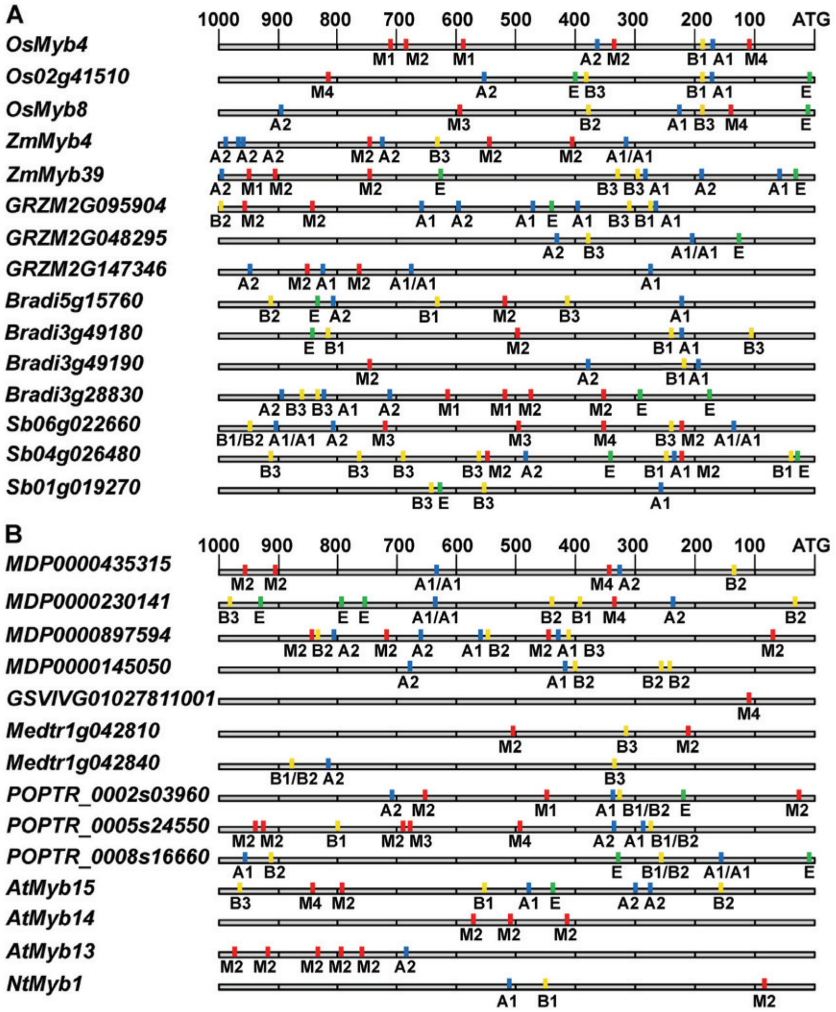

Fig. 2. *In silico* analysis of the *OsMyb4*-like gene putative promoters (1 000 bp) from monocot (*A*) and dicot (*B*) species. ABA-responsive (*blue lines*): ABRELATERD1 (ACGTG, A1); MYCATRD22 (CACATG, A2). Biotic stress-responsive (*yellow lines*): ELRECOREPCRP1 (TTGACC, B1); WBBOXPCWRKY1 (TTTGACT/C, B2); ASF1MOTIFCAMV (TGACG, B3). Environmental stress-responsive (*green lines*): LTRECOREATCOR15 (CCGAC, E). Myb binding sites (*red lines*): TATCCAOSAMY (TATCCA, M1); MYB1AT (WAACCA, M2); MYB2AT (TAACTG, M3); MYBPZM (CCWACC, M4). *Coloured lines* indicate the rough positions of *cis*-elements on the putative promoters.

AtMyb15, the observed responses to cold and wounding treatments are confirmed in the shoots, whereas no significant changes after the desiccation treatment are reported.

To determine whether the similar stress responses of some analysed genes might depend on analogous regulation mechanisms, the 5' upstream regions (1 000 bp from the start codon) of some of the *OsMyb4*-like genes were analysed for the presence of putative binding sites which are putatively involved in the stress response or in Myb binding (Fig. 2). This analysis showed the presence of several *cis*-elements shared by the analysed putative promoters. In addition, some *cis*-elements were present in a similar position on different 5' upstream regions especially for genes belonging to the same species. For instance, both the *ZmMyb4* and *ZmMyb39* putative promoters contained an ABA-responsive (MYCATRD22) MYC binding site located 986 and 994 bp from the ATG, respectively, and a MYB1AT MBS located 743 bp from the ATG. Moreover, two ABA-responsive (ABRELATERD1) partially overlapping binding sites were located in MDP0000435315 and MDP0000230141 putative promoters 635 and 641 bp from the ATG, respectively. The presence of a *W box* (*ELRECOREPCRP1*, TTGACC) in both *OsMyb4* and *Os02g41510* 5' upstream regions 184 and 189 bp from the ATG, respectively, is noteworthy. This sequence was situated the same distance from an ABA-responsive binding site (*ABRELATERD1*, ACGTG) in both putative promoters. These *cis*-elements were also present in the 5' upstream regions of *GRZM2G095904*, *Bradi3g49180*, *Bradi3g49190*, and *Sb04g026480* between 200 and 300 bp from the ATG. Finally, the W box was partially overlapping with a reverse MYB2AT (TAACTG) MBS in the sequence ATTGACC present in *OsMyb4*, *Os02g41510*, *Bradi3g49180*, *Bradi3g49190*, and *Bradi5g15760*. The conserved positions of these sequences suggested a possible common mechanism regulating these genes.

Based on the known ability of plant Myb TFs to auto-regulate their own transcription (Zhao *et al.* 2007, Espley *et al.* 2009), transient expression assays in tobacco protoplasts were performed to assess the transcriptional activity of OsMyb4 on the *OsMyb4* and *Os02g41510* putative promoters. The wild-type promoters and the corresponding forms mutated in the ELRECOREPCRP1/MYB2AT binding sites were used (Table 2). Both mutated promoters led to a 50 % reduction in the GUS activity compared to their wild-type forms. When the protoplasts were co-transformed with 10 µg of pCaMVMyb4, the OsMyb4 TF repressed the GUS activity driven by both wild-type promoters by 30 %, whereas it had no effect on the activity driven by both mutated promoters.

Table 2. Relative GUS activity [pmol(GUS) μg^{-1}(protein) min^{-1}] in transformed tobacco protoplasts. Each sample was transformed with 5 µg of pMyb4GUS, pMyb4mutGUS, pMyb41510GUS, or pMyb41510mutGUS. The amount of pCaMVMyb4 is indicated. Means ± SD of four independent experiments. Comparisons of differences between means were performed with Student's *t*-tests (ns - not significant, ** - $P \leq 0.01$, *** - $P \leq 0.001$).

	pCaMVMyb4 (0 µg)	pCaMVMyb4 (10 µg)	*P*
pMyb4GUS	90.6 ± 16.4	53.7 ± 3.0	**
pMyb4mutGUS	42.2 ± 3.8	38.9 ± 3.0	ns
pMyb41510GUS	52.7 ± 4.7	36.8 ± 1.5	***
pMyb41510mutGUS	26.7 ± 3.9	25.9 ± 2.9	ns

Discussion

The ectopic expression of the rice *OsMyb4* gene improves the stress tolerance/resistance in several species (Vannini *et al.* 2004, 2006, 2007, Mattana *et al.* 2005, Pasquali *et al.* 2008, Laura *et al.* 2010, Park *et al.* 2010, Soltész *et al.* 2012). The results from both monocot and dicot transgenic plants suggest a pivotal role for OsMyb4 in the stress signalling network and its conservation among species. Although the relationships between *OsMyb4* and other *OsMyb4*-like genes have previously been reported (Sugimoto *et al.* 2000, Fornalé *et al.* 2006, Gális *et al.* 2006, Mao *et al.* 2011, He *et al.* 2012, Katiyar *et al.* 2012, Zhang *et al.* 2012), our phylogenetic analysis suggests the presence of a single *OsMyb4* ancestor that originated prior to the separation of monocots and dicots. The putative ancestral gene most likely generated small gene families in various species through one or more duplications. In monocots, a direct relationships within three subgroups corresponding to OsMyb4, Os02g41510, and OsMyb8 were observed suggesting that the origin of the three Myb subgroups preceded the monocot speciation. Indeed, each rice protein corresponded to a single sorghum and to one or two *Brachypodium* and maize proteins suggesting a further duplication in these latter species. Due to the hexaploid nature of the *Triticum aestivum*, the relationship between rice and wheat is more complex. OsMyb4 corresponds to three wheat Myb proteins which are encoded by genes belonging to the three wheat genomes (A, B, D; Mao *et al.* 2011). Os02g41510 also corresponds to three wheat Myb factors; however, two of the factors (TaMyb32 and TaMyb33) were derived from the same D genome suggesting a further duplication in the wheat species (He *et al.* 2012, Qin *et al.* 2012, Zhang *et al.* 2012). To date, no putative homologous proteins of OsMyb8 have been reported, and it is expected that other wheat genes corresponding to *OsMyb8* and *Os02g41510* will be identified.

In dicots, the occurrence of duplication events was observed in most OsMyb4-like TFs. AtMyb13, AtMyb14, and NtMybJS1 clustered independently to the other OsMyb4-like TFs although the two corresponding *Arabidopsis* genes are widely reported as the putative orthologues of *AtMyb15*. This clustering suggests that a duplication event arose before *Arabidopsis* and tobacco diverged. AtMyb13 and AtMyb14 appear to have arisen from a further duplication. Due to the allopolyploidy of tobacco, it is expected that at least one or more tobacco genes homologous to *NtMybJS1* will be discovered (Sugimoto *et al.* 2000, Gális *et al.* 2006).

The expression analysis reported here indicates that the rice, wheat, and *Arabidopsis OsMyb4*-like genes are involved in the response to environmental stimuli. The induction of *OsMyb4* observed in all assayed conditions is in agreement with previous data (Vannini *et al.* 2004, 2006, Park *et al.* 2010). The similar induction shown by *Os02g41510* and the sequence similarity between the two TFs suggest that *Os02g41510* may be the paralogous gene of *OsMyb4*. In wheat, similar expression profiles under stress conditions were observed within each of the *OsMyb4*- and *Os02g41510*-like groups. The literature data indicate a role for some of these genes in the abiotic stress response. For instance, *TaMyb2/2A* confers an enhanced tolerance to multiple abiotic stresses (*i.e.*, drought, salinity, and freezing) when overexpressed in transgenic plants whereas *TaMyb73* confers salt tolerance and its expression is induced by jasmonic acid (Mao *et al.* 2011, He *et al.* 2012). *TaMyb33* is induced by NaCl, polyethylene glycol, and ABA treatments (Qin *et al.* 2012). With regards to *Arabidopsis*, our results about the strong induction of *AtMyb15* under desiccation and wounding are consistent with the literature data on the role of *AtMyb15* in the response to drought and pathogens (Chen *et al.* 2006, Ding *et al.* 2009, Liu *et al.* 2010). Some differences in the cold response of *Arabidopsis* genes were observed between our analyses and the literature data. Chen *et al.* (2013) demonstrated a down-regulation of *AtMyb14* under cold treatment and its involvement in freezing tolerance by affecting the expression of *CBF* genes. Agarwal *et al.* (2006) reported non-significant expression changes of *AtMyb13* and *AtMyb14* and a slight induction of *AtMyb15* under cold (4° C) stress. Moreover, Miura *et al.* (2007) reported that *AtMyb15* is strongly induced by freezing (0° C) and that its gene product acts as a CBF repressor, negatively affecting cold tolerance (Agarwal *et al.* 2006, Zhou *et al.* 2011). Conversely, the data on the abiotic stress response available in the *Arabidopsis eFP* browser indicate a down-regulation of *AtMyb15* in the shoots and an up-regulation in the roots up to 24 h of cold treatment. The specific experimental design used by various authors and differences in the analysed developmental stages and tissues of the plants may be responsible for the different characteristics reported in the stress response of *OsMyb4*-like genes. However, the involvement of the *Arabidopsis Osmyb4*-like genes in the cold stress response is clear. With regards to the other *OsMyb4*-like genes present in the phylogenetic tree, the involvement of tobacco genes in pathogen resistance has been thoroughly described (Yang *et al.* 1996, Sugimoto *et al.* 2000, 2003, Gális *et al.* 2006).

The *in silico* analysis on the 5’ upstream regions of the *OsMyb4*-like genes identified several *cis*-elements putatively responsible for a stress-driven expression pattern and for some conserved regulation mechanisms. In particular, the *OsMyb4* and *Os02g41510* putative promoters shared the sequence ATTGACC containing a W box, that is involved in the biotic stress response (Rushton *et al.* 2002) as well as a partially overlapping reverse MYB2AT binding site. Based on the known ability of reverse binding sites to interact with TFs (Kops *et al.* 2002, James *et al.* 2004), an involvement of this sequence in common regulation mechanisms can be envisaged. A comparison of our data with analogous analyses reported for the wheat genes (Mao *et al.* 2011, He *et al.* 2012, Qin *et al.* 2012) showed that the ELRECOREPCRP1 *cis*-element is also present in the putative promoter of the jasmonic acid-inducible *TaMyb73* gene (He *et al.* 2012). Mao *et al.* (2011) suggested that the expression of *TaMyb2* and other *OsMyb4*-like genes might be regulated not only through plant hormones and environmental stimuli but also through a “feedback control” driven by itself or by its family members. The transactivation experiments in tobacco protoplasts reported here showed that OsMyb4 is able to repress the activity of the *OsMyb4* and *Os02g41510* promoters. Using mutated promoters, we assessed whether the sequence ATTGACC could be related to this OsMyb4 activity. Both mutated promoters showed lower GUS activity than their wild-type forms in the absence of OsMyb4 indicating a role for this sequence in the transcriptional regulation of the two genes. Our hypothesis is that the sequence ATTGACC may be a binding site for an endogenous tobacco TF which positively regulates the two promoters. The repression activity exerted by the OsMyb4 TF on wild-type but not on mutated promoters suggests that OsMyb4 may undermine the tobacco TF from this binding site and negatively regulate the two wild-type promoters. Neither OsMyb4 nor the endogenous tobacco TF were able to regulate the transcriptional activity of both mutated promoters supporting the important role for this sequence in the transcriptional regulation of the *OsMyb4* and *Os02g41510* genes. The occurrence of the negative transcriptional regulation driven by OsMyb4 supports the hypothesis that an *in vivo* “feedback control” mechanism may regulate the expression of *OsMyb4*-like genes. The presence of the ELRECOREPCRP1/MYB2AT overlapping binding sites in the three genes *Bradi3g49180*, *Bradi3g49190*, and *Bradi5g15760* (the putative orthologues of *OsMyb4* and *Os02g41510*) also suggests that similar regulation mechanisms could be found in *Brachypodium*.

Transcriptional auto-regulation has been reported for *myb* genes in plants, namely a positive auto-regulation for the apple *Myb10* gene and a negative one for the *Arabidopsis Myb4* gene (Zhao *et al.* 2007, Espley *et al.* 2009). Moreover, the mammalian *c-Myb* gene has been demonstrated to be able to positively or negatively auto-regulate its own transcription through post-transcriptional modifications and/or cooperation with other TFs depending on the cellular context (Nicolaides *et al.* 1991, Guerra *et al.* 1995). Otherwise, the contrasting activity on the different promoters driven by *OsMyb4* in tobacco protoplasts suggests the involvement of distinct *cis*-elements that are responsible for this dual transcriptional action. Indeed, we previously demonstrated the ability of *OsMyb4* to activate the transcription of cold stress-related genes (Vannini *et al.* 2004), and in the present report, we demonstrate its ability to repress in the same cellular system its own transcription and that of *Os02g41510*. A double action (activator and repressor) has also been reported for the OsMyb4-cognate AtMyb15; this TF acts as a positive regulator of stress-responsive genes when induced by drought and salt stresses whereas it represses the transcription of some genes involved in cold tolerance under cold stress (Agarwal *et al.* 2006, Ding *et al.* 2009). Moreover, in *AtMyb15*-overexpressing *Arabidopsis* plants, *AtMyb13* and *AtMyb14* expression was reduced (Agarwal *et al.* 2006). These data further support the hypothesis that the expression of genes belonging to the *OsMyb4* family could be regulated by a “feedback control” mechanism.

Negative auto-regulation is a crucial knot in gene networks having a specific regulatory effect on the transcriptional dynamics; indeed, it has been demonstrated to speed up the transcriptional response time compared to simple regulation (Rosenfeld *et al.* 2002, Mejia-Guerra *et al.* 2012). The presence of a compensatory auto-regulation process is consistent with the high complexity of the OsMyb4-activated pathway. Similar mechanisms may regulate the expression level of other upstream acting genes; awareness of this possibility is critical for the successful application of transgenic approaches to crop improvement.

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