Two Cysteines in Plant R2R3 MYB Domains Participate in REDOX-Dependent DNA Binding

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ABSTRACT

Plant R2R3 MYB domain proteins comprise one of the largest known families of transcription factors. Discrete evolutionary steps have shaped the plant-specific R2R3 MYB family from the broadly distributed R1R2R3 MYB proteins. R1R2R3 MYB domains have a single Cys residue (Cys130) that needs to be reduced for DNA-binding and transcriptional activity. In contrast, most R2R3 MYB domains contain two cysteines, Cys49 and Cys53, with Cys53 at the equivalent position as Cys130 in R1R2R3 MYB. Using the maize P1 regulator of flavonoid biosynthesis as a typical R2R3 MYB-domain protein, we investigated here the in vitro REDOX requirement for DNA binding by P1. We show that the C53S mutation requires reducing conditions for DNA-binding, while C53A binds DNA under oxidizing and reducing conditions. Neither mutation impairs the in vivo regulatory activity of P1. The C49S and C49A mutants bind DNA in vitro irrespective of the REDOX conditions. A C49I mutant, which simulates the MYB domain of c-MYB, binds DNA only under reducing conditions and its binding is significantly affected by the C53S replacement. Interestingly, under non-reducing conditions, Cys49 and Cys53 form a disulfide bond that prevents the R2R3 MYB domain from binding DNA. Together, our results suggest that the evolutionary origin of Cys49 within the plants has provided R2R3 MYB domains with a regulatory feature not present in animal MYB domains, highlighting fundamental structural and functional differences between similar DNA-binding domains from plants and animals.
INTRODUCTION

MYB DNA-binding domains are formed by one to three or more imperfect repeats (R1, R2 and R3) containing periodic tryptophan residues (1-3). Each MYB repeat is defined by approximately 50 amino acids that form three $\alpha$-helices. The last two helices of each MYB repeat adopt a helix-turn-helix motif with the third helix of each MYB repeat involved in the main DNA contacts (4). The vertebrate Myb genes, which include c-Myb, A-Myb and B-Myb, encode proteins with MYB domains formed by three MYB repeats (R1R2R3 MYB). In contrast, the majority of plant Myb genes encode proteins with only two MYB repeats most similar to the vertebrate R2 and R3 MYB repeats (R2R3 MYB) (5-8). Plant R2R3 Myb genes are likely to have originated from an ancestral gene that is represented today by the B-Myb gene in vertebrates (6), and by the small pc-Myb (plant c-Myb) gene family in the plants (5). The evolutionary steps involved in the formation of the plant-specific R2R3 MYB domains from the broadly distributed R1R2R3 MYB domains involved the sequential i) loss of R1 to yield the “atypical R2R3 MYB domains”, ii) replacement of the first tryptophan of R3 by a hydrophobic amino acid, and iii) insertion of a Leu residue between the second and third helices of R2, to give the “typical R2R3 MYB” domains (9) (Fig. 1A). The loss of R1 and the replacement of the Trp residue probably had only a moderate effect upon the DNA-binding properties of the MYB domain, based on studies carried out in animal R1R2R3 MYB proteins (4,10,11). In contrast, the insertion of the Leu residue in v-
MYB, an oncogenic form of c-MYB containing only R2 and R3 (12), completely abolished binding to DNA (13). An extensive amplification of the R2R3 Myb gene family occurred within the plants 250-400 million years ago (14), which resulted in Arabidopsis encoding 125 R2R3 Myb genes (8,15) and maize and related monocots encoding over 200 R2R3 Myb genes (14,16). The amplification of the R2R3 Myb gene family in the plants provides a unique opportunity to understand how the evolutionary steps that shaped a family of transcription factors resulted in the functional differences displayed by these regulatory proteins throughout the plant kingdom.

A residue that has remained completely conserved in plants, fungi and animals during the evolution of MYB domains corresponds to a cysteine located in the DNA-recognition helix of R2 (Cys130 in c-MYB corresponding to Cys53 in Fig. 1B). This cysteine was proposed to serve as a REDOX sensor in vertebrate MYB transcription factors, and mutations of this residue significantly impaired DNA-binding and transcriptional activity of c-MYB and v-MYB (17,18). The NMR structure of the R2R3 MYB domain of c-MYB indicated that Cys130, the only cysteine present in c-MYB and related MYB factors from vertebrates, is included in the hydrophobic core, maintaining the three helices of R2 in an unbound conformation at room temperature (4). Consistent with this model, the replacement of Cys130 for Ser in c-MYB resulted in the loss of DNA binding (18). Interestingly, however, during the evolution of R2R3 MYB domains (Fig. 1A), a second cysteine appeared, located four residues N-terminal to the highly
conserved Cys53 (Cys49 in Fig. 1B). Cys49 is conserved in typical R2R3 MYB domains, but it is not present in the atypical R2R3 MYB domains or in the MYB domains of pc-MYB proteins (Fig. 1B).

Previously, we investigated the REDOX requirements of the P1 protein for DNA-binding. P1 encodes a typical R2R3 MYB transcriptional regulator of genes encoding biosynthetic enzymes of a branch of the maize flavonoid pathway (19). P1 activates transcription of the a1 gene by binding to the high- and low-affinity P1-binding sites (haPBS and laPBS, respectively) present in the a1 promoter (19,20). Similar to the DNA-binding activity of c-MYB, P1 requires a strong reducing environment to bind DNA (13). As is the case with other R2R3 MYB proteins, P1 contains two Cys residues in the MYB domain (Cys49 and Cys53, Fig. 1B), in addition to two cysteines in the short N-terminal region that precedes the MYB domain (21).

Here, we investigated the participation of the two MYB domain cysteines in the REDOX regulation of the DNA-binding activity of typical R2R3 MYB domains exemplified by the maize P1 transcription factor. We show that the conserved cysteine at position 53 (Cys53) is not essential for the DNA-binding activity of P1, in sharp departure to what has been established for vertebrate MYB proteins. Instead, Cys49 plays a much more important role in sensing the REDOX conditions, when Cys53 is replaced by Ser. However, in the C53A mutant, the DNA-binding activity of P1
becomes insensitive to oxidizing conditions. *In vivo*, the replacements of Cys53 to Ser or Ala have no effect on the transcriptional activity of P1. The replacement of Cys49 by Ser or Ala results in P1 proteins insensitive to the REDOX environment for DNA binding.

We also show that Cys49 and Cys53 form an intra-molecular disulfide bond in non-reducing conditions, providing an evolutionary new opportunity for the modulation of the DNA-binding activity of R2R3 MYB-domain proteins. Together, our studies suggest a novel mechanism for the REDOX control of DNA-binding by R2R3 MYB domains and suggest fundamental structural differences between plant and animal MYB domains.
EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of Recombinant Proteins - The P1 MYB domain (amino acids 1-118) was previously cloned as a N-terminal poly-histidine fusion (N10His-pMYB) (13). For the truncated version of the P1 MYB domain (N6His-pMYBΔ9), the coding region corresponding to amino acids 10-118 was generated by PCR from the full-length cDNA of P1 (21). The resulting PCR product was then introduced into the pKM260 (22) E. coli expression vector via NcoI and BamHI sites to generate N6His-pMYBΔ9. The amino acid sequence of N6His-pMYBΔ9 N-terminal to the MYB-domain sequence shown in Fig. 1 is NH2-MHHHHHHHASENLYFQGAM and the sequence C-terminal to the MYB domain is PAANKARKEAELAATAEQ-COO-. All point mutations were generated by site directed mutagenesis (Stratagene, Quick Change Mutagenesis Kit) of the pMYBΔ9 coding sequence in the pTAdv (Invitrogen) cloning vector. The MYB domain was then excised from the pTAdv vector and inserted into pKM260 E. coli expression vector through NcoI and BamHI sites.

For expression in E. coli, BL21 (DE3) Plys cells bearing the corresponding plasmids were grown, induced and purified essentially as previously described (13), with the following modifications. After induction of a 1 L culture, the cells were harvested by centrifugation and stored at –80 °C until further use. The cells were resuspended in 20
ml SB (50 mM sodium phosphate, pH 8.0; 100 mM NaCl; and 100 µg/ml phenylmethylsulfonyl fluoride) and passed twice through a French press. The cell lysate was centrifuged at 14,000x g for 20 minutes and the supernatant was filtered through two layers of Miracloth (Calbiochem). The Ni-NTA agarose resin (Qiagen) was equilibrated with SB and 1 ml of 50% slurry was added to the cell lysate supernatant and incubated for two hours with rocking at 4°C. The resin was gently recovered by centrifugation, resuspended in 5 ml of SB and loaded onto a column. The column was washed five times with five column volumes of SB and three times with five column volumes of WB (50 mM sodium phosphate, pH 8.0; 300 mM NaCl; 1% Tween 20; 5 mM 2-Mercaptoethanol; 10 mM EDTA and 10% glycerol). The protein was eluted with five washes of five column volumes of WB containing 50 mM imidazole. The elutions were then dialyzed against 60 volumes of A-0 buffer (10 mM Tris pH 7.5; 50 mM NaCl; 1 mM DTT; 1 mM EDTA; 5% glycerol) and stored at −80°C until further use. Each wash and elution fraction was collected and analyzed by SDS-PAGE, stained with Coomassie Brilliant Blue R-250 and quantified against a lysozyme protein standard. Purified proteins were used for each assay and were judged to be >90% pure by Coomassie Brilliant Blue staining of 15% SDS-PAGE gels.

*Electrophoretic Mobility Shift Assays* - Aliquots of purified proteins were
incubated with end-labeled double-stranded DNA generated by hybridization of complementary oligonucleotides. End labeling of synthetic oligonucleotide probes was carried out using T4-Polynucleotide Kinase (Invitrogen) in the presence of a two-molar excess of $\gamma^{32}$P-dATP (>8,000Ci/mmol, Amersham). The labeled oligonucleotides were then annealed to an equi-molar amount of complementary oligonucleotides by heating to 95°C and cooling to room temperature. A fraction of the double-stranded labeled oligonucleotides was precipitated on glass filters for quantification by scintillation of the radiation incorporated. The probes used contain APB1, the high affinity P1-binding sites (hapBS) from the $a1$ gene promoter, or APB5, a mutant in which the sites were destroyed (Fig. 2A) (19).

Protein-DNA incubations were performed essentially as described (19) with the following modifications. Approximately 35 ng of protein were incubated in A-0 buffer (10 mM Tris pH7.5; 50 mM NaCl; 1 mM EDTA; 5% glycerol) with 0.8 µg poly d(I)/d(C), and 1mM DTT, unless indicated otherwise. Protein-DNA complexes were resolved on a 8% polyacrylamide gels (80:1 acrylamide:bis-acrylamide) in 0.25X TBE (22.5 mM Tris-Borate and 0.5 mM EDTA) running buffer at 415 V for 55 minutes at 4°C. The gels were then dried onto Whatman paper and subjected to autoradiography at −70°C overnight.
Tryptophan fluorescence- Fluorescence experiments were carried out in a Horiba Fluoromax-3 Luminescence Spectrometer. An excitation wavelength of 295 nm was used with an emission and excitation slit width of 5 nm. Emission spectra were recorded at 0.5 nm intervals between 310 and 400 nm with a scanning speed of 300 nm per minute. Samples were analyzed in a quartz cuvette (1.0 cm x 1.0 cm) and contained 0.5 µM of purified recombinant proteins in 3 ml of A-0 buffer. To determine the fluorescence spectra of the proteins in denaturing conditions, the purified recombinant proteins were incubated with 6 M-guanidinium hydrochloride in A-0 buffer at room temperature for one hour prior to the fluorescence analysis. Each spectrum was combined as an average of five scans and corrected to the background of the A-0 buffer, with or without guanidinium hydrochloride.

DNA Constructs for Transient Expression Experiments - The p35SP1 construct was previously described (23). Other previously described constructs include pA1Luc, which contains 220 bp of the a1 promoter (19,20), p35SBAR (24) and pUbiGUS (19). p35SBAR was used for normalizing the concentration of CaMV 35S promoter sequences delivered in each bombardment and the pUbiGUS was used to normalize the efficiency of each bombardment. Mutants in the MYB domain of P1 were generated by PCR from their respective E. coli protein expression vectors and, after sequencing, were used to replace the MYB domain of P1 in the p35SP1 construct by digesting it with BamHI and
Microprojectile Bombardment and Gene Expression - Bombardment conditions
of suspension maize Black Mexican Sweet (BMS) cells and transient expression assays
for luciferase and GUS were performed essentially as previously described (23). Bombardments were performed in triplicate and each experiment was repeated at least
twice. The assays for luciferase and GUS and the normalization of the data were
performed as previously described (19). The fold activation results are expressed as the
ratio of arbitrary light units (luciferase) to arbitrary fluorescence units (GUS) of the
treatment with the transcriptional activator divided by the ratio of arbitrary light units
(luciferase) to arbitrary fluorescence units (GUS) of the reporter plasmid in the absence
of the regulator.

Detection of dimers and fluorescent labeling of recombinant proteins - Detection
of dimer formation was accomplished by incubating 700 ng of recombinant protein on ice
for 1 hour in the presence of either 10 mM DTT or 30 mM diamide. After incubation in
ice, 2X SDS-PAGE loading buffer without reducing agents (100 mM Tris-HCl pH 6.8;
4% SDS; 0.2% bromophenol blue and 20% glycerol) was added to each sample and
separated by electrophoresis on a 15% non-reducing SDS polyacrylamide gel.

Fluorescent labeling of reduced cysteine residues was accomplished by using the
thiol specific modifying agent 7-diethylamino-3-(4’-iodoacetylaminoaryl)-4-methylcoumarin (DCIA) (Molecular Probes). Approximately 350 ng of recombinant protein was incubated on ice in either the presence or absence of the reducing agent tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) (Molecular Probes). Samples in reducing conditions (1 mM TCEP) were incubated on ice for 30 minutes. DCIA was then added to all samples to a final concentration of 1 mM and incubated on ice for additional 30 minutes in the dark. After incubation with DCIA, TCEP was added to a final concentration of 3 mM in all samples. Samples were then mixed with 2X non-reducing SDS-PAGE loading buffer and separated on a 15% non-reducing SDS polyacrylamide gel. After separation, fluorescent imaging of the gel was accomplished using the Bio-RAD Gel Doc 2000 Documentation system. After fluorescent analysis, the gel was stained with Coomassie Brilliant Blue.
RESULTS

The MYB Domain of P1 Binds DNA Only When Reduced – Previously, we showed that the MYB domain of P1 binds DNA only in the presence of DTT (13). The MYB domain of P1 used in those studies (N_{10}His-p^{MYB}) contained four Cys residues, two at positions six and seven before the first helix of R2, and two others at positions 49 and 53 (Fig. 1B). To investigate which Cys residues in P1^{MYB} responds to the reducing conditions, we generated a truncated, poly histidine-tagged version of P1^{MYB}, in which the first nine amino acids of P1 were deleted (N_{6}His-p^{MYBΔ9}), removing the two Cys residues located in the N-terminal extension (21). To determine whether N_{6}His-p^{MYBΔ9} binds DNA similarly as the previously described N_{10}His-p^{MYB} protein (13), we tested total E. coli extracts, expressing equivalent amounts of the two proteins, by electrophoretic mobility shift assays (EMSA), using the APB1 probe (Fig. 2A). APB1 corresponds to the high-affinity P1-binding sites present in the promoter of al, one of the flavonoid biosynthetic genes regulated by P1 (19). N_{6}His-p^{MYBΔ9} binds APB1 as effectively as N_{10}His-p^{MYB} (Fig. 2B), indicating that the first 9 amino acids of P1 are dispensable for the DNA-binding activity of P1. The faster mobility of the N_{6}His-
pMYBΔ9-APB1 complex reflects the smaller size of N6His-pMYBΔ9, compared to N10His-pMYB. As previously shown for P1 and N10His-pMYB (13,19), N6His-pMYBΔ9 does not bind to the mutant APB5 DNA probe (Fig. 2D, lane 1). Similar to N10His-pMYB, the DNA-binding activity of N6His-pMYBΔ9 depends on the presence of DTT (Fig. 2C, compare lanes 1 and 4). These results suggest that Cys49 and/or Cys53 are responsible for the reducing conditions necessary for P1 to bind DNA.

To determine the participation of Cys53 in the REDOX regulation of DNA binding by P1, we replaced Cys53 with Ala or Ser in N6His-pMYBΔ9. The corresponding proteins (C53A and C53S respectively) were expressed in *E. coli*, affinity-purified on a Ni-NTA column and analyzed for DNA-binding activity to the APB1 probe by EMSA. In the presence of reducing conditions (1 mM DTT), wild type (WT, N6His-pMYBΔ9) and C53A bound with comparable strengths (Fig. 2C, lanes 1 and 2). In the presence of DTT (+DTT, Fig. 2C) the binding of C53S to APB1 was also comparable to WT (Fig. 2C, lane 3). In the absence of DTT however (-DTT, Fig. 2C), only C53A bound APB1 (Fig. 2C, lane 5), with neither the wild type nor C53S proteins showing any significant DNA binding. To determine whether the C53A or C53S mutations affected the sequence-specific DNA-binding activity of P1 (13,19), we tested wild type and the two mutants for binding to the APB5 probe. As shown in Fig. 2D,
none of these proteins bound to the mutant sites.

Next, we investigated the effect that the addition of the sulfhydryl oxidizing agent diamide had on the DNA-binding activity of these proteins. The DNA-binding activity of the wild type protein (N$_6$His-pMYB$\Delta$9) was abolished by the presence of 3 mM diamide (Fig. 2E, lane 2). The DNA-binding activity of this protein, however, is recovered by the addition of excess DTT (Fig. 2E, lane 3). The C53A protein (Fig. 2E, lanes 4-6) was not significantly affected by the treatment with diamide. Similar to wild type, binding of C53S to APB1 was completely abolished by the presence of diamide (Fig. 2E, lane 8), yet recovered when DTT was added (Fig. 2E, lane 9).

To better understand how these mutations affect the ability of the MYB domain of P1 to bind DNA, we made use of the intrinsic fluorescent properties of the five tryptophan residues present in R2R3 MYB domains (Fig. 1B). Using fluorescence spectroscopy, we compared the environments of the tryptophan residues in the wild type (WT, N$_6$His-pMYB$\Delta$9), C53A and C53S proteins. The fluorescence emission spectra for each of the three proteins was determined in 0 mM DTT, 1 mM DTT and 6 M guanidinium chloride (denaturing conditions), and the wavelength of the maximum emission fluorescence was recorded (Table 1). All the denatured proteins had similar emission maxima, between 357 nm and 359 nm, similar to the maximum of free tryptophan (360 nm) (Table 1). Compared to the denatured protein, the emission
maximum of the native wild type protein (in the presence of 1 mM DTT) was shifted

towards the shorter wavelengths (342 nm), a shift indicative of the tryptophan residues

being less exposed to the solvent, likely participating in the formation of a hydrophobic

core, as proposed for c-MYB (1-4). In the absence of DTT, the wild type protein
displayed a more open conformation (Table 1, 352 nm), when compared to the reduced
form (342 nm). C53A had a similar emission maximum in the absence of DTT as the
wild type protein in reducing conditions (Table 1, compare 343 nm with 342 nm). The
addition of 1 mM DTT shifted the emission maximum of C53A slightly further towards
the blue (Table 1, 338 nm). This result is consistent with the observation that C53A
binds efficiently DNA in both reducing and non-reducing conditions (Fig. 2C, lanes 2
and 5). In contrast, the C53S mutant had a similar emission maximum as wild type in
non-reducing conditions (Table 1, compare 350 nm with 352 nm). In the presence of 1
mM DTT, the maximum of C53S shifted towards the blue to 345 nm, consistent with the
ability of this protein to bind DNA in reducing conditions (Fig. 2C, lanes 3 and 6).

The ability of the C53S mutant to bind DNA only in the presence of DTT
suggested that Cys53 is not the only cysteine residue involved in the REDOX-dependent
DNA-binding activity of P1. Because C53S has only one additional cysteine residue
(Cys49, Fig. 1B), Cys49 or the interaction between Cys49 and Cys53 must be responsible
for this activity. However, the comparable binding of C53A to APB1 and the similar
tryptophan fluorescence spectra in the presence or absence of DTT suggests that the
replacement of C53 by the much smaller Ala residue overcomes the requirement for a reduced state of Cys49 for binding DNA.

**Cys53 is Dispensable for the In Vivo Regulatory Activity of P1** – The Cys53 residue is absolutely conserved in all plant and animal MYB transcription factors studied to-date (Fig. 1B). To investigate whether mutations of Cys53 would affect the transcriptional activity of P1 (19,23), we introduced the C53S and C53A mutations into the full-length P1 protein and compared to wild type P1 their ability to activate the *a1* promoter in the pA1Luc reporter construct. Transient expression experiments were carried out by bombarding the p35SP1, p35SP1C53A and p35SP1C53S plasmids with the pA1Luc reporter, in the presence of pUbiGUS as a normalizing control (see EXPERIMENTAL PROCEDURES). Luciferase activity was measured as an indication of the activation of the *a1* promoter, and normalized to the GUS activity. As observed in Fig. 3, mutant and wild type P1 activated the pA1Luc reporter construct at similar levels. Together, these results suggest that, despite its very high conservation and the essential role that the equivalent residue plays in animal MYB domains, Cys53 is not vital for the regulatory activity of P1.

**Effect of Cys49 Mutations on DNA-Binding Activity** – In contrast to animal
MYB domains, most plant R2R3 MYB domains contain a second Cys residue corresponding to position 49 in P1 (Fig. 1B). To investigate the role of Cys49 in the DNA-binding activity of P1, we replaced Cys49 in N6His-pMYBΔ9 with Ala, Ser and to the Ile residue present in the vertebrate c-MYB, A-MYB and B-MYB proteins (Fig. 1B) to give the C49A, C49S and C49I proteins, respectively. The DNA-binding activities of these proteins were investigated on the WT APB1 in the presence of 1 mM DTT (reducing conditions, Fig. 4A, lanes 1-6) or 3 mM diamide (oxidizing conditions, Fig. 4B, lanes 1-6). In the presence of DTT, the C49S and C49A proteins bound APB1 similar to wild type, while the DNA-binding activity of C49I was significantly reduced (Fig. 4A, compare lanes 4-6 with 1). Interestingly, however, the binding of C49S and C49A was not affected by the oxidizing environment provided by 3 mM diamide (compare Fig. 4A and 4B lanes 4-5), conditions in which the wild type protein or the C49I mutant gave no significant binding (Fig. 4A and 4B, lanes 1 and 6). Each of the Cys49 mutants (single and double) was also tested for DNA-binding to the APB5 probe in reducing conditions to ensure that they retained their specific DNA-binding activity. No DNA-binding was observed for any of these Cys49 mutant proteins on the APB5 probe (data not shown).

We compared the solvent exposure of the tryptophan residues in the C49A, C49S and C49I mutants to the corresponding wild type protein (Table 1 WT, corresponding to N6His-pMYBΔ9) by establishing the tryptophan fluorescence spectra of these proteins.
under reducing (1 mM DTT) and non-reducing (no DTT) conditions (Table 1). Consistent with the efficient binding of the C49A and C49S proteins to DNA in both reducing and oxidizing conditions (Fig. 4A and 4B), their fluorescence emission maxima was not affected by the presence of DTT (Table 1, compare 342 nm and 341 nm for C49A and 337 nm and 336 nm for C49S). In contrast, C49I, which shows reduced DNA-binding activity compared to wild type, displayed a fluorescence spectra in both reducing and non-reducing conditions more similar to the wild type (WT) in the absence of DTT (Table 1). Thus, the presence of more solvent-exposed tryptophans in the mutants invariably correlates with a decreased DNA-binding activity. Together, these findings emphasize the role of Cys49 in sensing the REDOX conditions preferred for high-affinity DNA-binding of P1 to DNA.

Effect of Double Mutations at Cys53 and Cys49 – To investigate the effect of amino acid replacements at both Cys49 and Cys53, double mutants were generated and tested for their ability to bind to APB1 in reducing (1 mM DTT, Fig. 5A) or oxidizing (3 mM diamide, Fig. 5B) conditions. As expected, given the absence of any other Cys residues in these mutants, all these proteins bind with the same affinity to APB1 in reducing or oxidizing conditions (Fig. 5A and 5B). The replacement of Cys49 and Cys53 by Ala in N6His-pMYBΔ9 (C49A/C53A) resulted in a protein with wild type levels of
DNA-binding activity in reducing or oxidizing conditions (Fig. 5A and B, lane 2). Interestingly, the double mutant C49A/C53S has its DNA-binding activity significantly impaired (Fig. 5A and B, lane 3), compared to either one of the single C49A or C53S mutants (Fig. 4A, lanes 4 and 3 respectively). In contrast, the C49I/C53A replacements permit the double mutant to bind DNA with a much higher affinity (Fig. 5A and B, lane 6) than the C49I single mutant does (Fig. 4A and B, lane 7). However, the C49I/C53S double mutant does not significantly bind the APB1 DNA in these assays (Fig. 5A and B, lane 7). Tryptophan fluorescence experiments carried out with the double mutants show similar fluorescence maxima in the presence and absence of DTT, and were suggestive of a more closed structure, resembling wild type protein in reducing conditions (Table 1) (not shown).

**Formation of intra-molecular disulfide bonds in the R2R3 MYB domain of P1**

To investigate whether the presence of Cys49 and/or Cys53 favors the formation of intra-molecular or inter-molecular disulfide bonds, we investigated by SDS-PAGE the formation of dimers in the wild type (N6His-pMYBD9), C53S, C49S and C49A/C53A proteins in reducing or oxidizing conditions (Fig. 6A). A protein of a molecular weight of 28 kDa was detected in the C53S single cysteine mutant protein following the treatment with diamide (Fig. 6A, filled arrow), but not in the wild type (WT), C49S or the double C49A/C53A mutant proteins (Fig. 6A, open arrow). These results suggest that the
absence of DNA binding by the C53S protein in oxidizing conditions might be in part a consequence of the formation of dimers. However, the presence of substantial amounts of monomeric C53S in the presence of diamide (Fig. 6A, lane 4), suggests that either the SDS-PAGE gels fail to represent exactly the monomer/dimer composition in the EMSA experiments (Fig. 4, lane 5), or that diamide induces other modifications not evident by SDS-PAGE. In contrast, the C49S mutant, which binds DNA irrespective of the REDOX conditions, does not form dimers. Interestingly, the wild type protein, which requires reducing conditions for binding DNA (Fig. 2E, lane 2), does not form dimers, suggesting the possibility of the formation of an intra-molecular disulfide bond that prevents DNA binding.

To directly investigate whether Cys49 and Cys53 form an intra-molecular disulfide bond, we used the fluorescent thiol specific probe DCIA, which covalently reacts with free sulphydryl groups to form a carbon-sulfur bond. Using DCIA, we fluorescently labeled the wild type MYB domain of P1 in the reduced environment provided by the non-thiol containing reducing agent TCEP (WT, Fig. 6B, lane 1). No fluorescence was detected in the absence of the reducing agent, consistent with the formation of an intra-molecular disulfide bond (WT, Fig. 6B, lane 2). DCIA labeled C53S under reducing conditions (Fig. 6B, lane 3), but the fluorescence was significantly reduced under non-reducing conditions (Fig. 6B, lane 4), consistent with the formation of an inter-molecular disulfide bond (Fig. 6A, lane 4). The observation that not all of
the C53S protein is involved in the formation of dimers (Fig. 6A, lane 4) is consistent with the decreased, yet significant, fluorescence observed (Fig. 6B, lane 4). Remarkably, however, C53S displays a very reduced DNA-binding activity in oxidizing conditions (Fig. 4B, lane 3), suggesting that the different conditions used for the EMSA and PAGE experiments significantly affect the environment of Cys49 in the C53S protein. Similarly, C49S, which shows similar DNA-binding activities under reducing and oxidizing conditions (Fig. 4A and B, lane 6) and does not form dimers (Fig. 6A, lane 6), shows a very reduced fluorescence in either reducing or oxidizing conditions (Fig. 6B, lanes 5 and 6), suggesting a poor accessibility of C53 to the DCIA label in the C49S mutant. As expected, a MYB domain in which both Cys residues were replaced by Ala (C49A/C53A), showed no fluorescence when treated with DCIA (Fig. 6B, lanes 7 and 8). Together, these results suggest the formation of a disulfide bond between C49 and C53 in the R2R3 MYB domain of P1.

**DISCUSSION**

Plant R2R3 MYB domain proteins comprise one of the largest families of transcription factors so far described. Discrete evolutionary steps have shaped the plant-specific R2R3 MYB family from the broadly distributed $R1R2R3$ $Myb$ genes (Fig. 1A). Vertebrate R1R2R3 MYB transcription factors, such as c-MYB, have a conserved cysteine in the DNA-recognition helix of R2 (Cys53 in Fig. 1B), which needs to be in the
reduced state to allow DNA-binding and transcriptional activation by this protein (17,18,25). Similarly as observed for c-MYB, the maize P1 transcription factor requires reducing conditions in order to bind DNA in vitro (13). Most typical R2R3 MYB transcription factors, to which P1 belongs, contain a second Cys in the MYB domain (Fig. 1B, Cys49). We show here, that different from what has been found in animal MYB domains, Cys53 is not essential for the in vitro DNA-binding or in vivo transcriptional activity of P1. We also provide evidence for the formation of an intra-molecular disulfide bond between Cys49 and Cys53, suggesting the possibility of a novel mechanism for the REDOX regulation of plant R2R3 MYB transcription factors.

The replacement of Cys53 by Ser (C53S) in P1 results in a protein that binds DNA only in reducing conditions (Fig. 2C, lanes 3 and 6), suggesting that, in the C53S protein, Cys49 needs to be reduced for efficient DNA-binding. Indeed, in oxidizing conditions Cys49 participates in the formation of disulfide linked dimers (Fig. 6A, lane 4). A similar replacement of Cys130 by Ser in c-MYB significantly reduces DNA binding (18,25) and in vivo transcriptional activity (17). However, the mechanism by which the C130S mutation impairs c-MYB function must be different from what we observed with the P1 C53S mutation, since there is no other Cys residue for the formation of an intra-molecular disulfide bond in c-MYB. In contrast to C53S, C53A binds DNA efficiently in both reducing and oxidizing conditions, similar to wild type in the presence of DTT (Fig. 2E, lanes 4-6). Tryptophan fluorescence experiments (Table 1) suggest a
conformation of C53A (in reducing or oxidizing conditions) more similar to that of the wild type protein in the presence of DTT, indicating a more “closed” environment for the tryptophan residues. In c-MYB, the replacement of Cys130 by the hydrophobic Ala or Val residues resulted in proteins with comparable DNA-binding activities as wild type (25). Our results also show that the replacement of Cys53 with Ala or Ser does not significantly affect the \textit{in vivo} regulatory activity of P1 (Fig. 3). We conclude from these findings that C53 in R2R3 MYB domains is not functionally equivalent to Cys130 in c-MYB.

Plant R2R3 MYB domains have a second conserved cysteine, Cys49. At the equivalent position c-MYB has an Ile residue (Fig. 1B). Thus, the C49I mutation in P1 is expected to mimic somehow the behavior of c-MYB. Indeed, in oxidizing conditions, C49I displayed no detectable DNA-binding activity (Fig. 4B, lane 6), yet DNA binding was partially restored by the addition of DTT (Fig. 4A, lane 6). These results suggest that, in the presence of the bulkier Ile residue at position 49, a significant strain is loaded on C53 to ensure that it is reduced, largely mimicking the DNA-binding properties of the MYB domain of c-MYB. Consistent with what has been observed for c-MYB (18,25), the C49I/C53S double mutant (equivalent to C130S in c-MYB) displayed a very poor DNA binding activity (Fig. 5A, lane 7), while the C49I/C53A bound DNA at levels comparable to wild type (Fig. 5A, lane 6). In contrast, the C49S and C49A mutants bind DNA equally well in reducing and oxidizing conditions (Fig. 4), suggesting that, in these
mutants, Cys53 is buried in the hydrophobic core, inaccessible for dimer formation (Fig. 6A). Thus, we conclude that the replacement of the bulky Ile49 by the smaller Cys49 during the evolution of R2R3 MYB proteins significantly decreased the availability of Cys53 to form inter-molecular disulfide bonds under non-reducing conditions. Instead, the presence of two Cys residues in R2R3 MYB domains open the opportunity for the formation of an intra-molecular disulfide bond under oxidizing conditions. The formation of this bond would be responsible for the REDOX-dependent DNA-binding activity of P1. The formation of an intra-molecular disulfide bond is consistent with the absence of observed dimers for N
6
His-pMYBΔ9 in oxidizing conditions (Fig. 6A, lane 2). The probing for free sulfhydryl groups using the fluorescent label DCIA confirmed the absence of free SH groups in N
6
His-pMYBΔ9. Thus, Cys49 and Cys53 form a disulfide bond in oxidizing conditions in P1, and this bond impairs DNA binding.

In the solved structure of c-MYB (Fig. 7), Ile126 (position equivalent to Cys49) comes just before the DNA-recognition helix of R2, facing away from Cys130. In fact, if Cys49 in P1 is positioned similarly as Ile126, it is difficult to explain the formation of an intra-molecular disulfide bond. Indeed, the SH groups from C49 and C53 would be approximately 7 Å apart, as this is the distance from Ile126 to Cys130, determined from the solved structure for c-MYB as shown in Figure 7, compared to the approximately 2 Å involved in a normal S-S bond (26). Based on the available structures of MYB
domains, it is unlikely that the insertion of Leu46, characteristic of typical R2R3 MYB domains (9), will significantly alter the relative positions of Cys49 to Cys53. Thus, it is more probable that the structures of animal and plant R2R3 MYB domains are significantly different in this region.

The regulation of protein activity by intracellular REDOX potential is a mechanism by which the activity of many proteins is regulated (18,27-32). Our results suggest the possibility that plant R2R3 MYB domain proteins are regulated in this fashion, and that the presence of two closely positioned cysteine residues permits the formation of intra-molecular disulfide bonds in non-reducing conditions that impair DNA binding. The participation of plant R2R3 MYB transcription factors in the control of metabolic pathways (8) often related to biotic or abiotic stress conditions that may result in the transitory increase of the oxidative potential of the cells suggests a possible physiological mechanism by which the activity of these transcription factors is regulated.

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Fig. 1. **Evolution and sequence alignment of MYB domains.** A, Evolutionary path of typical R2R3 MYB domains from broadly distributed R1R2R3 MYB domains, as described in Dias et al., 2003. B, An alignment of MYB domains made with ClustalW. Only the second (R2) and third (R3) MYB repeats are shown above with amino acid numbering corresponding to the P1 protein (21). Identical residues are shaded dark gray and similar residues are shaded light gray. The filled arrow indicates the absolutely conserved Cys53 in P1 corresponding to Cys130 in c-MYB. The open arrows indicate the insertion of Leu46, the position of Cys49, conserved in most plant R2R3 MYB domains, and the change to Ile70 from the highly conserved Trp. The predicted $\alpha$-helices of each repeat are indicated. The two MYB repeat (R2R3) proteins are from *Antirrhinum majus* AmMixta (CAA55725), *Arabidopsis thaliana* AtMYB1(AAF14022), AtMYB44 (BAB09015), AtMYB2 (AAB63819), AtMYB20 (AAG51765), AtMYB30 (BAB02134), At-TT2 (BAB08716), At-PAP1 (AAG09100), At-GL1 (AAC97387), AtMYB11 (CAB83111), *Oryza sativa* OsP (BAB64029), *Petunia hybrida* PhAn2 (AAF66727), *Physcomitrella patens* PpMYB2 (CAA47435) and *Zea mays* ZmC1 (P10290), ZmP1 (AAL24047). Three MYB repeat (R1R2R3) MYB domains are represented by the vertebrate c-MYB of *Homo sapiens* Hsc-MYB (AAA52030) and *Arabidopsis thaliana* Atpc-MYB1 (AAD46772).
Fig. 2. **DNA binding properties of the MYB domain of P1 and the Cys53 mutants.**  
A, sequence of the oligonucleotide probes used for the electrophoretic mobility shift assays (EMSA), corresponding to the P1-binding site in the $a1$ gene promoter (19). P1-binding and mutant sites are boxed in their respective oligos.  
B, EMSA with normalized crude lysates of bacterial extracts containing: lane 1, no protein extract; lane 2, N$_{10}$His-pMYB; and lane 3, N$_{6}$His-pMYB$_{\Delta 9}$.  
C, EMSA with purified proteins in the presence of the APB1 wild type DNA probe in the presence of DTT (+DTT) or in the absence of DTT (-DTT): lanes 1 and 4, N$_{6}$His-pMYB$_{\Delta 9}$; lanes 2 and 5, C53A; and lanes 3 and 6, C53S.  
D, EMSA with purified proteins in the presence of the mutant APB5 DNA probe and 1 mM DTT: lane 1, N$_{6}$His-pMYB$_{\Delta 9}$; lane 2, C53A; and lane 3, C53S.  
E, EMSA of purified proteins with the APB1 probe in the presence or absence of particular reagents before the incubation with DNA indicated by + and −, respectively. **Filled and open** arrows indicate protein-DNA complex and free DNA probe, respectively.

Fig. 3. **Cys53 is not essential for the P1 transcriptional activity.** Results of transient expression after co-bombardment of cultured maize BMS cells with wild type, C53A and C53S mutants driven from the constitutive CaMV 35S promoter (p35SP1, p35P1C53S and p35SP1C53S, respectively) together with the pA1Luc reporter construct. A
pUbiGUS construct was included in every bombardment as a normalization control. Each treatment was done in triplicate, and the LUC data were normalized for GUS activity. The fold activation was calculated as the ratio between each particular treatment and the treatment of pA1Luc without activator. The average values of triplicate experiments are shown and the error bars indicate the standard deviation of the samples.

Fig. 4. **Distinct effects of oxidizing conditions on the DNA-binding properties of the Cys49 and Cys53 mutants.** EMSA experiments with the APB1 probe and purified proteins. Samples were treated with the respective reagents for 30 minutes on ice prior to incubation with DNA. The names of the corresponding MYB domains are indicated. A, EMSA of purified single Cys mutants in the presence of 1 mM DTT  B, EMSA of purified single Cys mutants in the presence of 3 mM diamide. *Filled* and *open* arrows indicate protein-DNA complexes and free DNA probe, respectively.

Fig. 5. **DNA-binding properties of the double Cys49 and Cys53 mutants.** EMSA experiments with APB1 wild type DNA probe and purified proteins. Samples were treated with the respective reagents for 30 minutes on ice prior to incubation with DNA. The names of the corresponding MYB domains are indicated. A, EMSA of purified
double Cys mutants in the presence of 1 mM DTT. EMSA of purified double Cys mutants in the presence of 3 mM diamide. Filled and open arrows indicate protein-DNA complexes and free DNA probe, respectively.

Fig. 6. **Formation of inter-molecular and intra-molecular disulfide bonds.** A, Non-reducing 15% polyacrylamide gel stained with Coomassie Brilliant Blue using 700 ng of recombinant N6His-pMYBΔ9 and indicated mutant proteins incubated under reducing conditions (10 mM DTT) or oxidizing conditions (30 mM diamide). The filled arrow indicates a dimer of C53S (~28kDa), while the open arrow indicates the monomeric forms (~14 kDa). Apparent molecular weights are indicated, representing the Precision Plus All Blue Protein standard (Biorad) and are shown in kiloDaltons (kDa). B, Fluorescent labeling of free sulfhydryl groups using 350 ng of recombinant N6His-pMYBΔ9, and indicated mutant proteins incubated under reducing conditions (1 mM TCEP) or non-reducing conditions. After labeling, proteins were separated on a non-reducing 15% polyacrylamide gel. Fluorescently labeled proteins were visualized, after separation, by electrophoresis by ultraviolet light. Fluorescent imaging (lower panel) was accomplished using the Bio-RAD Gel Doc 2000 Documentation system. The Coomassie Brilliant Blue staining of the same gel is depicted in the upper panel.
Fig. 7. **Position of Cys130 in the solved structure of the R2 MYB c-MYB domain.** The solved structure of the *Homo sapiens* c-MYB proto-oncogene bound to DNA (PDB file 1H88) (33) was used along with DeepView/Swiss-PDB Viewer (v3.7) (34) to estimate the distance between the side chains of Cys130 (Cys53 in P1) and Ile126 (Cys49 in P1). A ribbon diagram of the peptide chain backbone of the c-MYB R2 MYB repeat is shown displaying the three $\alpha$-helices and residues addressed in the discussion are identified with their position in respect to Fig. 1B. Atom colors correspond to the following: blue = nitrogen, red = oxygen, white = carbon and yellow = sulfur.
Table 1. Wavelength (nm) of maximum fluorescence obtained from tryptophan fluorescence emission spectra of the truncated wild type P1 MYB domain (WT, N6His-pMYBΔ9) and single cysteine mutants in the presence of either 0 mM DTT, 1 mM DTT or 6 M guanidine HCl.

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Fig 3

- p35SPI
- p35SPI<sup>C53A</sup>
- p35SPI<sup>C53S</sup>

Fold Activation
Fig 4

A  DTT

B  Diamide

WT  C53A  C53S  C49A  C49S  C49I

1  2  3  4  5  6
Fig 5
Fig 6

A

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George F. Heine, Marcela J. Hernandez and Erich Grotewold

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