Expression of the MYB transcription factor gene  
*BplMYB46* affects abiotic stress tolerance and secondary cell wall deposition in *Betula platyphylla*

Huiyan Guo1,2, Yucheng Wang1, Liuqiang Wang1, Ping Hu1, Yanmin Wang1,3, Yuanyuan Jia1, Chunrui Zhang1, Yu Zhang1, Yiming Zhang1, Chao Wang1,** and Chuanping Yang1,**

1State Key Laboratory of Tree Genetics and Breeding, Northeast Forestry University, Harbin, China  
2Department of Life Science and Technology, Mudanjiang Normal College, Mudanjiang, China  
3Key Laboratory of Fast-Growing Tree Cultivating of Heilongjiang Province, Forestry Science Research Institute of Heilongjiang Province, Harbin, China

Received 28 January 2016; revised 12 June 2016; accepted 23 June 2016.  
*Correspondence (Tel/fax +86 451 82190607; email yangcp@nefu.edu.cn and Tel/ fax +86 451 82192218; email wangchao@nefu.edu.cn)

Keywords: *Betula platyphylla*, BplMYB46, abiotic stress, secondary wall deposition.

**Summary**  
Plant MYB transcription factors control diverse biological processes, such as differentiation, development and abiotic stress responses. In this study, we characterized BplMYB46, an MYB gene from *Betula platyphylla* (birch) that is involved in both abiotic stress tolerance and secondary cell wall biosynthesis. BplMYB46 can act as a transcriptional activator in yeast and tobacco. We generated transgenic birch plants with overexpressing or silencing of BplMYB46 and subjected them to gain- or loss-of-function analysis. The results suggest that BplMYB46 improves salt and osmotic tolerance by affecting the expression of genes including SOD, POD and P5CS to increase both reactive oxygen species scavenging and proline levels. In addition, BplMYB46 appears to be involved in controlling stomatal aperture to reduce water loss. Overexpression of BplMYB46 increases lignin deposition, secondary cell wall thickness and the expression of stress genes in secondary cell wall formation. Further analysis indicated that BplMYB46 binds to MYBCORE and AC-box motifs and may directly activate the expression of genes involved in abiotic stress responses and secondary cell wall biosynthesis whose promoters contain these motifs. The transgenic BplMYB46-overexpressing birch plants, which have improved salt and osmotic stress tolerance, higher lignin and cellulose content and lower hemicellulose content than the control, have potential applications in the forestry industry.

**Introduction**  
Plant growth and development are strongly influenced by various stresses, such as salinity, drought and extreme temperatures (Su et al., 2014). A large number of transcription factors (TFs) mediate stress responses in plants, including MYB (Oh et al., 2011), NAC (Mao et al., 2012), bZIP (Uno et al., 2000) and WRKY (Mare et al., 2004) family members. The MYB family is one of the largest families of TFs. *Arabidopsis* contains more than 198 MYB genes (Yanhu et al., 2006), cotton and Populus contain approximately 200 (Cedroni et al., 2003), maize contains 157 (Du et al., 2012a) and soybean contains 252 (Du et al., 2012b). The MYB family is divided into four classes, including 1R-, 2R3-, 3R- and 4R-MYB proteins, according to the number of MYB domains (Dubos et al., 2010). 2R3-MYB appears to be specific to plants and has been widely investigated (Kim et al., 2015; Li et al., 2006; Prouse and Campbell, 2012). MYBs bind to several cis-acting motifs, including the following: MBSI (T/C)/AAC (G/T)/G(A/C/T)/(A/C/T), which is involved in cell cycle control and resistance to low temperatures (Ma et al., 2009; Prouse and Campbell, 2012); MBSII (A/G)/(G/T)/TA(T)/G(T/G), which is involved in regulating secondary cell wall biosynthesis (Kim et al., 2012); MBSIIG, ACC(A/T)/ACC(A/C/T), which is related to flavonoid biosynthesis (Grotewold et al., 1994); MYBCORE, CAGTTA and CTGTTG, which are associated with drought tolerance (Ithal and Reddy, 2004); and AC-box, ACC(A/T)A/(A/C) (T/C), which is related to secondary cell wall deposition (Zhong et al., 2013).

Plant MYBs regulate cell differentiation, organ formation, leaf morphogenesis, secondary metabolism and abiotic stress responses (Ambawat et al., 2013; Qi et al., 2015; Sun et al., 2015; Xu et al., 2015). For instance, OsMYB44 overexpression enhances freezing tolerance in rice, and it also improves acclimation to cold and drought in transgenic apple (Pasquali et al., 2008; Vannini et al., 2004). TaMYB73 in wheat induces the expression of stress signalling genes and increases salinity tolerance in transgenic *Arabidopsis* (He et al., 2012). Overexpression of OsMYB48-1 induces the expression of stress-response genes and improves salinity and drought stress tolerance in rice (Xiong et al., 2014). The functions of MYBs in secondary wall biosynthesis have also been investigated. For example, *Arabidopsis* AtMYB46 and AtMYB83 function as master switches in a transcriptional network promoting secondary wall deposition that is regulated by the secondary wall-associated NAC domain protein 1 (SND1) (McCarthy et al., 2009; Zhong et al., 2007). PnMYB2, 3, 20 and 21 positively regulate secondary wall biosynthesis during wood formation in poplar trees (Zhong et al., 2013). Gain- and loss-of-function analysis showed that AtMYB61 regulates both stomatal aperture and the expression of genes involved in lignin deposition (Liang et al., 2005; Newman et al., 2004; Romano et al., 2012). AtMYB52 is a target gene regulated by AtMYB46 during secondary wall biosynthesis. Overexpression of AtMYB52 also improves drought tolerance by regulating the ABA signal transduction pathway, suggesting a possible connection between secondary wall deposition and ABA responses (Ko et al., 2009; Park et al., 2011). Although MYBs have been
investigated in diverse biological systems, their functional roles have not been fully elucidated. In addition, many members of the large MYB transcription factor family have not yet been characterized.

In this study, we cloned and functionally characterized an MYB transcription factor, BpMYB46, from birch (Betula platyphylla), a pioneer tree species widely distributed from Europe to Asia that has important applications for the paper, building and furniture industries (Zhang et al., 2012). Our results show that overexpression of BpMYB46 improves salt and osmotic stress tolerance and mediates secondary cell wall deposition in transgenic birch. This study increases our knowledge of the crosstalk between the abiotic stress and secondary cell wall biosynthesis pathways and provides insights into the functions of MYB proteins.

Results
BpMYB46 is an R2R3-type MYB protein

The MYB transcription factor gene BpMYB46 (GenBank accession number: KP711284) was isolated from B. platyphylla. Multiple sequence alignments (Figure S1) and phylogenetic analysis (Figure S2) indicate that BpMYB46 belongs to the R2R3-type MYB subfamily, with a highly conserved domain in the N-terminus containing R2 and R3 functional regions. Members of this subfamily, including VvMYB46, PtMYB2, PtMYB21 and AtMYB46, function in secondary cell wall biosynthesis.

Transactivating BpMYB46 localizes to the nucleus

We transformed 3SS : BpMYB46-GFP into onion epidermal cells by particle bombardment, using 3SS : GFP as the control. Green fluorescent signals from the 3SS : BpMYB46-GFP transformed cells were detected in the nuclei, which were stained using DAPI. By contrast, 3SS : GFP signals were uniformly distributed throughout the cell (Figure 1a), indicating that BpMYB46 is a nuclear protein.

To investigate whether BpMYB46 activated transcription and to identify the activation domain, a series of deletions of the BpMYB46 CDS were fused with the GAL4 DNA-binding domain sequence in pGBK7 (Clontech). The resulting constructs were, respectively, transformed into yeast cells for transcriptional activation analysis using the yeast two-hybrid system (Y2H). Yeast cells harbouring the full CDS of BpMYB46 grew normally on SD-/Trp-/His-/Ade/X-Gal medium, and xα-gal was activated (Figure 1b), suggesting that BpMYB46 is a transcriptional activator. Furthermore, analysis of deletions with truncated CDS of BpMYB46 suggested that the transcriptional activation domain is located in a region from amino acid 233 to 304 in BpMYB46 (Figure 1b).

Analysis of the expression of BpMYB46

To gain insight into the biological role of BpMYB46, we analysed the expression patterns of BpMYB46 in response to NaCl, ABA and mannitol treatment using real-time PCR. The results show that BpMYB46 expression was induced by NaCl, ABA and mannitol after 6–24 h of treatment (Figure 2a). In addition, the expression patterns of BpMYB46 in response to NaCl and ABA treatment were quite similar, that is induction after 6 to 24 h of treatment, reaching a peak at 24 h. Mannitol treatment increased BpMYB46 expression at 6 to 24 h, with a peak at 12 h. We further examined BpMYB46 expression in roots, leaves and various stem internodes of 6-month-old birch (Figure 2b). The expression of BpMYB46 was higher in the stem internodes than in roots or leaves, with 60-fold higher expression in the 18th internodes compared with leaves, whereas no significant difference in expression was observed between roots and leaves (Figure 2c). BpMYB46 expression was lowest in the 1st internodes, with an increasing gradient from the stem tip to base. The highest expression level was observed in the 18th internodes of stems, with levels approximately 18-fold those of the 1st internodes (Figure 2c). Therefore, BpMYB46 is predominantly expressed in stems and is more highly expressed in mature tissue (base of stem) than in juvenile tissue (tip of stem).

BpMYB46 binds to the MYBCORE and AC-box motifs

Previous studies have shown that MYB proteins bind to the MYBCORE and AC-box motifs (Ithal and Reddy, 2004; Zhong et al., 2013). To investigate the binding of BpMYB46 to these motifs, three tandem copies of these motif sequences (MYB-CORE: CAGTTA; AC-box: ACCACCT) were, respectively, cloned into pHis2 and their interactions with BpMYB46 were determined using Y1H analysis (Figure 3a). The results indicate that yeast cells cotransformed with BpMYB46-effector and different reporters grew on TD/3-AT medium, demonstrating that BpMYB46 also binds to the MYBCORE and AC-box motifs (Figure 3b).

To further verify the above interactions identified by Y1H, the pROK2-BpMYB46 construct was used as an effector, and three tandem copies of MYBCORE and AC-box, together with their mutants, were, respectively, fused with the minimal 3SS promoter (−46 to +1) to drive the GUS reporter gene (Figure 3c). GUS activity was detected in tobacco leaves following cotransformation of lines harbouring pROK2-BpMYB46 with the MYBCORE and AC-box motifs. However, very low GUS activity was detected in cotransformed lines harbouring pROK2-BpMYB46 and the respective mutant sequences (Figure 3d, e).

MYCORE is involved in drought stress responses (Ithal and Reddy, 2004), and the AC-box plays a role in secondary wall biosynthesis (Zhong et al., 2013).

Generation of transgenic birch plants with overexpression and knock-down of BpMYB46

We generated 16 BpMYB46-overexpressing (OE) and 15 RNAi-silenced BpMYB46 (SE) transgenic birch lines and examined the expression of BpMYB46 using real-time RT-PCR. The transgenic and wild-type (WT, nontransgenic) plants were generated from a single birch clone, which indicates that they have the same genetic background. In addition, the expression levels of endogenous BpMYB46 in the transgenic and WT plants were similar. Therefore, the expression levels of the transgene BpMYB46 and the functions of this gene could be investigated in the OE, SE and WT plants. Our results indicate that the expression of BpMYB46 was significantly increased in the OE lines, with levels 3-fold–38-fold higher than those of WT, but were significantly reduced in the SE lines, with a 50%–91% decrease relative to WT (Figure S3). BpMYB46-overexpressing transgenic lines 9 and 10 (termed OE9 and OE10, respectively), which showed moderate and high BpMYB46 expression, respectively, were selected for further study. Two RNAi-silenced BpMYB46 lines, lines 3 and 15 (termed SE3 and SE15), which exhibited a high degree of silencing of BpMYB46, were also employed for further study.

BpMYB46 confers salt and osmotic stress tolerance

Soil-grown transgenic birch plants, including OE, WT and SE lines, were exposed to salt or mannitol to evaluate their stress...
tolerance. There was no substantial difference in phenotype, growth rate, fresh weight or root length among OE, WT and SE lines under control conditions (Figure 4a), suggesting that BplMYB46 does not affect the growth phenotype or growth rate of the plants. Under NaCl or mannitol treatment, compared with WT plants, both OE9 and OE10 displayed significantly higher growth rates, were greener and exhibited less wilting, in addition to having significantly higher fresh weights and root lengths. By contrast, lines SE3 and SE15 exhibited more severe leaf rolling and wilting, significantly reduced fresh weights and root lengths and the loss of green coloration compared with WT (Figure 4b, c). In addition, the chlorophyll contents were similar among OE, WT and SE lines under control conditions. However, under salt and osmotic stress conditions, compared with WT plants, both OE9 and OE10 had significantly higher chlorophyll levels, while SE3 and SE15 had significantly lower chlorophyll levels (Figure 4d). Overall, these results suggest that BplMYB46 overexpression significantly improves abiotic stress tolerance in birch.

BplMYB46 affects reactive oxygen species (ROS) scavenging

In view of the key role of ROS in abiotic stress tolerance, we investigated whether BplMYB46 affects ROS scavenging. We evaluated the levels of H$_2$O$_2$, a main ROS species, using DAB in situ staining. No obvious difference in DAB staining was observed among OE, WT and SE lines under control conditions. However, under NaCl or mannitol treatment conditions, compared with WT plants, OE9 and OE10 exhibited reduced DAB staining, whereas the two SE lines displayed strongly increased DAB staining, indicating that BplMYB46 overexpression reduces H$_2$O$_2$ accumulation in plants (Figure 5a). We further measured...
H₂O₂ levels. Consistent with the DAB staining results, there was no difference among OE, WT and SE lines under control conditions. However, under salt and osmotic stress conditions, the H₂O₂ levels were highest in SE3 and SE15, followed by WT, whereas OE9 and OE10 had the lowest H₂O₂ levels (Figure 5b).

We also examined ROS levels in guard cells using DCFH-DA fluorescence staining (Figure 5c). When exposed to NaCl or mannitol, ROS strongly accumulated in guard cells in the two SE lines, while both OE lines had substantially reduced ROS levels compared with WT plants. As ROS levels were significantly different in the OE, WT and SE lines when exposed to NaCl or mannitol, we also examined the activities of superoxide dismutase (SOD) and peroxidase (POD), the two major ROS scavenging enzymes in plants. The OE lines had significantly higher SOD and POD activity than the other lines, followed by WT, whereas the SE lines had significantly lower SOD and POD activity than the other lines (Figure 5d, E).

As the SOD and POD activities in these lines were altered in response to NaCl and mannitol treatment, we examined the expression of six SOD and eight POD genes in these lines. Under NaCl or mannitol treatment, the expression of all SOD and POD genes was highest in the OE lines, followed by WT and the SE lines (Figure 5f). These results indicate that BpMYB46 enhances ROS scavenging by affecting the expression of SOD and POD.

**BpMYB46 affects proline biosynthesis**

The proline levels were similar among OE, WT and SE lines under control conditions. However, under NaCl or mannitol treatment, the OE lines had significantly higher proline levels, followed by the WT and SE lines (Figure 5g). We further analysed the expression of genes related to proline biosynthesis (including P5CS1 and P5CS2) and proline degradation (including P5CDH and ProDH). Compared with WT plants, the two P5CS genes were significantly more highly expressed in the OE lines but had reduced expression in the SE lines. Conversely, the proline degradation genes, including two P5CDH genes and one ProDH gene, were significantly down-regulated in the OE lines but significantly up-regulated in the SE lines compared with WT plants (Figure 5h).

**Overexpression of BpMYB46 reduces cell death**

Propidium iodide (PI) staining reflects cell membrane damage based on fluorescence levels. We used PI staining to investigate cell death under NaCl or mannitol stress conditions. Compared with WT plants, the OE lines displayed relatively weak fluorescence, indicating reduced cell death, while the SE lines exhibited stronger fluorescence, suggesting severe cell death (Figure 6a). Consistently, the OE lines had the lowest electrolyte leakage rates, but the SE lines had the highest rates, when exposed to NaCl or mannitol (Figure 6b). Together, these results suggest that BpMYB46 overexpression reduces cell death under salt and osmotic stress conditions.

**BpMYB46 overexpression decreases water loss and reduces stomatal apertures**

The OE lines showed significantly reduced water loss rates. The SE lines had elevated water loss rates compared with WT, with significantly higher water loss detected in detached leaves exposed to air (Figure 7a), indicating that BpMYB46 overexpression reduces the transpiration rate in plants. Under control conditions, the stomatal apertures were smaller in the OE lines.
than in WT plants, whereas the stomatal apertures in the SE lines were slightly larger than those of WT. Under salt and osmotic stress, the stomates in the OE lines were almost closed, in contrast to the stomates in the SE lines, which remained open (Figure 7b). In addition, the width/length ratios of stomatal apertures in the OE lines were significantly smaller than those of the WT and SE lines. However, under NaCl or mannitol stress, the width/length ratios of the stomatal apertures of the SE plants were significantly larger than those of the OE and WT plants (Figure 7c).

We also analysed the expression of BpMYB61 (GenBank number: KT344120), a gene homologous to Arabidopsis AtMYB61, which encodes a protein that regulates stomatal aperture (Liang et al., 2005). Under control conditions, the expression of BpMYB61 was significantly induced in the OE lines compared with WT plants, but no significant differences in BpMYB61 expression were detected between the WT and SE lines. Under salt or osmotic stress conditions, the expression of BpMYB61 was significantly induced in the OE lines. However, its expression was significantly reduced in the SE lines compared with WT plants (Figure 7d).

**BpMYB46 affects vessel dimension and secondary wall thickening in fibres**

We stained stem sections with phloroglucinol-HCl and toluidine blue, finding that the number of vessels was higher in the OE lines than in WT plants, whereas the dimensions of the vessels in the OE lines were smaller than those of WT. Conversely, the number of vessels in the SE lines was reduced, and the vessel dimension was larger, compared to WT (Figure 8a–c, g–i). We measured the ratios of the vessel area to total area in anatomical sections of plants. Compared with WT plants, the ratio of vessel area to total area was lower in the OE lines but higher in the SE lines (Figure 8n).

Additionally, toluidine blue staining showed no substantial difference in secondary wall thickening in vessels among OE, WT and SE lines (Figure 8g–i). However, the secondary wall thickening of xylem fibres in WT plants was lower than that in OE lines but higher than that in SE lines (Figure 8j–l). Measurements using Image J software further indicated that the secondary wall thickness of xylem fibres in the OE lines was approximately 2 μm (±0.07), approximately 1.45 μm (±0.1) in WT and almost 0.9 μm...
BpMYB46 controls secondary cell wall thickness in fibres. In the sections stained with phloroglucinol-HCl, which stains lignin, the red staining was more intense in the OE lines but less intense in the SE lines compared with WT (Figure 8a–c). These results indicate that higher lignin deposition occurred in the OE lines than in WT, but less lignin was deposited in the SE lines than in WT. Detection of lignin autofluorescence (Figure 8d–f) indicated a stronger fluorescent signal in OE (Figure 8d) than in WT (Figure 8e) but a weaker signal in the SE lines (Figure 8f) than in WT, suggesting that BpMYB46 promotes lignin deposition. We also found significantly increased lignin content in the OE lines and significantly reduced content in the SE lines compared with WT, as determined by chemical analysis (Table 1), which is consistent with the results of lignin autofluorescence analysis (Figure 8d–f). The chemical analysis also suggested that the cellulose content in the OE lines was higher than that of WT. However, the hemicellulose content in the OE lines was dramatically reduced compared with WT (Table 1). These results indicate that BpMYB46 has a positive effect on lignin and cellulose content but a negative effect on hemicellulose content.

BpMYB46 affects the expression of secondary wall biosynthesis genes

We investigated the expression of the birch genes phenylalanine ammonia lyase (PAL), caffeoyl-CoA O-methyltransferase (CCoAOMT), 4-coumarate-coa ligase (4CL), POD, Laccase (LAC), cinnamoyl-CoA reductase (CCR), cellulose synthase (CESA), fragile fibre (FRA) and irregular xylem (IRX), which are homologous to lignin, cellulose and hemicellulose biosynthesis genes, via real-time RT-PCR. We also investigate the expression of the birch genes BpMYB1, BpMYB2 and BpMYB3, which are homologous to AtMYB42, AtMYB103 and AtMYB52, respectively; these genes are involved in secondary cell wall formation in Arabidopsis. The expression of genes related to lignin and cellulose biosynthesis was significantly induced in the OE lines but significantly down-regulated in the SE lines. However, FRA and IRX, which are related to hemicellulose biosynthesis, were significantly down-regulated in the OE lines but significantly up-regulated in
**Figure 5** Detection of ROS scavenging and analysis of proline biosynthesis. (a) Detection of ROS using DAB in situ staining. (b) Assay of H$_2$O$_2$ levels. (c) Analyses of ROS production in intact guard cells by H$_2$DCF-DA staining. (d) SOD activity assay. (e) POD activity assay. (f) Expression analysis of POD and SOD genes under NaCl and mannitol treatment using qRT-PCR. The expressions of the genes in WT plants were used as calculators to normalize their expressions in OE and SE lines. (g) Analysis of proline levels in OE, WT and SE lines. (h) Expression analysis of proline biosynthesis and degradation-related genes following NaCl and mannitol treatment using qRT-PCR. The expressions of the genes in WT plants were used to normalize their expressions in OE and SE lines. Asterisk indicates $P < 0.05$. The error bars indicate the standard deviation (SD) from three biological replicates. ANOVA was used to determine statistically significant differences between results.
the SE lines (Figure 7o). These results suggest that BplMYB46 affects the expression of genes related to lignin, cellulose and hemicellulose biosynthesis in birch.

ChIP and promoter–reporter analyses indicate BplMYB46 binding

We performed ChIP analysis to determine whether BplMYB46 directly binds to the promoters of genes related to (1) abiotic stress (oxidative and osmotic stress), including POD, SOD and P5CS and (2) secondary wall deposition, including PAL, CcoAOMT, 4CL, POD, LAC, CESa and MYB. As BplMYB46 binds to MYBCORE and AC-box motifs in genes related to abiotic stress and secondary wall deposition (Figure 9), and these two motifs are present in the promoters of target genes of BplMYB46 (Table S1), the primers for ChIP were designed to amplify the regions containing the MYBCORE and/or AC-box motif. Quantitative ChIP-PCR showed that the promoters of these putative target genes were significantly enriched (>threefold; Figure 9a) in the chromatin immunoprecipitated with GFP antibody (ChIP+) when compared with those in Mock sample (ChIP−). These results indicate that BplMYB46 preferentially binds to the promoters of these genes, suggesting that BplMYB46 may directly regulate a set of genes that mediate abiotic stress responses and secondary wall biosynthesis.

To further verify that BplMYB46 binds to the promoters of this gene set as determined by ChIP, we cotransformed the pROK2-BplMYB46 and the truncated promoters containing the MYBCORE or AC-box motif into tobacco leaves, and cotransformation with 35S : LUC was used to normalize the transformation efficiency values (Figure 9b). GUS activity measurement suggested that BplMYB46 can bind to the promoters of all genes investigated (Figure 9c).

Discussion

Mette et al. (2000) showed that double-stranded promoter RNA hairpins cause trans-silencing of target genes triggered by methylation, which silences target genes more specifically than RNAi based on coding region. In the present study, as the genome of birch was not available early in our study, we employed RNAi-based silencing of the promoter sequence to specifically knock down the expression of BplMYB46. The results show that the expression of BplMYB46 was significantly reduced in the transgenic plants, with a minimum reduction of 90.77% (Figure S3), indicating that the RNAi-based promoter used in our study was also functional in birch plants.

Although determining the copy number of a transgenic cassette is important for characterizing transgenic plants, the aim of the present study was to generate transgenic birch lines suitable for gain- and loss-of-function analysis of BplMYB46. Therefore, we directly determined the expression levels of BplMYB46 in these plants, but we did not investigate the copy numbers of the cassette in OE and RNAi-silenced lines. The results show that the expression of BplMYB46 was significantly elevated...
in the OE lines and significantly reduced in the SE lines, indicating that the OE and SE lines were suitable for gain- and loss-of-function analysis, respectively.

In the present study, we used 200 mM NaCl or 300 mM mannitol solution for birch plant treatments. Sudden application of NaCl and mannitol in high concentration to plants would first cause osmotic shock rather than osmotic/salt stress, and followed by the imposition of an ionic shock (Shavrukov, 2013). Therefore, there is an overlap of changes in gene expression relating to ionic and osmotic responses, and these genes mainly involve in signal transduction, osmotic regulation, water loss and ionic component of salt stress response (Shavrukov, 2013).

In plants, Δ1-pyrroline-5-carboxylate synthetase (P5CS) catalyses the rate-limiting step in proline biosynthesis. P5CS overexpression greatly increases proline levels, while reducing P5CS expression abrogates the ability of the plant to accumulate proline (Deuschle et al., 2004). Two mitochondrial enzymes, pro-dehydrogenase (ProDH) and P5C-dehydrogenase (P5CDH), play sequential roles in catalysing proline degradation (Deuschle et al., 2004). In the current study, BpMYB46 expression was positively correlated with the expression of P5CS but negatively correlated with the expression of P5CDH and ProDH (Figure 5h). Therefore, proline levels were positively correlated with P5CS expression but negatively correlated with P5CDH and ProDH expression. These results suggest that BpMYB46 induces proline biosynthetic genes and inhibits the expression of proline degradation genes, resulting in elevated proline levels and improved abiotic stress tolerance.

The transpiration rate in a plant is closely related to water stress tolerance. Our results indicate that BpMYB46 expression reduced the transpiration rates of transgenic plants by controlling stomatal aperture to reduce water loss (Figure 7b, c). Indeed, overexpression of AtMYB61 confers resistance to drought in transgenic Arabidopsis by reducing stomatal aperture (Liang et al., 2005). Therefore, to investigate whether BpMYB46 controls stomatal

Figure 7 Analysis of water loss rate, stomatal aperture and BpMYB61 expression in OE, WT and SE BpMYB46 lines. (a) Water loss rates and (b, c) measurement of stomatal aperture under control conditions, 50 mM NaCl and 50 mM mannitol. (d) Expression of BpMYB61 (GenBank accession number: KT344120) in OE, WT and SE BpMYB46 lines under control conditions, 50 mM NaCl and 50 mM mannitol. Asterisk indicates *P < 0.05. The error bars indicate the standard deviation (SD) from three biological replicates. ANOVA was used to determine statistically significant differences between results.

© 2016 The Authors. Plant Biotechnology Journal published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 15, 107–121
aperture by regulating the expression of genes homologous to AtMYB61 in birch, we compared the sequences of BpMYB61 and AtMYB61, finding that they were highly homologous. We investigated the expression of BpMYB61 in the OE, WT and SE lines. Our results suggest that BpMYB46 positively regulates the expression of BpMYB61 (Figure 7d). These results suggest that
BplMYB46 reduces water loss by positively regulating BpMYB61, which reduces the stomatal aperture to prevent water loss. Overexpression of PtrMYB3 and PtrMYB20 in poplar increases the ectopic deposition of cellulose, xylan and lignin and regulates the expression of genes such as CCoAOMT1, 4CL, FRA8, IRX8, IRX9, CesA4, CesA7 and CesA8, which are related to cellulose, xylan and lignin biosynthesis (McCarthy et al., 2010), suggesting that MYB transcription factors play an important role in secondary wall biosynthesis. In the current study, BplMYB46 was more highly expressed in stems than in other tissues (Figure 2). Furthermore, overexpression of BplMYB46 increased secondary wall thickening (Figure 8m). Knock-down of BplMYB46 reduced the secondary wall thickness in xylem fibres and decreased the lignin content (Figure 8m, Table 1). RT-PCR analysis showed that

Table 1 Secondary cell wall composition of transgenic birch and WT plants

| Line       | Lignin (%) | Cellulose (%) | Hemicellulose (%) |
|------------|------------|---------------|-------------------|
| BplMYB46-OE| 25.80 ± 0.11| 44.34 ± 0.89  | 21.46 ± 1.3       |
| Wild type  | 24.56 ± 0.16| 42.17 ± 0.97  | 25.09 ± 1.0       |
| BplMYB46-SE| 22.52 ± 0.31| 40.49 ± 0.37  | 29.10 ± 2.8       |

Measurements were conducted on transgenic birch plants overexpressing BplMYB46, wild-type (WT) plants and RNAi-silenced BplMYB46 plants. Values represent mean and standard deviation. All transgenic lines displayed significantly different values compared to WT (P < 0.05%). n = 11 (12 biological replicates).

Figure 9 The regulation of target genes by BplMYB46, as determined by ChIP and GUS activity analyses. (a) Real-time quantitative PCR analysis showing the enrichment of the promoter sequences of genes after chromatin immunoprecipitation. ChIP+: The sonicated chromatin was immunoprecipitated with GFP antibody; mock: the sonicated chromatin was immunoprecipitated with anti-hemagglutinin (HA); input: the sonicated chromatin used as positive control. Three biological replicates were performed. Chromatin from whole seedlings was isolated from pROK2-35S: BplMYB46-GFP birch plants produced by Agrobacterium tumefaciens-mediated transient transformation. The tubulin sequence was used as an internal control. After normalization against tubulin, the values of the enrichment of the promoter sequences of target genes in ChIP- were set to 1. The error bars indicate the standard deviation (SD) from three biological replicates. (b) Schematic diagram of the reporter and effector constructs used for coexpression in tobacco plants. (c) Relative GUS activity of the truncated promoters of genes. Asterisk indicates P < 0.05. The error bars indicate the standard deviation (SD) from three biological replicates. ANOVA was used to determine statistically significant differences between results. The GenBank accession numbers of promoters used in (a) and (c): KX373440–KX373458.
overexpression and silencing BplMYB46 alter the expression of lignin, cellulose and hemicellulose biosynthesis-related genes, including PAL, CCoAOMT, 4CL, POD, CCR, LAC, CESAs, FRA and IRX (Figure 8b). In addition, ChIP-qPCR shows BplMYB46 preferentially binds to these same promoters (Figure 9). These results strongly suggest that BplMYB46 may regulate a set of lignin, cellulose and hemicellulose biosynthesis-related genes.

In the present study, we studied the bindings of BplMYB46 to promoters using ChIP method (Figure 9). However, the evidence of the bindings between the BplMYB46 and promoters was from plant lines with overexpression of the BplMYB46-GFP fusion protein and therefore could be an artefact of ectopic expression. Additionally, a set of promoters were selected and used for ChIP-PCR to investigate their bindings to BplMYB46, which does not provide a full picture of the impact of the native BplMYB46 on global steady state transcripts like RNA-seq would. Therefore, to study the native BplMYB46 on global steady state transcripts in the future, ChIP-Seq will be performed using BplMYB46 antibody to immunoprecipitate the chromatin bound by endogenous BplMYB46 in WT birch plants.

Birch plants are self-sterile, meaning that it is impossible to generate homozygote plants by selfing. Additionally, birch trees do not reach the reproductive stage to produce seeds until they are more than 12 years old. Therefore, as it is impossible to produce homozygous transgenic plants by selfing, hemizygous transgenic plants were used in this study. In the future, when the transgenic plants reach the flowering stage, we plan to perform anter culture to generate haploid plants and to generate transgenic homozygous plants by performing chromosome doubling of the haploid plants.

Experimental procedures

Cloning and subcellular localization of BplMYB46

The cDNA sequence of BplMYB46 was obtained from the birch transcriptome (Wang et al., 2014). The CDS of BplMYB46 without the stop codon fused in-frame to the N-terminus of green fluorescent protein (GFP) was transformed into the pROK2 vector under the control of the CaMV 35S promoter (35S : MYB-GFP; primer sequences are shown in Table S2). The GFP protein under the control of the 35S promoter was used as a control (35S : GFP). The 35S : MYB-GFP and 35S : GFP constructs were introduced into onion epidermal cells by particle bombardment (Bio-Rad laboratories, Inc. Hercules, California, USA). After incubation for 48 h, the transformed onion epidermal cells were stained with DAPI (100 ng/mL) and visualized under an LSM700 confocal laser microscope (Zeiss, Jena, Germany).

Transactivation assay

The complete and various truncated versions of the CDS of BplMYB46 were PCR amplified (using the primers listed in Table S3) and fused in-frame to the GAL4 DNA-binding domain in the pGBK7 vector to generate the pGBK7-BplMYB46 construct (Clontech laboratories, Inc. Mountain View, California, USA). The pGBK7-BplMYB46 construct was transformed into AH109 yeast cells, which were incubated on SD-/Trp or SD-/Trp/-His/-Ade/X-a-Gal medium at 30 °C for 3–5 days.

Plant materials and stress treatments

Birch seeds were planted in pots with a 12-cm diameter containing a mixture of perlite/vermiculite/soil (1 : 1 : 4) in a greenhouse under controlled conditions (16/8 h light/dark, 25 °C and 70%–75% relative humidity). Each pot, which contained four seedlings, was thoroughly watered with deionized water every day. After 2 months, the plants were watered with 200 mM NaCl, 100 μM ABA or 300 mM mannitol solution, which was applied to the top of the soil. All seedlings were collected at the same time after treatment for 6, 12 or 24 h, and seedlings watered with deionized water were used as a control. Three independent biological replicates were performed, and each replicate included four seedlings. The roots, leaves and internodes of stems from 6-month-old birch plants were harvested, including the 1st, 4th, 8th, 12th, 16th and 18th stem internodes.

Real-time PCR

Total RNA was isolated from birch using the CTAB method (Chang et al., 1993) and treated with DNase I to remove DNA contamination. Total RNA was reverse transcribed into cDNA using a PrimeScript™ RT reagent Kit (Takara Bio Inc. Kusatsu, Shiga, Japan) for RT-PCR analysis. Real-time RT-PCR was performed with a TransStart Top Green qPCR SuperMix kit (TransGen Biotech, Beijing, China) using the primer sequences listed in Table S4. The amplification procedure was conducted using the following parameters: 94 °C for 30 s; 45 cycles at 94 °C for 12 s, 58 °C for 30 s and 72 °C for 45 s; and 79 °C for 1 s for plate reading. Three independent experiments were performed in triplicate. The tubulin (GenBank accession number: FG067376) and ubiquitin (GenBank accession number: FG065618) genes were used as the internal controls. The relative expression level of each gene was calculated using the delta–delta CT method (Livak and Schmittgen, 2001).

Examining the binding of BplMYB46 to the MYBCORE and AC-box motifs using Y1H

Three tandem copies of MYBCORE (CAGTTA) and AC-box (ACCAACT) were inserted into pHIS2 (Clontech) upstream of the reporter gene HIS3, respectively. The CDS of BplMYB46 was cloned into pGAD7-Rec2 (Clontech) as the effector (pGAD7-BplMYB46). The constructs were cotransformed into Y187 cells, which were plated onto SD-/Trp/-His (DDO) and SD-/Trp/-His/-Leu/ (TDO) medium supplemented with 50 mM 3-AT (3-amino-1, 2, 4-triazole) and incubated at 30 °C for 3–5 days. All primers used are listed in Table S5.

Binding of BplMYB46 to motifs and promoters

Three tandem copies of MYBCORE, AC-box, their mutated sequences and the truncated promoter were, respectively, fused with the 35S CaMV minimal promoter (−46 bp to +1) to drive the GUS gene in a modified pCAMBIA1301 vector (in which the 35S hygromycin region was deleted) using the primers listed in Tables S6 and S7. The full CDS of BplMYB46 was cloned into pROK2 under the control of the 35S promoter (35S : BplMYB46) as the effector. The effector was co-transformed with each reporter into tobacco leaves by Agrobacterium tumefaciens-mediated transient transformation (Ji et al., 2014). The firefly luciferase (LUC) gene driven by the 35S promoter was co-transformed into tobacco leaves as a control for normalization of transformation efficiency. GUS and luciferase activities were determined as previously described (Gampala et al., 2001).

Generation of plants with overexpression and silencing of BplMYB46

To silence BplMYB46, a truncated promoter of BplMYB46 (223 bp in length) with sense and antisense sequences was inserted into the pGBKT7 vector to generate the pGBKT7-BplMYB46 construct (Clontech laboratories, Inc. Mountain View, California, USA). The pGBK7-BplMYB46 construct was transformed into AH109 yeast cells, which were incubated on SD/-Trp or SD/-Trp/-His/-Ade/X-a-Gal medium at 30 °C for 3–5 days. All primers used are listed in Table S5.
Effects of BplMYB46 expression on birch

Water loss, stomatal aperture measurements and BplMYB61 expression

The fresh weights (FW) of detached leaves were determined, and the leaves were then desiccated under normal atmospheric conditions. The leaves were weighed (desiccated weight) after exposure to air for 0.5, 1, 1.5, 2, 3, 5 and 8 h, dried overnight at 80 °C and their dry weights (DW) determined. The water loss rates (WLR) were calculated using the formula: WLR (%) = ([FW – desiccated weight]/[FW – DW]) × 100.

Epidermal peels were stripped from the leaves of 2-month soil-grown OE, SE and WT plants and floated in a solution of 30 mM KCl and 10 mM MES-KOH (pH 6.15), followed by incubation for 2 h in the light at 22 °C to induce stomatal opening (Cheng et al., 2013). Then, 50 mM NaCl or 50 mM mannitol was added to the buffer solution. The samples were incubated for an additional 2 h. Stomatal apertures were photographed using light microscopy (Olympus BX43, Olympus Corporation, Shinjuku-ku, Tokyo, Japan). The ratios of the widths and lengths of stomatal apertures under different treatments were calculated.

The expression of BplMYB61, a gene homologous to AtMYB61, encoding a protein that regulates stomatal aperture in Arabidopsis, was investigated in the OE, WT and SE BplMYB46 lines under control conditions, 50 mM NaCl and 50 mM mannitol stress using the primers listed in Table S9.

Histological analysis

Stems of 6-month soil-grown OE, WT and SE BplMYB46 birch plants were fixed in FAA solution (70% ethanol: glacial acetic acid: formaldehyde: 90: 5: 5, v/v) and embedded in frozen sectioning medium (OCT; Thermo Scientific, Waltham, MA) to obtain 25-μm-thick stem base sections using a Microtome Cryostat (Thermo Scientific HM560). The stem sections were stained with phloroglucinol-HCl and toluidine blue and examined by light microscopy (Zhong et al., 2006). Lignin autofluorescence was observed under a confocal laser microscope (Zeiss, Jena, Germany). For scanning electron microscopy (SEM), 0.2-cm-thick sections of 12-month soil-grown OE, WT and SE BplMYB46 birch plants were obtained manually and observed under a scanning electron microscope (S-4800, HITACHI, Tokyo, Japan). The ratio of vessel area to total area was measured from 12 anatomical sections representing each genotype after staining with phloroglucinol-HCl. The secondary wall thickness of xylem fibres in the SEM micrographs was quantified in 45 cells using Image J software (http://rsbweb.nih.gov/ij/).

Determination of secondary wall composition

The lignin, cellulose and hemicellulose levels of stems from the OE, WT and SE lines grown in soil for 12 months were determined according to the Klason procedure (Whiting et al., 1981) using an automatic fibre analyser (Ankom 2000; Ankom, Macedon, NY). Twelve biological replicates were performed in this experiment.

Expression analysis of BplMYB46 target genes

For stress tolerance-related gene expression analysis, birch plants grown for 2 months in soil were treated with 200 mM NaCl or 300 mM mannitol for 24 h; plants watered with deionized water only were harvested at the same time and used as controls. For wood formation-related gene analysis, stems of the OE, WT and SE BplMYB46 birch plants grown for 6 months in soil were harvested. All primers used are listed in Table S10. Three independent experiments were performed, with three biological replicates.
ChIP assay

The 35S: BpIMYB46-GFP construct was transformed into birch plants for the ChIP assay. The ChIP assay was performed according to the published method (Haring et al., 2007). Briefly, after protein and chromatin were cross-linked, the chromatin was sheared into 0.2–0.8 kb fragments by sonication, and 1/10 (volume) of each sample was used for the input control. Sonicated chromatin was immunoprecipitated with GFP antibody (Abmart) (ChIP+), and chromatin immunoprecipitated with anti-heatagglutinin (HA) antibody was used as a negative control (Mock). The antibody-bound complex was precipitated with protein A + G agarose beads. The immunoprecipitated DNA was purified by chloroform extraction. The enrichment of the truncated promotors in immunoprecipitated samples were determined by real-time PCR, and the tubulin sequence was used as an internal control. Three biological replications were performed. The primer sequences used for ChIP amplification are listed in Table S11.

Statistical analysis

Histological indices involving stress responses and measurement of wood characters related to secondary wall deposition were analysed using ANOVA. All statistical analyses were performed using SPSS software (IBM, Chicago, IL, USA), version 18.0. Differential analysis of wood characters was performed using the DUNCAN method.

Acknowledgements

This work was supported by Innovation Project of State Key Laboratory of Tree Genetics and Breeding (Northeast Forestry University, 2013A05) and National Natural Science Foundation of China (No. 31270703 and No. 31470671).

Conflict of interest

The authors have no conflict of interest to declare.

References

Ambawat, S., Sharma, P., Yadav, N.R. and Yadav, R.C. (2013) MYB transcription factor genes as regulators for plant responses: an overview. Physiol. Mol. Biol. Plants, 19, 307–321.
Bates, L.S., Waldren, R.P. and Teare, J.D. (1973) Rapid determination of free proline for water stress studies. Plant Soil, 39, 205–207.
Cedroni, M.L., Cronn, R.C., Adams, K.L., Wilkins, T.A. and Wendel, J.F. (2003) Evolution and expression of MYB genes in diploid and polyploid cotton. Plant Mol. Biol. 51, 313–325.
Chang, S., Puryear, J. and Cairney, J. (1993) A simple and efficient method for isolating RNA from pine trees. Plant Mol. Biol. Rep. 11, 113–116.
Cheng, M.C., Liao, P.M., Kuo, W.W. and Lin, T.P. (2013) The Arabidopsis ETHYLENE RESPONSE FACTOR1 regulates abiotic stress-responsive gene expression by binding to different cis-acting elements in response to different stress signals. Plants Physiol. 162, 1566–1582.
Deuschle, K., Funck, D., Forlani, G., Stransky, H., Biehl, A., Leister, D., van der Graaff, E. et al. (2004) The role of [Delta]-pyrroline-5-carboxylate dehydrogenase in proline degradation. Plant Cell, 16, 3431–3452.
Du, H., Feng, B.R., Yang, S.S., Huang, Y.B. and Tang, Y.X. (2012a) The R2R3-MYB transcription factor gene family in maize. PLoS ONE, 7, e37463.
Du, H., Yang, S.S., Liang, Z., Feng, B.R., Liu, L., Huang, Y.B. and Tang, Y.X. (2012b) Genome-wide analysis of the MYB transcription factor superfamily in soybean. BMC Plant Biol. 12, 106.
Dubos, C., Stracke, R., Grotewold, E., Weisshaar, B., Martin, C. and Lepiniec, L. (2010) MYB transcription factors in Arabidopsis. Trends Plant Sci. 15, 573–581.
Gampaia, S.S., Hagenbeek, D. and Rock, C.D. (2001) Functional interactions of lantanum and phospholipase D with the abisic acid signaling effectors VP1 and ABI1-1 in rice protoplasts. J. Biol. Chem. 276, 9855–9860.
Grotewold, E., Drummond, B.J., Bowen, B. and Peterson, T. (1994) The MYB-homologous P gene controls phlophane pigment in maize floral organs by directly activating a flavonoid biosynthetic gene subset. Cell, 76, 543–553.
Haring, M., Offermann, S., Daniker, T., Horst, I., Petersbanzel, C. and Stam, M. (2007) Chromatin immunoprecipitation: optimization, quantitative analysis and data normalization. Plant Methods, 3, 11.
He, Y., Li, W., Li, J., Jia, Y., Wang, M. and Xia, G. (2012) Ectopic expression of a wheat MYB transcription factor gene, TaMYB73, improves salinity stress tolerance in Arabidopsis thaliana. J. Exp. Bot. 63, 1511–1522.
Ithal, N. and Reddy, A.R. (2004) Rice flavonoid pathway genes, OsDfr and OsAn, are induced by dehydration, high salt and ABA, and contain stress responsive promoter elements that interact with the transcription activator, Os1-CMYB. Plant Sci. 166, 1505–1513.
Ji, X., Zheng, L., Liu, Y., Nie, X., Liu, S. and Wang, Y. (2014) A transient transformation system for the functional characterization of genes involved in stress response. Plant Mol. Biol. Rep. 32, 732–739.
Kim, W.C., Ko, J.H. and Han, K.H. (2012) Identification of a cis-acting regulatory motif recognized by MYB46, a master transcriptional regulator of secondary wall biosynthesis. Plant Mol. Biol. 78, 489–501.
Kim, J.H., Hyun, W.Y., Nguyen, H.N., Jeong, C.Y., Xiong, L., Hong, S.W. and Lee, H. (2015) AtMYB7, a sub-group 4 R2R3 Myb, negatively regulates ABA-induced inhibition of seed germination by blocking the expression of the ZIP transcription factor ABI5. Plant, Cell Environ. 38, 559–571.
Ko, J.H., Kim, W.C. and Han, K.H. (2009) Ectopic expression of MYB46 identifies transcriptional regulatory genes involved in secondary wall biosynthesis in Arabidopsis. Plant J. 60, 649–665.
Li, J., Li, X., Guo, L., Lu, F., Feng, X., He, K., Wei, L. et al. (2006) A subgroup of MYB transcription factor genes undergoes highly conserved alternative splicing in Arabidopsis and rice. J. Exp. Bot. 57, 1263–1273.
Liang, Y.K., Dubos, C., Dodd, I.C., Holroyd, G.H., Hetherington, A.M. and Campbell, M.M. (2005) AtMYB61, an R2R3-MYB transcription factor controlling stomatal aperture in Arabidopsis thaliana.Curr. Biol. 15, 1201–1206.
Lichtenthaler, H.K. (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol. 148, 350–382.
Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods, 25, 402–408.
Ma, Q., Dai, X., Xu, Y., Guo, J., Liu, Y., Chen, N., Xiao, J. et al. (2009) Enhanced tolerance to chilling stress in OsMYB3R-2 transgenic rice is mediated by alteration in cell cycle and ectopic expression of stress genes. Plant Physiol. 150, 244–256.
Mao, X., Zhang, H., Qian, X., Li, A., Zhao, G. and Jing, R. (2012) TaNAC2, a NAC-type wheat transcription factor conferring enhanced multiple abiotic stress tolerances in Arabidopsis. J. Exp. Bot. 63, 2933–2946.
Mare, C., Mazzucotelli, E., Crosati, C., Francia, E., Stanca, A.M. and Cattivelli, L. (2004) Hv-WRKY38: a new transcription factor involved in cold- and drought-response in barley. Plant Mol. Biol. 55, 389–416.
McCarthy, R.L., Zhong, R. and Ye, Z.H. (2009) MYB83 is a direct target of SNID1 and acts redundantly with MYB46 in the regulation of secondary cell wall biosynthesis in Arabidopsis. Plant Cell Physiol. 50, 1950–1964.
McCarthy, R.L., Zhong, R., Fowler, S., Lyskowski, D., Pyaensia, H., Carleton, K., Spicer, C. et al. (2010) The poplar MYB transcription factors, PtMYB83 and PtMYB20, are involved in the regulation of secondary wall biosynthesis. Plant Cell Physiol. 51, 1084–1090.
Mette, M.F., Auersat, W., van der Winden, J., Matzke, M.A. and Matzke, A.J. (2000) Transcriptional silencing and promoter methylation triggered by double-stranded RNA. The EMBO journal, 19, 5194–5201.
Newman, L.I., Perazza, D.E., Juska, J. and Campbell, M.M. (2004) Involvement of the R2R3-MYB, ATMYB61, in the ectopic lignification and dark-photomorphogenic components of the del3 mutant phenotype. Plant J. 37, 239–250.
Oh, J.E., Kwon, Y., Kim, J.H., Noh, H., Hong, S.W. and Lee, H. (2011) A dual role for MYB60 in stomatal regulation and root growth of Arabidopsis thaliana under drought stress. Plant Mol. Biol. 77, 91–103.
Park, M.Y., Kang, J.Y. and Kim, S.Y. (2011) Overexpression of AtMYB52 confers ABA hypersensitivity and drought tolerance. Mol. Cells, 31, 447–454.
Pasquali, G., Bricolli, S., Locatelli, F., Baldoni, E. and Mattana, M. (2008) Osmor, expression improves adaptive responses to drought and cold stress in transgenic apples. Plant Cell Rep. 27, 1677–1686.

Prouse, M.B. and Campbell, M.M. (2012) The interaction between MYB proteins and their target DNA binding sites. Biochim. Biophys. Acta, 1819, 67–77.

Qi, T., Huang, H., Song, S. and Xie, D. (2015) Regulation of jasmonate-mediated stamen development and seed production by a bHLH-MYB complex in Arabidopsis. Plant Cell, 27, 1620–1633.

Romano, J.M., Dubos, C., Prouse, M.B., Wilkins, O., Hong, H., Poole, M., Kang, K.Y. et al. (2012) AtMYB61, an R2R3-MYB transcription factor, functions as a pleiotropic regulator via a small gene network. New Phytolog. 195, 774–786.

Sergeev, I., Alexieva, V. and Karanov, E. (1997) Effect of spermine, atrazine and 2,4-D on zucchini seed germination and seedling emergence. Compt. Rend. Acad. Bulg. Sci. 50, 121–124.

Shavrukov, Y. (2013) Salt stress or salt shock: which genes are we studying? J. Exp. Bot. 64, 119–127.

Su, L.T., Li, J.W., Liu, D.Q., Zhai, Y., Zhang, H.J., Li, X.W., Zhang, Q.L. et al. (2014) A novel MYB transcription factor, GmMYB11, from soybean confers drought and cold tolerance in Arabidopsis thaliana. Gene, 538, 46–55.

Sun, X., Gong, S.Y., Nie, X.Y., Li, W., Huang, H.Q. and Li, X.B. (2015) A R2R3-MYB transcription factor that is specifically expressed in cotton (Gossypium hirsutum) fibers affects secondary cell wall biosynthesis and deposition in transgenic Arabidopsis. Physiol. Plant. 154, 420–432.

Uno, Y., Furihata, T., Abe, H., Yoshida, R., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2000) Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. Proc. Natl Acad. Sci. USA, 97, 11632–11637.

Vannini, C., Locatelli, F., Bracale, M., Magnani, E., Marsoni, M., Osnato, M., Mattana, M. et al. (2004) Overexpression of the rice OsMYB1 gene increases chilling and freezing tolerance of Arabidopsis thaliana plants. Plant J. 37, 115–127.

Wang, L., Xu, C., Wang, C. and Wang, Y. (2012) Characterization of a eukaryotic translation initiation factor 5A homolog from Tamarix androssowii involved in plant abiotic stress tolerance. BMC Plant Biol. 12, 118.

Wang, C., Zhang, N., Gao, C., Cui, Z., Sun, D., Yang, C. and Wang, Y. (2014) Comprehensive transcriptome analysis of developing xylem responding to artificial bending and gravitational stimuli in Betula platyphylla. PLoS ONE, 9, e87566.

Whiting, P., Favis, B., St-Germain, F. and Goring, D. (1981) Fractional separation of middle lamella and secondary wall tissue from spruce wood. J. Wood Chem. Technol. 1, 29–42.

Xiong, H., Li, J., Liu, P., Duan, J., Zhao, Y., Guo, X., Li, Y. et al. (2014) Overexpression of OsMYB48-1, a novel MYB-related transcription factor, enhances drought and salinity tolerance in rice. PLoS ONE, 9, e92913.

Xu, R., Wang, Y., Zheng, H., Lu, W., Wu, C., Huang, J., Yan, K. et al. (2015) Salt-induced transcription factor MYB74 is regulated by the RNA-directed DNA methylation pathway in Arabidopsis. J. Exp. Bot. 66, 5997–6008.

Yanaihara, K., Xiaoyuan, Y., Kun, H., Meihua, L., Jigang, L., Zhaofeng, G., Zhiquiang, L. et al. (2006) The MYB transcription factor superfamily of Arabidopsis: expression analysis and phylogenetic comparison with the rice MYB family. Plant Mol. Biol. 60, 107–124.

Zhang, X., Wang, L., Meng, H., Wen, H., Fan, Y. and Zhao, J. (2011) Maize ABP9 enhances tolerance to multiple stresses in transgenic Arabidopsis by modulating ABA signaling and cellular levels of reactive oxygen species. Plant Mol. Biol. 75, 365–378.

Zhang, R., Yang, C., Wang, C., Wei, Z., Xia, D., Wang, Y., Liu, G. et al. (2012) Time-course analyses of abscisic acid level and the expression of genes involved in abscisic acid biosynthesis in the leaves of Betula platyphylla. Mol. Biol. Rep. 39, 2505–2513.

Zhong, R., Demura, T. and Ye, Z.H. (2006) SND1, a NAC domain transcription factor, is a key regulator of secondary wall synthesis in fibers of Arabidopsis. Plant Cell, 18, 3158–3170.

Zhong, R., Richardson, E.A. and Ye, Z.H. (2007) The MYB46 transcription factor is a direct target of SND1 and regulates secondary wall biosynthesis in Arabidopsis. Plant Cell, 19, 2776–2792.

Zhong, R., McCarthy, R.L., Hahngight, M. and Ye, Z.H. (2013) The poplar MYB master switches bind to the SMRE site and activate the secondary wall biosynthetic program during wood formation. PLoS ONE, 8, e69219.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Sequence alignments of BplMYB46 with other plant MYBs.

Figure S2 Phylogenetic comparison of BplMYB46 with other plant MYBs.

Figure S3 Relative expression of BplMYB46 in the OE and SE lines.

Table S1 Promoter motifs of genes mediating abiotic stress responses and lignin biosynthesis regulated by BplMYB46.

Table S2 Primer sequences used in the construction of pROK2-BplMYB46-GFP.

Table S3 Primer sequences used to amplify the whole or truncated CDS of BplMYB46 in the transactivation assay.

Table S4 Primer sequences of genes analyzed by real-time RT-PCR.

Table S5 Primer sequences used in the Y1H assay.

Table S6 Primer sequences used in the construction of the reporter constructs analyzed in tobacco plants.

Table S7 Primer sequences used in the construction of the reporter constructs to verify the results of the ChIP assay.

Table S8 Primer sequences used in the construction of BplMYB46 overexpression and silencing lines.

Table S9 Primer sequences used in the analysis of BplMYB46 expression in BplMYB46 overexpression and silencing lines.

Table S10 Primer sequences used in the analysis of BplMYB46 target genes using real-time RT-PCR.

Table S11 Primer sequences used in ChIP-PCR analysis.