Bean Metal-Responsive Element-Binding Transcription Factor Confers Cadmium Resistance in Tobacco

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Cadmium (Cd) is highly toxic to plants. Modulation of Cd-responsive transcription is an important way for Cd detoxification in plants. Metal-responsive element (MRE) is originally described in animal metallothionein genes. Although functional MREs also exist in Cd-regulated plant genes, specific transcription factors that bind MRE to regulate Cd tolerance have not been identified. Previously, we showed that Cd-inducible bean (Phaseolus vulgaris) stress-related gene 2 (PsSR1) produces a short (S-PsSR1) transcript (S-PsSR2) driven by an intronic promoter. Here, we demonstrate that S-PsSR2 encodes a bean MRE-binding transcription factor 1 (PvMTF-1) that confers Cd tolerance in tobacco (Nicotiana tabacum). PvMTF-1 expression was up-regulated by Cd at the levels of RNA and protein. Importantly, expression of PvMTF-1 in tobacco enhanced Cd tolerance, indicating its role in regulating Cd resistance in plants. This was achieved through direct regulation of a feedback-insensitive Anthranilate Synthase α-2 chain gene (ASA2), which catalyzes the first step for tryptophan biosynthesis. In vitro and in vivo DNA-protein interaction studies further revealed that PvMTF-1 directly binds to the MRE in the ASA2 promoter, and this binding depends on the zinc finger-like motif of PvMTF-1. Through modulating ASA2 up-regulation by Cd, PvMTF-1 increased free tryptophan level and subsequently reduced Cd accumulation, thereby enhancing Cd tolerance of transgenic tobacco plants. Consistent with this observation, tobacco transiently overexpressing ASA2 also exhibited increased tolerance to Cd. We conclude that PvMTF-1 is a zinc finger-like transcription factor that links MRE to Cd resistance in transgenic tobacco through activation of tryptophan biosynthesis.

Cadmium (Cd) is a nonessential metal that negatively affects plant growth and development. At molecular level, plants change transcription profiles in response to Cd; for example, upon exposure of Arabidopsis (Arabidopsis thaliana) to Cd, the expression of those genes responsible for Cd uptake, transport, and detoxification is significantly changed (Weber et al., 2006). Thus, modulation of Cd-responsive transcription is an important way for Cd detoxification in plants. In animals, Cd-mediated transcription has been well characterized in metallothionein (MT) genes. These genes contain metal-responsive elements (MREs), which largely determine their transcription level upon Cd treatment (Stuart et al., 1985). MRE consists of a highly conserved heptanucleotide core (5'-TGCGNC-3'; Culotta and Hamer, 1989). Zinc finger-containing MRE-binding transcription factor 1 (MTF-1) is capable of binding MRE to drive Cd-induced expression of MT (Radtke et al., 1993). However, how Cd mediates transcription in plants largely remains unknown.

Interestingly, MRE also exists in the promoter regions of many Cd-responsive plant genes, such as Pseudotsuga menziesii metallothionein-like gene (PmMT; Chatthai et al., 2004), rice (Oryza sativa) metallothionein gene (ricMT; Lü et al., 2007), rice class I-4b metallothionein gene (OsMT-I-4b; Dong et al., 2010), and bean (Phaseolus vulgaris) stress-related gene 2 (PsSR2; Qi et al., 2007a). Moreover, in rice, many Cd-responsive miRNA-encoding genes contain MREs in their promoters (Ding et al., 2011), suggesting that MREs may also be involved in Cd-induced transcription of noncoding genes in plants.

Several studies have defined plant MREs as functional heavy metal-responsive elements. In the context of a minimal Cauliflower mosaic virus 35S promoter, the 35-bp PsSR2 promoter fragment containing a MRE can activate the expression of the GUS reporter gene in a metal-inducible manner in tobacco (Nicotiana tabacum) protoplasts (Qi et al., 2007a). A 137-bp ricMT promoter fragment containing a MRE is required for copper-activated expression of GUS in transgenic Arabidopsis seedlings (Lü et al., 2007). The OsMT-I-4b promoter contains four copies of MREs and can confer heavy metal-inducible expression of GUS in Arabidopsis plants (Dong et al., 2010). Although no plant MRE-binding proteins have been identified yet, these evidences strongly suggest that there must be some transcription factors directly binding MREs in these plants. In this study, we sought to identify such transcription factors that link MRE to Cd tolerance and thus extend our understanding of MRE-mediated transcription upon Cd exposure in plants.

In plants, one strategy for improving tolerance to heavy metals, such as Cd, is to alter cellular metabolism leading to accumulation of amino acids and amino acid-derived molecules (for review, see Sharma and Dietz, 2006). Recently, the Trp biosynthesis involved in
Cd detoxification has emerged in plants. Increasing Trp biosynthesis in vivo or application of Trp in vitro can significantly improve Cd tolerance in Arabidopsis and tomato (Solanum lycopersicum) plants (Sanjaya et al., 2008). This is achieved by reducing Cd accumulation and enhancing antioxidant defense in plants. Trp is synthesized through a five-enzyme pathway. Anthranilate synthase (AS) mediates the first step of Trp synthesis, but its activity can be inhibited by Trp through a negative feedback loop. Interestingly, in some plant species such as tobacco, a feedback-insensitive form of AS (ASA2) is identified (Song et al., 1998). Overexpression of ASA2 gene results in high level of free Trp in transgenic tobacco (Tsa et al., 2005). Thus, ASA2 is likely a Cd tolerance-related gene in tobacco through regulation of Trp biosynthesis.

Our previous study demonstrates that bean PsoSR2 is a heavy metal-inducible gene that confers Cd tolerance in tobacco plants (Zhang et al., 2001; Chai et al., 2003; Qi et al., 2007a). Recently, we showed that PsoSR2 produces a short (S) PsoSR2 transcript (S-PsoSR2) driven by an intronic promoter (Qi et al., 2007b). In this study, we demonstrated that the expression of S-PsoSR2 is regulated by Cd treatment and alternative polyadenylation, respectively. S-PsoSR2 encodes a Cd-resistant transcription factor that specifically binds to a MRE of ASA2 in transgenic tobacco plants. Through modulating up-regulation of ASA2 by Cd, this MRE-binding transcription factor increases Trp biosynthesis and thus enables transgenic tobacco plants to accumulate less Cd compared with the wild-type tobacco plants. These findings provide a new plant MRE-binding transcription factor that links MRE to Cd tolerance in planta and cast a new light in understanding plant MRE-mediated transcription upon Cd exposure.

RESULTS

S-PsoSR2 Expresses Two Alternatively Polyadenylated Isoforms

Our previous studies demonstrated that bean PsoSR2 gene, which contains a single intron, generates two alternative transcripts (PsoSR2 and S-PsoSR2) by alternative transcription start site (TSS; Qi et al., 2007a, 2007b). PsoSR2 transcript originates from the upstream TSS and encodes a 200-amino acid PsoSR2 protein (Qi et al., 2007b), whereas S-PsoSR2 is produced by an intronic TSS (Fig. 1A). Cd has been shown to activate additional TSS in maize (Zea mays) Bronze2 (Bz2) gene (Mars and Walbot, 1997). Thus, we were interested to know whether Cd stress changes the TSS of PsoSR2. By using 5′ RACE analysis, we found that unlike Bz2 gene, Cd stress did not affect the usage of TSS in PsoSR2 (Supplemental Fig. S1). Although no additional TSS was identified in PsoSR2, the intronic TSS of PsoSR2 defined in this 5′ RACE is located 54 bp upstream of the originally mapped one (Supplemental Fig. S2). This suggested that the original intronic TSS, which was determined in the transgenic tobacco (Qi et al., 2007b), did not represent the true 5′ end of S-PsoSR2 in its native species.

Furthermore, we cloned the full-length PsoSR2 and S-PsoSR2 complementary DNAs (cDNAs), respectively, which allowed us to identify two S-PsoSR2 isoforms (S-PsoSR2-1 and S-PsoSR2-2; Fig. 1A). S-PsoSR2-1 and S-PsoSR2-2 use the distal and proximal polyadenylation site (PAS) in their 3′ untranslated regions, respectively. Relative to S-PsoSR2-2, S-PsoSR2-1 was preferentially expressed even under Cd stress, suggesting that S-PsoSR2 mainly used the distal PAS and Cd stress did not influence the PAS utilization. Meanwhile, unlike S-PsoSR2, PsoSR2 transcript only uses the distal PAS (Fig. 1A). These findings indicated that S-PsoSR2 but not PsoSR2 is regulated by alternative polyadenylation.

S-PsoSR2 Encodes a New Cd-Responsive Protein

Compared with the PsoSR2 transcript, S-PsoSR2 transcript excludes the exon 1 and contains a 126-nucleotide alternative intron fragment as its first exon (lowercase letters in Supplemental Fig. S2). Although two alternatively polyadenylated S-PsoSR2 transcripts are presented in bean, they contain distinct 3′ untranslated region while sharing the same open reading frame (ORF; +65 to +247, red letters in Supplemental Fig. S2). This ORF encodes a 60-amino acid small protein (Fig. 1A). Because this protein can function as a MRE-binding transcription factor, we hereafter named it PvMTF-1. BLASTP analysis showed no protein with significant homology to PvMTF-1 in the GenBank database, suggesting that PvMTF-1 is a newly identified small protein.

To further characterize this new PvMTF-1 protein, we generated its antibody against a synthetic peptide (underlined residues in Fig. 1A) corresponding to its C terminus. By using this antibody, we were able to detect an approximately 7-kD weak band representing PvMTF-1(6.86 kD) with the protein extract of bean leaf (Fig. 1B), demonstrating that S-PsoSR2 transcript can be translated into a protein. However, we cannot rule out other proteins encoded by S-PsoSR2. In fact, S-PsoSR2 transcript also contains other two ORFs (Supplemental Fig. S2), i.e. ORF2 (+196 to +699) and ORF3 (+557 to +796), which may encode a N-terminal truncated PsoSR2 protein and a 79-amino acid protein, respectively. Future studies will be interesting to verify whether S-PsoSR2 can be translated into these two proteins.

Interestingly, PvMTF-1 protein accumulation in Cd-treated bean leaves was strongly enhanced (Fig. 1B). This may be due to high accumulation of S-PsoSR2 transcript after Cd treatment. To test this, we performed quantitative reverse transcription (qRT)-PCR analysis of S-PsoSR2 and S-PsoSR2 transcripts on bean seedlings without or with Cd treatment (Fig. 1C). Similar to the positive control PsoSR2 (19-fold induction by Cd), S-PsoSR2 accumulation exhibited a 14-fold induction by Cd stress.

To rule out the possibility of qRT-PCR products of S-PsoSR2 resulted from PsoSR2 splicing variants such as unspliced transcript and/or contaminating genomic DNA, we performed reverse transcription (RT)-PCR splicing analysis combining with a minus RT control PCR.
The result showed that only one band corresponding to the full-length PvSR2 was detected both in normal and Cd-treated bean plants (Supplemental Fig. S3), suggesting that PvSR2 gene did not undergo alternative splicing and S-PvSR2 is not a PvSR2 splice variant. This is consistent with our previous finding that Cd exposure fails to inhibit the PvSR2 splicing in bean seedlings (Qi et al., 2009). Moreover, no amplification products in the minus RT control PCR verified the absence of genomic DNA contamination (Supplemental Fig. S3).
**PvMTF-1 Functions as a Transcriptional Activator**

The subcellular localization of a protein indicates its biological function. Therefore, we fused PvMTF-1 with GFP and found that it was exclusively localized in the nucleus, whereas the mock GFP control was restricted to the cytoplasm (Fig. 1D). This finding supported that PvMTF-1 may be involved in transcriptional regulation.

To further confirm whether PvMTF-1 plays a role as a transcriptional activator, we performed a transcriptional activity assay in yeast (Saccharomyces cerevisiae) cells (Fig. 1E). The C-terminal activation domain (CTAD) of Arabidopsis heat-shock transcription factor A2 was used as a positive control (Liu et al., 2013). Similar to CTAD, PvMTF-1 was able to activate the expression of HIS3 or LacZ reporter gene. Combining all these data, we concluded that it acts as a transcription activator.

**Expression of PvMTF-1 Enhances Cd Tolerance in Tobacco**

Because PvMTF-1 functions as a Cd-responsive transcription activator, we further examined whether PvMTF-1 has a functional relevance in Cd tolerance of plants. To achieve this, 35S::PvMTF-1 was introduced into tobacco plants, and two individual transgenic T1 lines (L2 and L4) with a single insert were selected for further analysis. Western-blot analysis using anti-PvMTF-1 antibody confirmed the exogenous expression of PvMTF-1 protein in L2 and L4 but not in wild-type control (Fig. 2A). In addition, L2 and L4 exhibited no phenotypic differences compared with wild-type plants under normal growth condition. However, after CdCl₂ treatment, both survival rate (Fig. 2B) and chlorophyll content (Fig. 2C) of L2 or L4 were significantly higher than those of wild-type plants. This finding demonstrated the capability of PvMTF-1 in conferring tobacco plants Cd resistance.

**Identification of PvMTF-1 Target Genes in Tobacco by ChIP-Cloning Assay**

To elucidate the molecular mechanism as to how PvMTF-1 mediates Cd resistance in tobacco plants, we sought to identify the downstream targets of PvMTF-1 in tobacco and determined which of these are involved in Cd resistance. To address this, we generated transgenic tobacco plants constitutively expressing GFP-tagged PvMTF-1 protein driven by 35S (35S::PvMTF-1-GFP) and chose a homozygous line to perform chromatin immunoprecipitation (ChIP)–cloning assay. Western-blot analysis using anti-GFP antibody confirmed the expression of PvMTF-1-GFP fusion protein in this line but not in wild-type control (Fig. 3A).

The DNA fragments immunoprecipitated by anti-GFP antibody were blunted and then subjected to cloning. Through sequencing inserted DNA of 113 positive clones, we obtained 16 known tobacco genomic DNA sequences (Supplemental Table S1). Three out of the 16 sequences were matched to the promoter regions of three genes, including ASA2 (~1,222/−1,081, relative to the TSS; Song et al., 1998), Nicotiana plumbaginifolia nectarin 1

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**Figure 2.** Expression of PvMTF-1 improves Cd tolerance in tobacco plants. A, An immunoblot (IB) of leaf proteins with anti-PvMTF-1 antiserum confirmed the presence of PvMTF-1 protein in L2 and L4 but not in wild-type (WT) tobacco. Large unit of Rubisco (Rubisco L) stained with Coomassie Brilliant Blue served as a loading control. B, The 3-week-old seedlings were sowed on a one-half-strength MS liquid medium without (Control) and with 450 μM CdCl₂ for survival rate assay after 5 d. C, The 4-week-old seedlings were sowed on a one-half-strength MS liquid medium without (Control) and with 400 μM CdCl₂ for chlorophyll content analysis after 2 d. The data represent means ± SD of three independent experiments. Significance between experimental values was assessed by Student’s t test (*P < 0.05; **P < 0.01). FW, Fresh weight.
precursor (NEC1) gene (−1,233/−1,041, relative to the translational start codon), and *Nicotiana tabacum extracellular invertase Nin88* (Nin88) gene (−2,457/−2,694, relative to the translational start codon; Goetz et al., 2001). Because Trp biosynthesis is related to Cd tolerance in plants (Sanjaya et al., 2008) and ASA2 is a key enzyme involved in Trp biosynthesis (Song et al., 1998; Tsai et al., 2005), we selected ASA2 as a potential Cd tolerance-related gene for further study.

**PvMTF-1 Modulates ASA2 Up-Regulation by Cd**

Next, we investigated the regulation of ASA2 expression by PvMTF-1. The mRNA levels of ASA2 were
measured by qRT-PCR in L2, L4, and wild-type control. As shown in Figure 3B, treatment of tobacco seedlings with 300 μM CdCl₂ resulted in a more than 4-fold increase of ASA2 in L2 and L4 compared with wild-type control, although ASA2 expression was unaltered under normal condition. The increased ASA2 expression mediated by PvMTF-1 was not due to Cd treatment, because ASA2 was not Cd inducible (Fig. 3B).

Overexpression of ASA2 in tobacco confers resistance to the toxic Trp analog 5-methyl-tryptophan (5-MT; Tsai et al., 2005). Thus, we examined whether ASA2-elevated L2 or L4 is conferred ability to resist 5-MT stress (Fig. 3C). Under normal condition, similar with wild-type seedlings, the root growth of L2 or L4 seedlings exhibited increased sensitivity to 200 or 400 μM 5-MT stress. However, in the presence of 100 μM CdCl₂, 5-MT-treated L2 and L4 seedlings showed no statistical difference in root length compared with untreated controls. Under the same conditions, the root growth of wild-type seedlings was also more sensitive to 5-MT stress. These results suggested that L2 and L4 confer tolerance to 5-MT in a Cd-dependent manner. This 5-MT tolerance phenotype together with qRT-PCR results suggested that PvMTF-1-activated ASA2 expression requires Cd stress.

Cd Stress Fails to Enhance the Binding of PvMTF-1 to the ASA2 Promoter

To test whether Cd-dependent up-regulation of ASA2 driven by PvMTF-1 is due to an increased binding of PvMTF-1 to the ASA2 promoter, we performed ChIP-PCR experiment in the 35S::PvMTF-1-GFP transgenic tobacco line treated with or without Cd stress (Fig. 3D). PCR analysis showed that the promoter region mapping at −1,222 and −1,081 (named ASA2p and confirmed in ChIP-cloning assay), but not the coding region of ASA2 (+1600/+1790, named ORF), was detected in chromatin DNA immunoprecipitated by the anti-GFP antibody. There was no ASA2p or ORF detected in the mock IgG control. Furthermore, quantitative PCR (qPCR) analysis revealed significant enrichment of PvMTF-1 at the ASA2p (4- and 3.65-fold enrichment compared with that of the ORF in control and Cd-treated samples, respectively). For the enrichment of PvMTF-1 binding on ASA2p, no statistically significant difference was observed between control and Cd-treated samples, suggesting that Cd stress did not significantly increase the binding of PvMTF-1 to the ASA2p in vivo. These findings demonstrated that Cd stress is not capable of enhancing the binding of PvMTF-1 to the ASA2 promoter.

PvMTF-1 Specifically Recognizes the MRE of ASA2 Promoter

Next, we determined the binding sites of PvMTF-1 in the ASA2p. We noted a MRE (−1,111 to −1,105, 5′-TGCA-CAC-3′) within the ASA2p (Fig. 4A). Because PvMTF-1 is a Cd-resistant transcription factor, we speculated that PvMTF-1 might bind to the ASA2p via the MRE. To address this, we performed yeast one hybrid (Y1H) combining electrophoretic mobility shift assay (EMSA) to check whether PvMTF-1 directly interacts with the MRE of ASA2p. Results showed that PvMTF-1 was able to bind to the ASA2p (Fig. 4B), which is consistent with our ChIP-PCR data (Fig. 3D). MREd is the strongest MRE of the mouse MT-I promoter (Stuart et al., 1985). Point mutations within MREd (5′-TGCACTC-3′ mutated to 5′-aatAaT-3′) completely abolish its regulatory activity (Culotta and Hamer, 1989). Therefore, we introduced the same point mutations within the MRE of ASA2p (5′-TGCA-CAC-3′ mutated to 5′-aatAaT-3′, named REMut) and found that the ASA2p with REMut (named mASA2p; Fig. 4A) was not recognized by PvMTF-1 in Y1H assay (Fig. 4B). This result suggested that PvMTF-1 interacts directly with the ASA2p via its MRE. Moreover, we used three tandem repeats (named 3×MRE) of 20-bp MRE-containing promoter region (−1,118/−1,099, boxed in Fig. 4A) as bait and found that PvMTF-1 could bind to 3×MRE but not to its mutated sequence (named 3×REMut; Fig. 4C). Thus, Y1H combined with mutation analysis confirmed a direct binding of PvMTF-1 to the MRE within the ASA2p.

We constructed a fusion protein with a His tag (His-6-PvMTF-1, 11.88 kD) in Escherichia coli for EMSA. The biotin-labeled 3×MRE and 3×REMut were used as the probes. A weak band with similar size to that of His-6-PvMTF-1 was detected by Coomassie Brilliant Blue staining in the mock control proteins extracted from E. coli expressing empty vector (Fig. 4D), suggesting that the endogenous E. coli proteins were copurified with His-6-PvMTF-1. Thus, we use it as a negative control in EMSA to avoid possible false-positive results. Western-blot analysis confirmed the presence of His-6-PvMTF-1 protein in purified proteins (Fig. 4D). Shifted complex was observed between His-6-PvMTF-1 and 3×MRE probes (Fig. 4E, lane 5) but not 3×REMut probes (Fig. 4E, lane 8). This binding was completely abolished by 50-fold molar excess of unlabeled competitor probes (Fig. 4E, lane 7), demonstrating a specific binding. As expected, mock proteins produced no bands with 3×MRE or 3×REMut probes (Fig. 4E, lanes 3 and 4). This in vitro evidence, together with results obtained by ChIP and Y1H in vivo, demonstrate that PvMTF-1 is an MRE-binding transcription factor.

Zinc Finger-Like Motif Is Required for the MRE-Binding of PvMTF-1

Next, we characterized the structure of PvMTF-1 involved in MRE-binding. Using PSleed server (http://bioinf.cs.ucl.ac.uk/psipred), we found that PvMTF-1 is a predicted helix-coil-helix protein (Fig. 5A). Interestingly, it appears to contain a putative Cys-4-zinc finger-like motif (Cys8-X11-Cys20-X45-Cys36-X8-Cys45). We therefore examined whether this putative zinc finger-like motif is necessary for the binding of PvMTF-1 on MRE. When we mutated the PvMTF-1 Cys-8 and Cys-45 residues to Ser-8 and Ser-45 residues, this mutated PvMTF-1 (PvMTF-1mut) failed to bind to the 3×MRE in Y1H (Fig. 5B) and EMSA analysis (Fig. 5C). These results
clearly indicate that zinc finger-like motif is essential for the MRE binding of PvMTF-1.

**PvMTF-1 Improves Cd Tolerance in Tobacco by Activation of Trp Biosynthesis**

We further investigated how PvMTF-1 confers tolerance to Cd in transgenic tobacco by regulating ASA2 expression. It has been shown that increasing free Trp content improves Cd tolerance in some plants such as Arabidopsis and tomato by reducing Cd accumulation (Sanjaya et al., 2008). Overexpression of ASA2 in tobacco increases free Trp content (Tsai et al., 2005). Given PvMTF-1-modulated ASA2 up-regulation by Cd, we hypothesized that PvMTF-1 improves Cd tolerance of tobacco by activating Trp biosynthesis. Under normal growth conditions, we did not detect any difference of free Trp content between wild-type and transgenic tobacco plants L2 or L4. Upon exposure to Cd, however, L2 or L4 had 3.5-fold higher level of free Trp than the wild type (Fig. 6A). This is consistent with the result that PvMTF-1-activated ASA2 expression requires Cd stress (Fig. 3, B and C). Moreover, L2 or L4 accumulated less Cd in shoots and especially in roots than the wild type (Fig. 6B). These results suggested that through modulating up-regulation of ASA2 by Cd, PvMTF-1 activates Trp biosynthesis and then reduces Cd accumulation, thus allowing Cd tolerance of transgenic tobacco. In agreement with these results, exogenous addition of Trp can also favor the growth of wild-type plants in Cd-containing medium (Fig. 6C).

In addition to ASA2, Trp synthase b subunit (TSB1) is another key enzyme mediating Trp biosynthesis, and it can confer tolerance to Cd by activation of Trp biosynthesis in plants (Sanjaya et al., 2008). Our qRT-PCR experiments revealed that PvMTF-1 did not significantly alter TSB1 expression (Supplemental Fig. S4), suggesting that PvMTF-1-mediated Trp biosynthesis in transgenic tobacco plant is not achieved by activating TSB1 expression.

**Transient Expression of ASA2 Improves Cd Tolerance in Tobacco Seedlings**

To provide evidence for a direct correlation between ASA2 protein and Cd tolerance in tobacco, we constructed C-terminal His-6-tagged ASA2 protein driven by 35S (35S:ASA2-His-6) and verified its functional relevance in Cd tolerance of tobacco seedlings by Agrobacterium tumefaciens-mediated transient expression. GUS reporter gene under the control of 35S (35S:GUS) was used to

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**Figure 4.** PvMTF-1 directly binds to the MRE of ASA2 promoter. A. The sequence of ASA2p surrounding a MRE (red case). Mutations introduced into the mASA2p are shown in lowercase. B. Y1H binding assay of PvMTF-1 to ASA2p or mASA2p bait. Interaction was indicated by the ability of cells to grow on synthetic defined (SD) medium lacking Trp/Leu (SD-WL) and lacking Trp/Leu/Fus (SD-WLH) containing the indicated concentrations of 3-amino-1,2,4-triazole (3-AT), respectively. The Gal4 activation domain (AD) or fused with PvMTF-1 (AD-PvMTF-1) was used as prey. C. As in B, except that 3×MRE or 3×MREmut was used as bait. D. Immunoblot (IB) with anti-PvMTF-1 antiserum confirmed the production of His-6-PvMTF-1 in the purified Ni-NTA-bound His-6-PvMTF-1 proteins (His-6-PvMTF-1) but not in mock proteins (empty vector control). Staining gels ran in parallel with Coomassie Brilliant Blue (CBB) served as a loading control (bottom). E. EMSA assay performed using biotin-labeled 3×MRE and 3×MREmut as probes with His-6-PvMTF-1 or mock proteins. The arrowhead indicates the DNA-protein complex. All experiments were performed at least twice showing similar results.
showed similar phenotype under the same Cd stress conditions (Fig. 7D). These results suggested that expression of ASA2 increases Cd tolerance in tobacco plants.

**DISCUSSION**

**PsR2 Gene Is an Interesting Cd-Resistant Plant Gene Unit**

PsR2 is a Cd-regulated plant gene. Alternative TSS coupled with alternative PAS result in a full-length

![Figure 5. A zinc finger-like motif is essential for the PvMTF-1/MRE interaction. A, The predicted helix-coil-helix structure of PvMTF-1, showing a Cys-4 zinc finger-like motif. Cys residues are underlined. Mutations introduced into the PvMTF-1mut are shown in red. B, Y1H binding assay of AD-PvMTF-1mut to 3×MRE or 3×MREmut bait. AD-PvMTF-1 and AD were used as a positive control and a negative control, respectively. C, Immunoblot (IB) with an anti-His-6 antibody confirmed the production of His-6-PvMTF-1mut in the purified Ni-NTA-bound His-6-PvMTF-1mut proteins from *E. coli* cell lysate. His-6-PvMTF-1 was used as a positive control, whereas mock proteins (empty vector control) were used as a negative control. Staining gels ran in parallel with Coomassie Brilliant Blue (CBB) served as a loading control. These proteins were subjected to EMSA assay using biotin-labeled 3×MRE and 3×MREmut as probes. The arrowhead indicates the DNA-protein complex. All experiments were performed twice showing similar results.

![Figure 6. PvMTF-1 confers tobacco Cd tolerance by activation of Trp biosynthesis. A and B, Ten-week-old tobacco seedlings were grown in liquid MS media (Control) or same media with 600 μM CdCl₂ for 5 d (CdCl₂) and then were subjected to free Trp content and Cd content analysis, respectively. Data are means ± SD (*n* = 10) of three independent experiments. C, Two-week-old wild-type tobacco seedlings grown in MS agar media without (Control) or with 400 μM CdCl₂ (CdCl₂) or with 400 μM CdCl₂ and 100 μM Trp (CdCl₂ + Trp). The photographs were taken 7 d after treatment and then subjected to chlorophyll content analysis. Data are means ± SD (*n* = 6) of two independent experiments. Significance between experimental values was assessed by Student’s *t*-test (*P* < 0.05 and **P** < 0.01). FW, Fresh weight; DW, dry weight.

Figure 5. A zinc finger-like motif is essential for the PvMTF-1/MRE interaction. A, The predicted helix-coil-helix structure of PvMTF-1, showing a Cys-4 zinc finger-like motif. Cys residues are underlined. Mutations introduced into the PvMTF-1mut are shown in red. B, Y1H binding assay of AD-PvMTF-1mut to 3×MRE or 3×MREmut bait. AD-PvMTF-1 and AD were used as a positive control and a negative control, respectively. C, Immunoblot (IB) with an anti-His-6 antibody confirmed the production of His-6-PvMTF-1mut in the purified Ni-NTA-bound His-6-PvMTF-1mut proteins from *E. coli* cell lysate. His-6-PvMTF-1 was used as a positive control, whereas mock proteins (empty vector control) were used as a negative control. Staining gels ran in parallel with Coomassie Brilliant Blue (CBB) served as a loading control. These proteins were subjected to EMSA assay using biotin-labeled 3×MRE and 3×MREmut as probes. The arrowhead indicates the DNA-protein complex. All experiments were performed twice showing similar results.

monitor gene expression in transient expression assay. Detectable GUS activity in tobacco seedlings confirmed the successful transformation (Fig. 7A). We further confirmed the expression of ASA2-His-6 fusion proteins by western blot with anti-His-6 antibody (Fig. 7B). The size of the immunoblot band was smaller than the predicted one of ASA2-His-6 fusion protein (about 70 kD). This may be due to loss in N-terminal chloroplast transit peptide, because ASA2 is a chloroplast-localized protein and contains a 60-amino acid chloroplast transit peptide in its N terminus (Zhao and Last, 1995; Song et al., 1998). After Cd treatment, chlorophyll content in the seedlings expressing ASA2-His-6 was much higher than that of 35S:GFP vector control plants (Fig. 7C). Under the growth condition without Cd stress, however, no differences between chlorophyll content of two transgenic tobacco seedlings were detected. In agreement with this observation, PvMTF-1 transgenic tobacco seedlings (L2 and L4)

![Control](https://example.com/control.png) ![CdCl₂](https://example.com/cdcl2.png) ![CdCl₂+Trp](https://example.com/cdcl2+trp.png)

**Figure 6.** PvMTF-1 confers tobacco Cd tolerance by activation of Trp biosynthesis. A and B, Ten-week-old tobacco seedlings were grown in liquid MS media (Control) or same media with 600 μM CdCl₂ for 5 d (CdCl₂) and then were subjected to free Trp content and Cd content analysis, respectively. Data are means ± SD (*n* = 10) of three independent experiments. C, Two-week-old wild-type tobacco seedlings grown in MS agar media without (Control) or with 400 μM CdCl₂ (CdCl₂) or with 400 μM CdCl₂ and 100 μM Trp (CdCl₂ + Trp). The photographs were taken 7 d after treatment and then subjected to chlorophyll content analysis. Data are means ± SD (*n* = 6) of two independent experiments. Significance between experimental values was assessed by Student’s *t*-test (*P* < 0.05 and **P** < 0.01). FW, Fresh weight; DW, dry weight.
PvSR2 transcript and two alternatively polyadenylated S-PvSR2 transcripts from a single PvSR2 gene (Fig. 1A). Previous study has shown that the expression of a truncated PvSR2 protein lacking its N-terminal 33 amino acid residues improves Cd tolerance in tobacco plants (Chai et al., 2003). In this study, we revealed that PvMTF-1 encoded by S-PvSR2 also confers Cd tolerance in tobacco. Thus, through alternative promoter, PvSR2 gene produces generates two functionally related proteins that can confer Cd tolerance in transgenic tobacco plants.

Of note, PvSR2 expression is regulated at multiple levels including alternative TSS and PAS. Cd stress fails to change the TSS and PAS choice in PvSR2 gene, although Cd stress significantly increases accumulation level of PvSR2 transcripts. Moreover, Cd exposure does not inhibit the PvSR2 splicing. Therefore, Cd appears to involve several limited steps in PvSR2 expression such as the mRNA accumulation.

PvMTF-1 Is a New MRE-Binding Transcription Factor in Plants

Through examining the biological roles of S-PvSR2 transcript, we found that S-PvSR2 encodes a Cd-responsive and Cd-resistant transcription factor PvMTF-1 that specifically binds to a plant MRE. Several lines of evidence established in this work support our conclusion. First of all, PvMTF-1 is a new protein identified in bean, which is confirmed by immunoblot with anti-PvMTF-1 antibody. Secondly, PvMTF-1 is localized in the nucleus of plant cells and displays transactivation activity both in yeast and tobacco cells. Thirdly, PvMTF-1 specifically binds to the MRE of ASA2 promoter both in vivo and in vitro. Fourthly, expression of PvMTF-1 in transgenic tobacco enhances Cd tolerance through transcriptional regulation of Trp biosynthesis. Finally, PvMTF-1 expression is up-regulated by Cd stress.

Similar to the MRE of animal MT genes, several plant MREs are responsible for heavy metal-induced expression of reporter gene (Lü et al., 2007; Qi et al., 2007a; Dong et al., 2010). This indicates that MRE is a highly conserved MRE among plants and animals. MT genes are best understood examples of MRE-mediated transcription units in animals. However, how MRE mediates metal-inducible transcription of genes remains unclear in plants. And no MRE-binding transcription factors have been identified yet. PvMTF-1 may be a new plant MRE-binding transcription factor. It specifically binds to a MRE and confers Cd tolerance of tobacco. Thus, our findings in this study further extend the understanding of MRE-mediated transcription upon Cd exposure in plants.

MTF-1 is a unique MRE-binding transcription factor in animals. It contains six C2H2 zinc finger motifs that are essential for its MRE binding. Identifying MTF-1 homology through comparing sequence similarity in plants does not seem feasible. This indicates that plant MRE-binding transcription factors differ from animal MTF-1, although MRE is a conserved MRE between

Figure 7. Cd tolerance analysis of ASA2 in tobacco seedlings by A. tumefaciens-mediated transient expression. A, GUS staining in the seedlings expressing 35S:GUS but not untransformed control confirming the validity of transformation. B, Immunoblot (IB) with anti-His-6 antibody confirming the expression of His-6-tagged ASA2 proteins in transformed tobacco seedlings. Untransformed tobacco seedlings were used as a negative control. Coomassie Brilliant Blue (CBB) staining of protein was used to verify the loadings. C, Phenotype analysis of tobacco seedlings transformed with 35S:ASA2-His6 or with vector control (35S:GFP) in response to Cd. The transformed seedlings were sowed on a one-quarter-strength MS liquid medium without (-Cd) and with 200 μM CdCl2 (+Cd). The photographs were taken 3 d after treatment and then subjected to chlorophyll content analysis. Data are means ± so of three independent experiments. Asterisk indicates Student’s t test significant at P < 0.05. D, As in C, except that 2-week-old L2, L4, and wild-type (WT) control seedlings were used. FW, Fresh weight.
plants and animals. PvMTF-1 can functionally bind to a plant MRE but has no homology to MTF-1. Interestingly, it also contains a zinc finger-like motif, and this motif is essential for its MRE binding. Thus, PvMTF-1 appears to share similar structural features with MTF-1, but it is not a plant ortholog of MTF-1.

Of greater interest, however, is the Cd-regulated transcriptional activity of PvMTF-1. In the presence of Cd, PvMTF-1 activates the ASA2 expression (Fig. 3B), subsequently enhances 5-MT tolerance (Fig. 3C), and increases free Trp level (Fig. 6A) in transgenic tobacco plants. These data clearly suggest Cd stress activating PvMTF-1 transcriptional activity in tobacco. The molecular mechanism of how Cd activates the transcriptional activity of PvMTF-1 remains unknown. Cd stress fails to enhance the binding of PvMTF-1 to the ASA2 promoter (Fig. 3D). This indicates that PvMTF-1 activates the ASA2 expression through an unidentified mechanism rather than enhancing binding by Cd. One possibility is that PvMTF-1 protein binds Cd, which subsequently triggers the initiation of transcriptional activation. Alternatively, Cd exposure leads to PvMTF-1 modification (such as phosphorylation), which recruits PvMTF-1 to the ASA2 gene and thus regulates its transcription in tobacco. Identification and characterization of proteins interacting with PvMTF-1 in Cd-stressed tobacco may explain this hypothesis.

In addition to PvMTF-1, there are other Cd-responsive element-binding transcription factors in plants. The pas element of tobacco auxin-regulated gene parA is a known Cd-responsive element, and a nuclear protein complex called as-1-binding factor (ASF-1) could bind to this element (Kusaba et al., 1996). A tobacco basic leucine zipper transcription factor TGA2.2 was identified as the main component of ASF-1 (Niggeweg et al., 2000), suggesting that it is a potential pas element-binding transcription factor involved in Cd-inducible expression of parA gene.

**PvMTF-1 Is a New Cd-Resistant Transcription Factor**

Some transcription factors are Cd inducible in plants (Weber et al., 2006); however, few of them have been characterized in regulating Cd tolerance. Wheat (Triticum aestivum) heat shock transcription factorA4a and its rice ortholog confer Cd tolerance in transgenic rice seedlings by regulating MT gene expression (Shim et al., 2009). Indian mustard Brassica juncea Cd-regulated gene15 and its Arabidopsis ortholog TGA3 improve Cd tolerance in transgenic Arabidopsis and tobacco plants through the regulation of Cd uptake by roots (Farinati et al., 2010). In Arabidopsis plants, co-overexpression of basic helix-loop-helix (bHLH) transcription factor bHLH29 with bHLH38 or bHLH39 can enhance Cd tolerance via increasing Cd sequestration in roots (Wu et al., 2012). These Cd-resistant transcription factors confer Cd tolerance through different mechanisms, generally due to regulating different downstream target genes. There is no evidence that these transcription factors could bind to a MRE, indicating that they may recognize the cis-regulatory promoter elements rather than MRE. Unlike these Cd tolerant transcription factors, PvMTF-1 represents a new MRE-binding transcription factor and confers Cd tolerance through a new molecular mechanism (i.e. regulation of Trp biosynthesis).

**Transcription Factor Interacting with Metal-Responsive Elements Is of Universal Importance for Metal Homeostasis in Plants**

Several studies have suggested that transcription factors interacting with metal-deficiency response element is of universal importance for essential metal (i.e. iron, zinc, or copper) homeostasis in plants. In rice, the iron deficiency response element-binding transcription factor1, a cellular iron status sensor, regulates iron deficiency-induced genes for iron homeostasis (Kobayashi et al., 2007, 2012). In Arabidopsis, two basic leucine zipper transcription factors, bZIP19 and bZIP23, regulate the adaptation to zinc deficiency by binding to the zinc deficiency element of target genes (Assunção et al., 2010). In single-celled algae Chlamydomonas reinhardtii, copper response regulator CRR1 senses copper status and then activates genes necessary in periods of copper deficiency by binding to the copper-responsive element (Kropat et al., 2005; Sommer et al., 2010). SQUAMOSA PROMOTER BINDING PROTEIN-LIKE7 (SPL7), CRR1 ortholog in Arabidopsis, directly binds to copper-responsive elements within the promoter regions of copper homeostasis genes, such as microRNA398 (Yamasaki et al., 2009), FERRIC REDUCTASE OXIDASE4 (FRO4)/FRO5 (Bernal et al., 2012), and COPPER TRANSPORTER6 (COP6; Jung et al., 2012). Copper in vitro inhibits the DNA binding activity of SPL7 (Sommer et al., 2010), indicating that SPL7 can also act as a copper sensor in Arabidopsis cells.

Little is known about how transcription factors interact with MRE to regulate toxic metal detoxification in plants. One example well studied is that plant tolerance to aluminum toxicity on acid soils is transcriptionally regulated by zinc finger-containing transcription factors (Arabidopsis sensitive to proton rhizotoxicity1 and rice Al resistance transcription factor1) and Arabidopsis WRKY46 transcription factor (Iuchi et al., 2007; Yamaji et al., 2009; Ding et al., 2013). The cis-acting element of ART1 was identified in its 29 target genes involved in aluminum tolerance in rice (Tsutsui et al., 2011). WRKY46 negatively regulates the transcription of ALMT1, which encodes a malate efflux transporter responsible for Arabidopsis aluminum resistance, by directly binding to the known W-boxes of the ALMT1 promoter (Ding et al., 2013). Thus, at least for ART1 and WRKY46, these two aluminum resistance-related transcription factors are involved in aluminum detoxification by interacting with known cis-elements in plants.

In this study, we characterized an MRE-binding transcription factor PvMTF-1 in transgenic tobacco plants. Tobacco expressing PvMTF-1 has higher free Trp level and a reduced Cd accumulation, resulting in Cd tolerance of transgenic tobacco. Thus, PvMTF-1 links MRE to Cd resistance in transgenic tobacco. Our findings provide
strong evidence that interaction between Cd-responsive transcription factors (such as PvMTF-1) and Cd-responsive elements (such as MRE) is also involved in Cd detoxification in planta.

**MATERIALS AND METHODS**

**Primers**

Oligonucleotide primers used for PCR, RT-PCR, qPCR, construction of expression vectors, and sequencing are listed in Supplemental Table S2.

**Genetic Transformation**

For the 35S:PvMTF-1-GFP and the 35S:PvMTF-1, the ORF of PvMTF-1 was PCR amplified from bean (Phaseolus vulgaris) genomic DNA and cloned into the Ncol/SpeI of pCAMBIA1302 and the XhoI/SacI of pBI121, respectively.

Insert DNA sequences were verified by sequencing using T320 reverse primer and pBI121 forward primer, respectively. The resulting construct was introduced into tobacco (Nicotiana tabacum) by Agrobacterium tumefaciens-mediated method. Transferred DNA inheritance of transgenic tobacco lines (T0) was scored by hygromycin or kanamycin segregation analysis in the T1 generation.

**Plant Materials and Treatments**

Wild-type tobacco ‘W38’ plants or transgenic tobacco were grown in Murashige and Skoog (MS) medium under a 16-h-light (25°C)/8-h-dark (20°C) cycle. For Cd stress, 2-week-old 35S:PvMTF-1 transgenic lines (L2 and L4) and wild-type tobacco seedlings were transferred onto MS liquid medium (control) or the same medium supplemented with 300 μM CdCl₂ for 24 h in the greenhouse. For ChIP assay, the 35S:PvMTF-1-GFP transgenic seedlings were treated without (control) or with 300 μM CdCl₂ for 12 h.

The surface-sterilized bean ‘Saxa’ seeds were germinated on 1.5% (w/v) water agar at 25°C. When two true leaves expanded, the bean seedlings were transferred onto one-half-strength MS liquid medium for 5 d and then were subjected to the analysis of root length.

For the effects of Trp on tobacco growth, 2-week-old wild-type seedlings were transferred onto MS agar medium without or with 400 μM CdCl₂ or with 400 μM CdCl₂ and 100 μM Trp (Sigma). The photographs were taken 7 d after stress and then were subjected to the analysis of chlorophyll content.

**RNA Isolation and cDNA Synthesis**

Total RNA was extracted from plant material using the RNAprep pure plant kit with on-column DNase digestion (Tiangen Biotech) according to the manufacturer’s instructions. Bean Actin (Wen et al., 2005) and tobacco Actin (Rotenberg et al., 2006) were used as an internal control for bean gene (PvSR2 and S-ProtSR2) and tobacco gene (ASA2 and TSB1), respectively. The relative expression levels were analyzed using a delta-delta cycle threshold method.

**qRT-PCR Analysis**

qRT-PCR analysis was performed on a IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) with SYBR premix Ex-Taq kit (Takara) according to manufacturer’s instructions. Bean Actin (Wen et al., 2005) and tobacco Actin (Rotenberg et al., 2006) were used as an internal control for bean gene (PvSR2 and S-ProtSR2) and tobacco gene (ASA2 and TSB1), respectively. The relative expression levels were analyzed using a delta-delta cycle threshold method.

**5’ RACE and Cloning of Full-Length cDNAs**

The 5’ RACE of ProtSR2 and S-ProtSR2 were identified by using a SMARTer RACE cDNA amplification kit (Clontech). The following gene-specific primers were used: E2-R and E1/2-R for 5’ RACE of ProtSR2 and E2-R and AE1-R for 5’ RACE of S-ProtSR2. Cloning of full-length cDNA was performed by nested RT-PCR using E1-F or E1/2-F (for ProtSR2) or AE1-F or AE1/2-F (for S-ProtSR2) and oligo(dT)18. PCR products were cloned into the pMD18T-vector (Takara) and sequenced.

**Tolerance Assay of Transgenic Tobacco Plants**

For survival rate assay, 3-week-old L2, L4, and wild-type seedlings were sowed on a one-half-strength MS liquid medium without (as a control) and with 450 μM CdCl₂ for 5 d.

To analyze chlorophyll content, 4-week-old L2, L4, and wild-type seedlings were sowed on a one-half-strength MS liquid medium without (as a control) and with 400 μM CdCl₂ for 2 d. Chlorophyll was extracted with 80% (v/v) acetone from the whole seedlings. Chlorophyll content was determined at 665 and 645 nm according to Lichtenthaler (1987).

To determine free Trp and Cd content, 10-week-old seedlings were transferred onto one-half-strength MS liquid medium (control) or the same medium supplemented with 600 μM CdCl₂ for 5 d in the greenhouse. Free Trp analysis was performed on1290 uHPLC-6460 QqQ LC/MS (Agilent) at the physical and chemical analysis and testing center of the Beijing Institute of Technology according to Tsai et al. (2005). Shoots and roots of the CdCl₂-treated seedlings were harvested separately, washed three times with ice-cold water, and then digested with 11 N HNO₃ at 200°C overnight. Digested samples were diluted with 0.1 N HNO₃. Cd content of samples was determined by using an inductively coupled plasma-mass spectrometry at the Beijing Center for Physical and Chemical Analysis.

For 5-MT (Sigma) tolerance, 2-week-old seedlings were grown in vertical, solid 5-MT-containing (0, 200, or 400 μM 5-MT) MS medium without or with 100 μM CdCl₂ for 5 d and then were subjected to the analysis of root length.

To detect Trp or Cd content, 2-week-old wild-type seedlings were transferred onto MS agar medium without or with 400 μM CdCl₂ or with 400 μM CdCl₂ and 100 μM Trp (Sigma). The photographs were taken 7 d after stress and then were subjected to the analysis of chlorophyll content.

**Subcellular Localization**

The 35S:PvMTF-1-GFP or pCAMBIA1302 empty vector (35S:GFP) constructs were transformed into rice (Oryza sativa) leaf protoplasts. Preparation and transformation of rice leaf protoplasts were performed as previously described (Bart et al., 2006). The florescence signal was observed through a Zeiss 5 Live laser scanning confocal microscope 12 h after transformation.

**Transcriptional Activity Assay in Yeast**

The coding sequence of PvMTF-1 was PCR amplified and cloned into pGBKKT (Clontech) to generate BD-PvMTF-1. The transcriptional activation assay activity in yeast (Saccharomyces cerevisiae) was performed as previously described (Liu et al., 2013).

**Site-Directed Mutagenesis of PvMTF-1**

Site-directed mutagenesis of PvMTF-1 was PCR amplified from 35S:PvMTF-1 using a pair of primers (AD-PvMTF-1mut-F/AD-PvMTF-1mut-R) containing a mismatched nucleotide at amino acid residues 8 and 45. Two mismatched nucleotides changed Cys-8 (TGT) and Cys-45 (TGT) to Ser-8 (TCT) and Ser-45 (TCT). The resulting PCR fragments were digested by BamHI and SphI and ligated in frame into the pGAD424 vector to yield AD-PvMTF-1mut. For the pET-28a(+)–PvMTF-1mut, PvMTF-1mut fragments were PCR amplified from AD-PvMTF-1mut using a pair of primers (pET-28a-PvMTF-1mut-F/pET-28a-PvMTF-1mut-R) and then cloned into pGEMH and Saff of pET-28a(+) (Novagen).

**YIH Assay**

For bait construction, the ASA2p, mASA2p, 3×MRE, and 3×MREmut were synthesized by Shanghai Sangon Biotechnology and then cloned into pHIS2.1 (Clontech). The resulted plasmids were sequenced using a pHIS2.1 sequencing primer. The coding sequence of PvMTF-1 was amplified by PCR and cloned into pCAD424 (Clontech) to yield AD-PvMTF-1. AD-PvMTF-1 and AD-PvMTF-1mut were used as prey. The YIH assays were performed as previously described (Liu et al., 2013).

**Total Protein Extraction and Western Blot**

Total protein extraction from the 35S:PvMTF-1-GFP transgenic tobacco seedlings, the 35S:PvMTF-1 transgenic tobacco leaves, the 35S:ASA2-His-6 transgenic tobacco seedlings, and bean leaves were performed as previously described (Liu et al., 2013).

To detect PvMTF-1 and ASA2-His-6 fusion protein, total proteins were loaded onto 12% (w/v) SDS-PAGE, immunoblotted onto polyvinylidene difluoride membrane, and then probed with the primary anti-PvMTF-1 rabbit peptide (VAPSTLTPRNA) antisem (1:400 diluted; Beijing Protein Innovation) and an
anti-His-6 antibody (Abcam), respectively. A gel ran in parallel was stained by Coomassie Brilliant Blue to show equal loading. The secondary antibody was used on horseradish peroxidase-linked anti-rabbit IgG (diluted 1:5,000 in blocking buffer; Cell Signaling Technology). Chemiluminescence was performed on a Fujifilm LAS-3000 imager with ECL Prime Western Blotting detection reagent (Amersham Biosciences).

For the expression of PvMTF-1-GFP fusion proteins in the 3SS:PlacMTF-1-GFP transgenic tobacco seedlings, western blot was performed using anti-GFP antibody (Abcam) according to the procedure described previously (Liu et al., 2013).

ChIP-Cloning Analysis

Four-week-old 3SS:PlacMTF-1-GFP transgenic tobacco seedlings were subjected to ChIP assay using anti-GFP antibody (ab290; Abcam) according to the instruction of the EpiQuik Plant ChIP Kit (Epigentek). Immunoprecipitated DNA products were blunted using DNA Blunting Kit (Takara), and then one A tail was added to the 3' end of blunt DNA according to the instructions for the DNA A-Tailing Kit (Takara). The resulting DNA products were cloned into pMD18T-vector (Takara) and sequenced. The inserted DNA was used as a query to BLASTN analysis.

ChIP-PCR Analysis

Four-week-old 3SS:PlacMTF-1-GFP transgenic tobacco seedlings were grown on one-half-strength MS liquid medium without or with 300 μM CdCl2 for 12 h and then were subjected to ChIP assay using anti-GFP antibody (ab290; Abcam) and normal mouse IgG according to the instruction of the EpiQuik Plant ChIP Kit (Epigentek).

PCR was performed on 1 μL of the immunoprecipitated DNA using the ExTaq (Takara). The amplification began with 94°C for 4 min, followed by 40 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, with a final elongation at 72°C for 7 min. The PCR products were detected on 1.2% (w/v) agarose gels and visualized by ethidium bromide staining.

qRT-PCR was performed on a PikoReal 96 cycler (Thermo Scientific) with a SYBR premix Ex Taq kit (Takara) according to manufacturer’s instructions. One microtiter of 20-fold dilution immunoprecipitated DNA was analyzed by qRT-PCR. Enrichment of the ChIP target was expressed as a binding ratio between GFP antibody-immunoprecipitated samples and those immunoprecipitated with IgG control. The binding ratio was calculated by using a delta-delta cycle threshold method as previously described (Mukhopadhyay et al., 2008).

Fusion Protein Preparation and EMSA

To generate the pET-28a (+)–PvMTF-1, the coding sequence of PvMTF-1 was cloned into pET-28a and pET-28a (+) using the T-A cloning cassette into pET-28a (+)–PvMTF-1, or pET-28a (+)–PvMTF-1mut was transformed into Echerichia coli BL21 and induced with 0.5 mm isopropylthio-β-galactoside at 28°C for 4 h. The cells were harvested by centrifugation and lysed by sonication. The proteins in the lysate were purified by nickel-nitrilotriacetic acid (Ni-NTA) agarose beads (CwBio, China) according to the instruction. Protein concentrations were determined by using the Bradford reagent (Shanghai Sangon and valuable suggestions.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. 5’-RACE confirms the TSS of PvSR2 gene.

Supplemental Figure S2. Nucleotide and the predicted amino acid sequence of 5’-PvSR2 cDNA.

Supplemental Figure S3. RT-PCR splicing analysis of PvSR2 gene.

Supplemental Figure S4. qRT-PCR analysis of TSB1 expression.

Supplemental Table S1. The tobacco genomic DNA fragments immunoprecipitated by PvMTF-1.

Supplemental Table S2. A list of primers used in this study.

ACKNOWLEDGMENTS

We thank Weivei Zhang (Capital Normal University) for critical reading and valuable suggestions.

Received November 3, 2014; accepted January 25, 2015; published January 26, 2015.

LITERATURE CITED

A. tumefaciens-Mediated Transient Expression

For 3SS:ASA2-His-6, the coding region of ASA2 was PCR amplified from tobacco cDNA using 1302-ASA2-F/1302-ASA2-R and cloned into the Blunt II/NheI sites of pCAMBIA1302 infused with His-6 tag. The insert ASA2 DNA sequences were verified by sequencing using a series of 1302-ASA2 reverse primers. 3SS:ASA2-His-6, pCAMBIA1302 (3SS-GFP), or pBl212 (3SS-GUS) was introduced into A. tumefaciens GV3101. Two-week-old tobacco seedlings were vacuum infiltrated with A. tumefaciens as described by Marioni et al. (2008), followed by 3-d incubation in darkness. Western blot was performed with anti-His-6 antibody (Abcam) to verify expression of ASA2-His-6 fusion proteins in transformed tobacco seedlings. GUS staining of tobacco seedlings transiently expressing 3SS:GUS was monitored for transformation efficiency. Transformed tobacco seedlings were transferred to a filter paper soaked with one-quarter-strength MS solution (control) or with the same solution containing 200 μM CdCl2 for 3 d and then were subjected to analysis of chlorophyll content. As a control, 2-week-old L2, L4, and wild-type seedlings were sown on a one-quarter-strength MS liquid medium without (as a control) and with 200 μM CdCl2 for 3 d and then were subjected to the analysis of chlorophyll content.

Statistical Analysis

Statistical analysis was performed using SigmaStat Version 3.5. Significance (P < 0.05, P < 0.01) was assessed by the Student’s t test.

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GénBank/EMBL databases under the accession numbers ALM1 (AT1G08390), HHL18S (AT5G9670), HZJ19 (AT1G3040), HJZ12 (AT2G6790), HHL19 (AT3G546980), C07P6 (AT2G26975), HHL12A (AT2G81600), FRO4 (AT5G29980), FRO5 (AT5G22990), SPL7 (AT5G18830), STOP1 (AT1G34770), TGA3 (AT1G22070), and WRKY46 (AT2G46400).

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