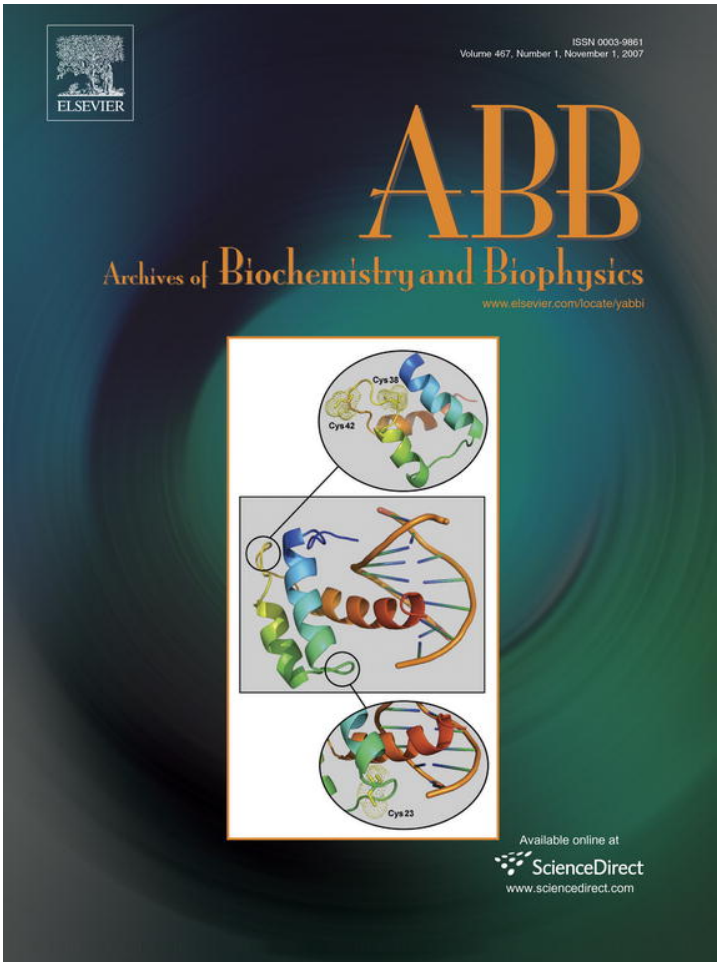

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# Conserved homeodomain cysteines confer redox sensitivity and influence the DNA binding properties of plant class III HD-Zip proteins

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**Abstract**

The role of four cysteines present within the homeodomain of the homeodomain-leucine zipper (HD-Zip) class III protein Athb-9 has been studied. DNA binding by the Athb-9 HD-Zip domain was only observed after incubation in the presence of reducing agents or the thioredoxin system, suggesting that the protein is sensitive to redox conditions. A similar behavior was observed for proteins that show the same binding specificity of Athb-9 present in nuclear extracts. The use of single and double mutants indicated that two out of three of the cysteines at positions 23, 38 and 42 are required for redox sensitivity, while Cys58 is not involved. A role of Cys23 and Cys58 in determining the DNA binding efficiency and specificity, respectively, of the reduced Athb-9 HD-Zip domain was also evident from these studies. It can be postulated that redox conditions may modulate the function of Athb-9 in plant development. Sequence conservation suggests that the results can be extended to all HD-Zip III transcription factors.


*Keywords:* Redox regulation; Plant transcription factor; DNA binding; Homeodomain; *Arabidopsis thaliana*

The homeodomain (HD)[1] is a 60-amino acid motif found in a group of eukaryotic transcription factors usually involved in regulating developmental processes [1–3]. It folds into a characteristic three-helix structure that is able to specifically interact with DNA. Helices I and II are separated by an extended loop while helices II and III (the DNA recognition helix) are connected by a four-amino-acid turn.

The model plant *Arabidopsis thaliana* contains about 100 HD proteins that can be divided into different subfamilies [4,5]. One of the subfamilies, termed HD-Zip, is composed of proteins that contain a leucine zipper dimerization motif adjacent to the HD and bind DNA as dimers. The removal of the leucine zipper causes a complete loss of binding, indicating that dimerization is essential for efficient recognition of DNA [4,6,7].

There are four classes of HD-Zip proteins, I–IV, each composed of several members in different plant species [6,8,9]. Athb-9, and probably all other HD-Zip III proteins, bind the 11-bp dyad symmetric sequence GTAAT(G/C)ATTAC, which can be regarded as composed of two partially overlapping TNATTAC sequences containing the TNAT core recognized by most HDs [9]. Accordingly, it has been postulated that each monomer interacts with one of these half-sequences in a way that resembles the interaction of monomeric animal HDs with DNA. HD-Zip III proteins differ from other HD-Zip proteins in the relative orientation of the leucine zipper respective to the HD [9,10]. The HD of HD-Zip III proteins also presents a four-amino-acid insertion between helices II and III and a set of conserved cysteines at defined positions.

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[1] *Abbreviations used:* HD, homeodomain; GST, glutathione *S*-transferase; GSSG, oxidized glutathione; DTT, dithiothreitol; GSH, glutathione; EMSA, electrophoretic mobility shift assays.

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In the present work, we have analyzed the role of cysteines present in the HD of the Arabidopsis HD-Zip III protein Athb-9, also known as PHAVOLUTA (PHV), involved in determining the radial patterning in shoots [11]. The results indicate that specific cysteines are involved in determining the DNA binding activity, specificity and redox sensitivity of the HD-Zip III domain.

## Materials and methods

### *Cloning, expression and purification of recombinant proteins*

The Athb-9 HD-Zip domain was expressed as a fusion with glutathione *S*-transferase (GST) from *Schistosoma japonicum* [12]. For this purpose, a fragment encoding amino acids 7–119 of Athb-9 was amplified by RT-PCR from total leaf RNA and cloned in frame in vector pGEX-3X. Mutants with Cys to Ala changes within the HD were constructed by the overlap extension method [13] using complementary oligonucleotides (Table 1). All constructions were checked by DNA sequence analysis.

Purification by affinity chromatography was carried out essentially as described by Smith and Johnson [12], with modifications described by Palena et al. [14]. Purified proteins (>95% as judged by Coomassie brilliant blue staining of denaturing polyacrylamide gels) were used for the assays. Protein amounts were measured as described by Sedmak and Grossberg [15] and verified by inspection of the corresponding bands in polyacrylamide gels. DNA binding assays were performed with the proteins fused to GST. Controls made with proteins obtained after cleavage with factor Xa indicated that the GST moiety does not affect the behavior of the recombinant proteins.

*Escherichia coli* thioredoxin was expressed from plasmid pET-32a(+) (Novagen, Inc.) and purified by nickel affinity chromatography. *Escherichia coli* thioredoxin reductase was expressed from plasmid pTrR301 and purified as described by Mulrooney [16].

### *Treatment of proteins with redox agents*

Purified proteins were dialyzed overnight at 4 °C in 50 mM Tris–HCl (pH 8.0). Treatments with redox agents were performed in this buffer during 1 h at room temperature. Reagents were dissolved in the same buffer.

### *Electrophoresis of proteins*

Non-reducing SDS–polyacrylamide gels were performed using standard procedures, except that β-mercaptoethanol was omitted from the loading buffer. Samples (1 μg protein) were preincubated at room temperature in 50 mM Tris–HCl (pH 8.0) plus the indicated additions, mixed with loading buffer, boiled during 5 min and loaded onto a 10% (w/v) polyacrylamide gel. After electrophoresis, gels were stained with Coomassie brilliant blue.

Table 1
Oligonucleotides used for PCR amplification and site-directed mutagenesis

| Name | Sequence (5′ → 3′) |
|---|---|
| H9-1 | GGCGGATCCCCATGGACGATAGAGACT |
| H9-2 | GCGGATCCCGTGTGGATTCGATGTTTCAT |
| C58A-F | AATCGCAGAGCACGAGAGAG |
| C58A-R | CTTCTCTCGTGCTCTGCGATT |
| C23A-F | TATGCTGAGGCTCCTAAACCT |
| C23A-R | AGGTTTAGGAGCCTCAGCATA |
| C38A-F | ATTCGTGAAGCTCCCATTCTC |
| C38A-R | GAGAATGGGAGCTTCACGAT |
| C42A-F | CCCATTCTCGCTAACATCGAG |
| C42A-R | CTCGATGTTAGCGAGAATGGG |

### *DNA binding assays*

For electrophoretic mobility shift assays (EMSA), aliquots of purified proteins or nuclear extracts prepared from cauliflower buds as described by Maliga et al. [17] were incubated with double stranded DNA generated by hybridization of the complementary oligonucleotides 5′-GAT CCTCGTAATCATTACGATCTG-3′ and 5′-AATTCAGATCGTAAT GATTACGAG-3′. Binding reactions were performed in the absence or presence of reducing agents as described [7] and immediately loaded onto a running gel (5% acrylamide, 0.08% bis-acrylamide in 0.5× TGE plus 2.5% glycerol; 1× TGE is 90 mM Tris, 190 mM glycine, 1 mM EDTA, pH 8.0). The gel was run in 1× TGE at 30 mA for 1.5 h and dried prior to autoradiography. When comparing binding to different oligonucleotides, equal amounts of DNA (as judged by absorbance at 260 nm before labeling, and by scintillation counting after labeling) were used. The assays shown are representative of at least three experiments that produced essentially the same results.

## Results

### *Athb-9 binds DNA only after incubation with reducing agents*

Amino acid sequence comparisons of the HD-Zip domains of HD-Zip III proteins showed the presence of three conserved cysteines located in the loop that separate helices I and II, the turn between helices II and III and the recognition helix, respectively (Fig. 1). Athb-9 contains an additional cysteine located between helices II and III, forming a CPILC motif with one of the conserved cysteines. The presence of conserved cysteines and the CxxxC motif, which has been implicated as a redox switch in other proteins [18,19], led us to investigate the behavior of Athb-9 under reducing and oxidizing conditions.

To test the role of the oxidation state of cysteines on the properties of Athb-9, we incubated the recombinant protein in the presence of the oxidants diamide and oxidized glutathione (GSSG) and the reducing agents dithiothreitol (DTT) and reduced glutathione (GSH) and analyzed the extent of DNA binding using EMSA (Fig. 2a). Only after incubation in the presence of reducing agents a shifted band was observed, indicating that cysteines in the reduced state are required for efficient DNA binding by Athb-9. Interestingly, a sample incubated in Tris buffer alone showed no DNA binding, suggesting that the protein is spontaneously oxidized during dialysis or incubation without reducing agents. In addition, protein that was not dialyzed after purification showed the same behavior as dialyzed protein, even if 5 mM GSH was used for elution (not shown).

### *Reduced thioredoxin catalyzes the activation of Athb-9*

The fact that the reduction of disulfide bonds promotes the activation of Athb-9 raises the possibility that a similar reaction is used under physiological conditions to regulate the properties of this transcription factor. Redox changes in protein cysteines are usually catalyzed *in vivo* by thioredoxin, a small protein that is in turn reduced by NADPH

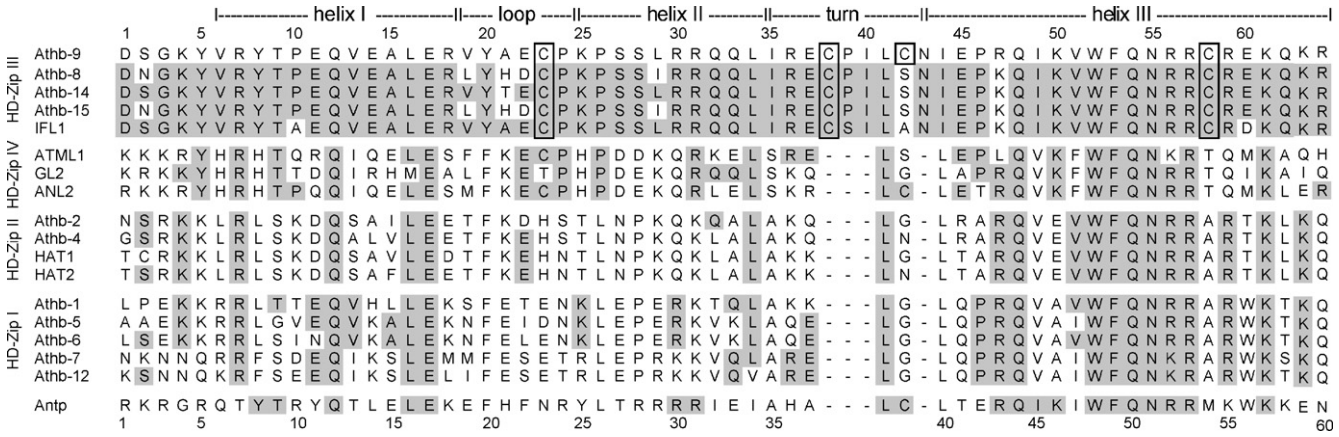


Fig. 1. Alignment of the HDs of HD-Zip proteins from different classes. The HD sequences of representative class I to IV HD-Zip proteins are shown. The HD of *Drosophila* Antennapedia (Antp) is shown for reference. Gaps were introduced to maximize the alignment. Amino acids conserved in other HDs respective to Athb-9 are shadowed. Cysteines conserved in HD-Zip III proteins and an additional cysteine present only in Athb-9 are boxed. Numbers below and above the alignment indicate amino acid positions of canonical and HD-Zip III HDs, respectively.

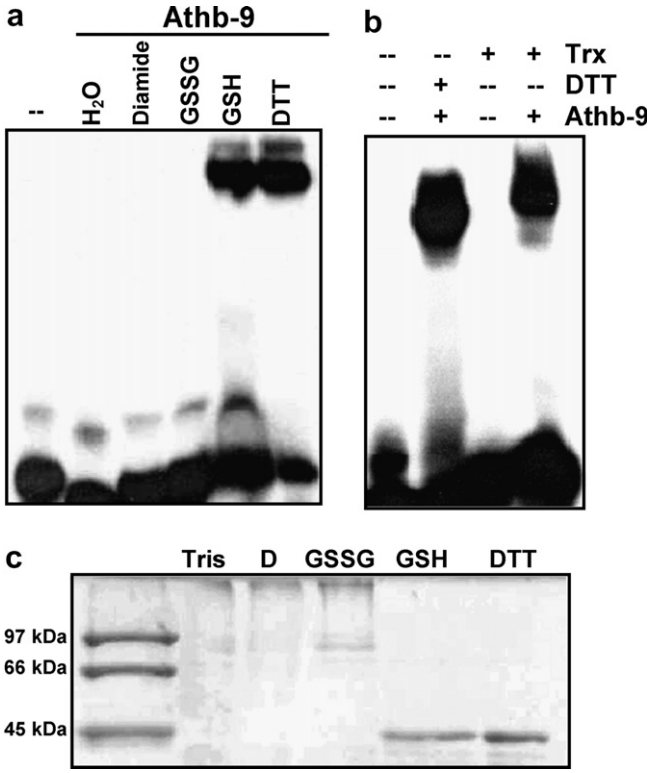


Fig. 2. Redox changes modulate Athb-9 DNA binding activity. (a) EMSA of Athb-9 binding to DNA after incubation in the presence of either 10 mM diamide, 25 mM GSSG, 25 mM GSH, 25 mM DTT, or in the absence of redox agents ($H_2O$) during 1 h at room temperature. (b) A similar assay as in (a) after incubation in the presence of 0.013 μg/μl each thioredoxin and thioredoxin reductase plus 0.55 mM NADPH (Trx) or 25 mM DTT. (c) Non-reducing SDS–polyacrylamide gel electrophoresis of recombinant Athb-9 incubated as described in (a); D indicates incubation in the presence of diamide.

in the presence of thioredoxin reductase [20]. We have then analyzed the thioredoxin-dependent activation of Athb-9 using recombinant thioredoxin and thioredoxin reductase purified from *E. coli*. As shown in Fig. 2b, this system was as efficient as DTT in the activation of Athb-9, suggesting that thiol/disulfide exchanges may be catalyzed by the same proteins *in vivo*.

### *Oxidized Athb-9 forms intermolecular disulfide bonds*

The fact that Athb-9 binds DNA as a dimer suggests that the cysteines may form intermolecular disulfide bonds when oxidized. This possibility was analyzed by performing denaturing polyacrylamide gels under non-reducing conditions of proteins subjected to different treatments. As shown in Fig. 2c, Athb-9 formed species with significantly reduced mobility under oxidizing conditions, while proteins treated with either GSH or DTT behaved as monomers. Thus, the formation of interchain disulfide bonds may be related with the decrease in affinity for DNA.

### *Native proteins present in plant nuclear extracts also show redox-sensitive DNA binding*

Binding of proteins present in plant nuclear extracts to the Athb-9 DNA target sequence was also tested under different redox conditions. For this purpose, nuclear protein extracts were prepared from cauliflower (*Brassica oleracea*), a species closely related to *Arabidopsis*. As shown in Fig. 3, only a faint retarded band was observed with an extract that has been previously incubated in the presence of the oxidant diamide. Incubation in the presence of DTT, in turn, produced a net retarded band of lower mobility. This band was also observed when the extract was incubated in the presence of the thioredoxin/thioredoxin reductase system, but not upon incubation in the absence of redox agents (Fig. 3).

Competition experiments showed that binding of nuclear proteins to the Athb-9 target site was efficiently competed by an excess of unlabeled DNA containing the same sequence, but not by DNA carrying variants of this sequence (Fig. 3). This result indicates that the redox-sen-

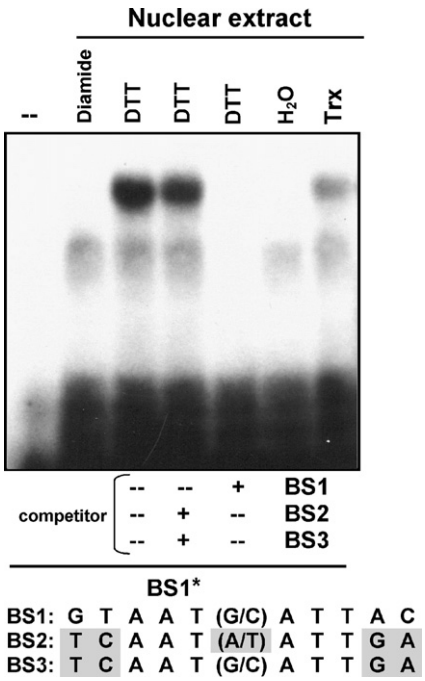


Fig. 3. Nuclear proteins that recognize the Athb-9 target sequence show redox-sensitive DNA binding. EMSA of the binding of plant nuclear proteins (10 μg) to labeled DNA containing the Athb-9 target site (BS1). The nuclear extract was previously incubated in the presence of either 10 mM diamide, 25 mM DTT, or in the absence of redox agents ($H_2O$) during 1 h at room temperature. Incubation was also performed in the presence of 0.013 μg/μl each thioredoxin and thioredoxin reductase plus 0.55 mM NADPH (Trx). Where indicated, a 10-fold molar excess of unlabeled DNA carrying the Athb-9 target site (BS1) or variants of this sequence (BS2, BS3) was also included. The relevant sequences of the oligonucleotides used for competition are shown below; positions that do not match the Athb-9 target site are shadowed.

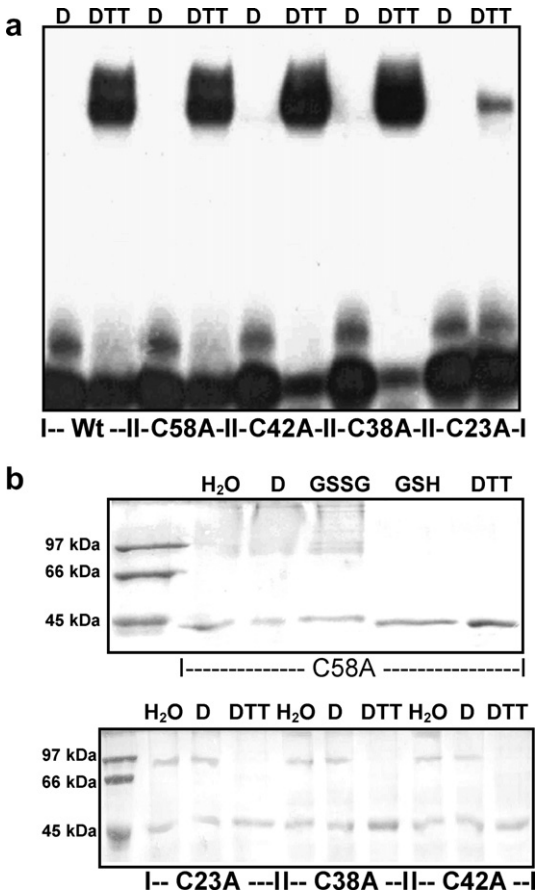


Fig. 4. Single cysteine mutants of Athb-9 are sensitive to oxidation. (a) EMSA of Cys-to-Ala mutants of Athb-9 after incubation in the presence of either 10 mM diamide (D) or 25 mM DTT. (b) Non-reducing SDS–polyacrylamide gel electrophoresis of mutant proteins incubated as in (a).

sitive proteins present in nuclear extracts have the same DNA binding specificity of Athb-9, suggesting that they are members of the HD-Zip III family. We conclude, then, that native HD-Zip III proteins present in plant nuclear extracts undergo the same redox transitions as the recombinant protein expressed and purified from *E. coli*.

*Role of individual cysteines in Athb-9*

Single mutations of cysteines in the HD of Athb-9 to alanine produced proteins still sensitive to oxidation, suggesting that different cysteine pairs can originate a redox-sensitive protein (Fig. 4a). Non-reducing gels indicated the presence of monomers and covalently bound dimers under oxidizing conditions for all the proteins under study (Fig. 4b). Since oxidized proteins showed no binding to DNA under the conditions tested, it can be speculated that intramolecular disulfide bonds also originate proteins unable to bind DNA.

Mutation of cysteine at position 23 (within the loop that connects helices I and II) produced a protein still sensitive to oxidation but with a considerable lower DNA binding capacity under reducing conditions (Fig. 4a). This cysteine, in the reduced state, seems then to be involved in either the interaction with DNA or the correct folding of the HD.

Further analysis of Athb-9 redox sensitivity was performed by constructing the six possible double-mutants in the four cysteines. Analysis of DNA binding under reducing and oxidizing conditions indicated that proteins that conserved two of the three cysteines located between the HD helices (cysteines 23, 38 and 42) were still sensitive to oxidation, while those that contained Cys58 and another of the cysteines were insensitive (Fig. 5a). Again, proteins in which Cys23 was mutated showed reduced binding activity under reducing conditions.

All proteins, with the sole exception of the mutant that conserved cysteines 23 and 38, produced monomers and covalently linked dimers under oxidizing conditions (Fig. 5b). The mentioned mutant formed only dimers under the same conditions, suggesting that only intermolecular disulfide bonds are formed. Cysteines within the loops are then responsible for the redox sensitivity of Athb-9, through the formation of inter- and intramolecular disulfide bonds, while Cys58 is not involved in redox sensitivity.

The fact that Cys58 is not involved in redox sensitivity may suggest that this residue does not participate in the

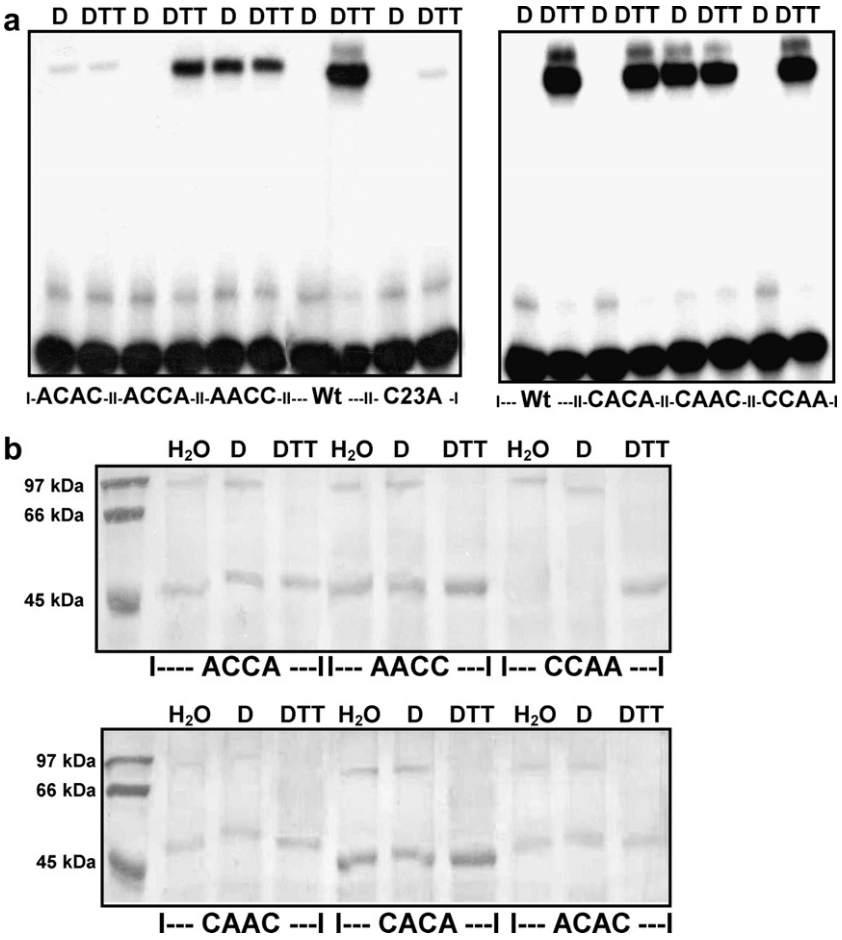


Fig. 5. Redox sensitivity of Athb-9 binding to DNA is abolished in some double cysteine mutants. (a) EMSA of the binding of Athb-9 double mutants to DNA after incubation in the presence of redox agents, as described in Fig. 2. Letters A or C (from left to right) indicate the presence of Ala or Cys at positions 23, 38, 42 and 58, respectively. (b) Non-reducing SDS–polyacrylamide gel electrophoresis of mutant proteins incubated as in (a).

formation of disulfide bonds. Mutation of Cys58 to Ala, however, produces an increase in the amount of monomers under oxidizing conditions (i.e. compare Figs. 2c and 4b). The most likely explanation for this behavior is that Cys58 forms disulfide bonds within the context of the native protein, but that this behavior is lost upon mutation of other cysteines.

Residues at position 54 establish contacts with DNA and determine the binding specificity in several HDs [21]. In HD-Zip IV proteins, Thr54 together with Phe47 originate a preference for the half-sequence TAAATG instead of TAATTG [22], bound by HD-Zip I proteins. These proteins, in turn, contain Ala54. Cys58 in Athb-9 is equivalent to residue 54 in other HDs due to the presence of a four-amino-acid insertion in HD-Zip III proteins. As shown above, mutation of Cys58 to Ala in Athb-9 does not produce a significant change in DNA binding to the Athb-9 recognition sequence. Since this mutation approximates the primary structure of the recognition helix to that of HD-Zip I and HD-Zip II proteins (Fig. 1), we tested binding of the mutant to DNA sequences containing the preferred binding sites of proteins from these classes. Fig. 6 shows that the native Athb-9 HD-Zip domain interacts very inefficiently with these sequences. The Ala58 mutant, in turn, is able to bind oligonucleotides with the half sequences TAATTG, TGATTG and TAATTA with significant efficiency, although it conserves the preference for TGATTA. Mutation of Cys58 seems then to confer a relaxed DNA binding specificity to the Athb-9 HD. Similar experiments using the mutants at positions 23, 38 or 42 showed that the DNA binding specificity of the respective proteins was not altered (not shown).

## Discussion

In the present work, we show that the plant HD transcription factor Athb-9 undergoes redox modifications that produce changes in its DNA binding properties. Athb-9 belongs to the HD-Zip III class which members participate in processes related with meristem development, determination of organ polarity and vascular differentiation [11,23–25]. It is noteworthy that two of the cysteines involved in redox modulation are conserved in all members of the HD-Zip III family, suggesting that all HD-Zip III proteins undergo similar changes. Indeed, we have shown that nuclear proteins with the same DNA

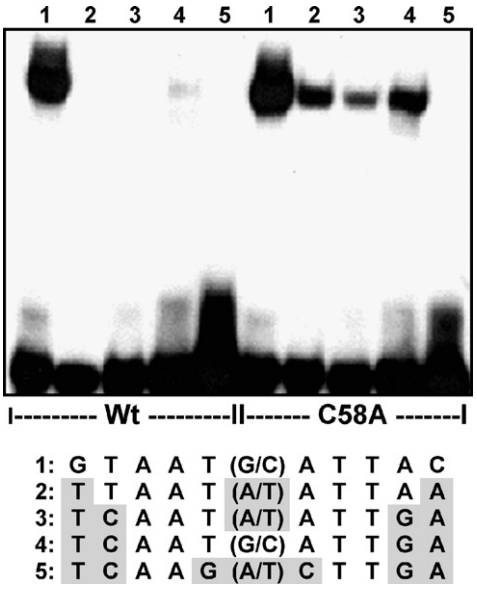


Fig. 6. Ala58-Athb-9 shows reduced selectivity for different DNA sequences. Either wild-type or Ala58-Athb-9 were analyzed by EMSA for binding to the Athb-9 preferred target site (1) or to oligonucleotides with related pseudopalindromic sequences (2–5). Below, the relevant sequences of the different oligonucleotides are shown.

binding specificity of Athb-9 show redox-sensitive DNA binding.

Considering the nature of the structural modifications that render HD-Zip III proteins inactive under oxidation conditions, it seems that both inter- and intramolecular disulfide bonds are involved. If Cys42, present only in Athb-9, is not considered, it can be postulated that both Cys23 and Cys38, conserved in all HD-Zip III proteins, are required for redox sensitivity. These cysteines seem to form only intermolecular disulfide bonds, suggesting that they may participate in the establishment of covalent links between monomers. These links may cause a distortion in the HD structure, thus rendering it inactive for DNA binding. Modeling of HD-Zip III protein structure suggests it is unlikely that disulfide bonds may be established between adjacent monomers of a dimer bound to DNA, since the residues involved would be at a considerable distance (Fig. 7). Covalent links may rather occur between members of different dimers. Alternatively, both loops may be flexible enough to get close to each other in monomers of the same dimer. The presence of a four-amino-acid extension between helices II and III may be functional for this behavior.

Besides its involvement in conferring redox sensitivity to the Athb-9 HD, Cys23 also seems to be required for efficient binding to DNA. A direct interaction with DNA of loop residue Tyr25 has been observed in the engrailed and Antennapedia HDs [26,27]. Modeling of the Athb-9 HD (Fig. 7) shows that Cys23 is located near the DNA backbone and may establish contacts with DNA since it is located in a flexible region. Alternatively, Cys23 may interact with helix III amino acids helping to stabilize the

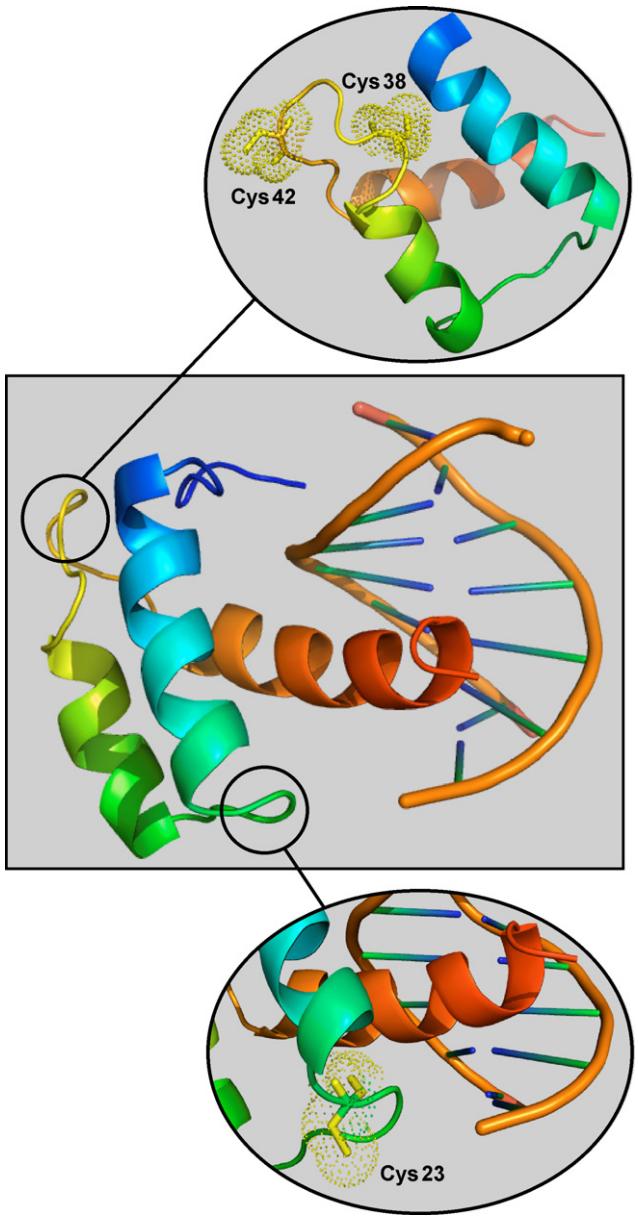


Fig. 7. A model of the Athb-9 HD complex with DNA. The homology model of the Athb-9 HD was made in the Swiss-Model Workspace, using the rat Insulin gene enhancer protein ISL-1 (PDB ID:1bw5) as template. The model was refined by energy minimization using Gromos Force Field, included in the software DeepView/Swiss Pdb Viewer v3.7 SP5 [34], and then fitted with the HD from the *Drosophila* paired protein bound to DNA (PDB ID: 1fjl). The RMSD between the aligned parts (50 residues) was 1.19 A°. The drawings were made with PyMOL v0.99rc6. Yellow spheres represent van der Waals radii of cysteines.

folding of the recognition helix, as described for residues 24 and 25 in mutants of the Mata1 HD with increased binding affinity [28].

*In vivo*, GSH and thioredoxin catalyze the reduction of disulfide bonds in proteins [20,29]. Our results using these reagents *in vitro* indicate that these systems are able to promote the activation of Athb-9. The thioredoxin/thioredoxin reductase system was also able to activate native proteins present in nuclear extracts using NADPH as sub-

strate. We propose, then, that the intracellular levels of GSH and reduced thioredoxin may operate to influence the activation state of HD-Zip III proteins *in vivo*. Regarding the physiological significance of our observations, it should be mentioned that redox agents are known to influence several aspects of development in plants. As an example, it has been shown that GSH modulates tracheary element differentiation, meristem formation and embryogenesis [30–32]. The influence of redox conditions on some of these processes may operate through changes in the activity of HD-Zip III transcription factors. Another indication that HD-Zip III proteins may be modulated by redox conditions comes from the finding that these proteins contain a conserved domain known as the MEKHLA domain, related to the PAS domain found in redox sensitive prokaryotic proteins [33].

In conclusion, we demonstrate that cysteines in the Athb-9 HD have different functions, modulating its DNA binding activity and specificity and conferring redox responsiveness to the protein. These properties may be extended to other members of the HD-Zip III class suggesting that redox conditions may be important modulators of the action of these transcription factors within plant cells.

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