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Redox Regulation of Plant Homeodomain Transcription Factors*

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Adriana E. Tron‡, Carlos W. Bertoncini§, Raquel L. Chan¶, and Daniel H. Gonzalez¶‖

From the Cátedra de Biología Celular y Molecular, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, CC 242 Paraje El Pozo, 3000 Santa Fe, Argentina

Several families of plant transcription factors contain a conserved DNA binding motif known as the homeodomain. In two of these families, named Hd-Zip and glabra2, the homeodomain is associated with a leucine zipper-like dimerization motif. A group of Hd-Zip proteins, namely Hd-ZipII, contain a set of conserved cysteines within the dimerization motif and adjacent to it. Incubation of one of these proteins, Hahb-10, in the presence of thiol-reducing agents such as dithiothreitol or reduced glutathione produced a significant increase in DNA binding. Under such conditions, the protein migrated as a monomer in non-reducing SDS-polyacrylamide gels. Under oxidizing conditions, a significant proportion of the protein migrated as dimers, suggesting the formation of intermolecular disulfide bonds. A similar behavior was observed for the glabra2 protein HAHR1, which also contains two conserved cysteines within its dimerization domain. Site-directed mutagenesis of the cysteines to serines indicated that each of them has different roles in the activation of the proteins. Purified thioredoxin was able to direct the NADPH-dependent activation of Hahb-10 and HAHR1 in the presence of thioredoxin reductase. The results suggest that redox conditions may operate to regulate the activity of these groups of plant transcription factors within plant cells.

The homeodomain (HD)[1] is a 61-amino acid protein motif found in eukaryotic transcription factors generally involved in the regulation of developmental processes (1–3). It folds into a characteristic three-helix structure that interacts specifically with DNA (1, 4–6). Helices II and III form a structure that resembles the helix-turn-helix motif found in many prokaryotic transcription factors. Helix III (the recognition helix) fits into the major groove of DNA, making extensive contacts with specific bases and the sugar-phosphate backbone (7–10). Despite the resemblance in structure between the HD and the helix-turn-helix motif, a striking difference is that HDs usually bind DNA as monomers with high affinity (11, 12). This fact has been explained by the presence of extended contacts along the recognition helix and the N-terminal arm of the HD.

HDs are present in almost every eukaryotic organism that has been investigated. In plants, several families of HD proteins have been described (13). One of these families, named Hd-Zip, comprises proteins with a typical leucine zipper motif adjacent to the C-terminal end of the HD (14, 15). As expected, these proteins bind DNA as dimers (16). The removal of the leucine zipper or the introduction of extra amino acids between the HD and the zipper significantly reduces binding affinity, indicating that the leucine zipper is responsible for the correct positioning of the HD for efficient binding (16). The analysis of binding at different protein concentrations suggests that dimer formation is a prerequisite for DNA binding (17). It has been suggested that Hd-Zip proteins may be involved in regulating developmental processes associated with the response of plants to environmental conditions (13, 18, 19).

A different family of plant HD proteins, named glabra2, consists of larger proteins with an N-terminal HD. These proteins also bind DNA as dimers and possess a dimerization motif that resembles a leucine zipper truncated by a loop (20, 21). Most members of the glabra2 family are expressed specifically in epidermal cells, and the first member to be identified (glabra2) is involved in the development of trichomes, root hairs, and the seed coat mucilage (22–26).

The HD and dimerization motif constitute the most conserved part of the different members of each family. Sequence analysis also revealed the presence of conserved cysteine-containing motifs within variable regions (*i.e.* within the variable loop in glabra2 proteins and near the C terminus of the leucine zipper in Hd-ZipII proteins). Because conserved cysteines have been reported to be involved in the redox regulation of the properties of several transcription factors (27–33), we have tested the effect of oxidants and reductants on DNA binding and quaternary structure of two proteins from these families. Our results indicate that redox conditions are a key factor determining the binding of these proteins to DNA and the formation of covalent oligomeric structures. We propose that a redox-dependent mechanism may operate *in vivo* to modulate the activity of these transcription factors in response to metabolic and/or environmental signals.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of Recombinant Proteins—An *Nhe*I/*Xba*I fragment containing sequences coding for the entire HD and dimerization motif plus additional amino acids (positions 86–325) from the sunflower glabra2 protein HAHR1 (34) was excised from pHX1 (which contains this fragment in vector pUC119) with *Hin*cII and *Eco*RI and cloned into the *Sma*I and *Eco*RI sites of pGEX-3X as described (35). For the production of a protein with only the first half of the dimerization motif (amino acids 86–184), a *Kpn*I deletion of this clone was used. Fragments encoding proteins with either Cys or Ser at positions 185 and 188 were obtained by PCR on native HAHR1 and oligonucleotide 5′-GGCGAATTCTTGGTGATGCTCCCTGTG-3′ in combination with ei-

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‡ A fellow of Consejo Nacional de Investigaciones Científicas y Técnicas.

§ An undergraduate fellow of Universidad Nacional del Litoral.

¶ Members of Consejo Nacional de Investigaciones Científicas y Técnicas.

‖ To whom correspondence should be addressed. Tel./Fax: 54-342-4575219; E-mail: dhgonza@fbcb.unl.edu.ar.

[1] The abbreviations used are: HD, homeodomain; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; GSSG, oxidized glutathione; GSH, reduced glutathione.

ther 5′-GGGCTGGCAAGCCACGTTTGGTG-3′ (for CC-HAHR1), 5′-AAGGGTACCAGTACCAAC-3′ (for SC HAHR1), or 5′-GCGGGTACCAGTACCAACAGTGGTTTC-3′ (for SS-HAHR1). For CS HAHR1, oligonucleotides 5′-GGCGAATTCTTGGTGATGCTCCCTGTG-3′ and 5′-AAGGGTACCTGTACCAAC-3′ were used with DNA from SS-HAHR1 as template. Amplified products were digested with *Kpn*I and *Eco*RI and cloned into similar sites in the HAHR1-expressing plasmid. These constructs express proteins bearing the entire HD and dimerization motif (amino acids 86–234).

An *Spe*I fragment encoding amino acids 81–231 from the sunflower Hd-ZipII protein Hahb-10 (36), previously cloned into the *Xba*I site of pMAL-c2, was used as the source for expression in pGEX4T-3 using *Bam*HI and *Sal*I sites for excision and cloning. Fragments encoding the entire Hd-Zip domain plus the CPSCE motif (amino acids 81–208) were amplified and cloned in-frame into the *Bam*HI and *Eco*RI sites of pGEX4T-3. Amplifications were performed using oligonucleotides pGEX1 (5′-GGGCTGGCAAGCCACGTTTGGTG-3′) and CC10 (5′-CCGAATTCCCGATCTGTTCACACGAC-3′ for CCCCC-Hahb-10), SC10 (5′-CCGAATTCCCGATCTGTTCACACGACGGAGACACG-3′ for CCC-SC-Hahb-10), or CS10 (5′-CCGAATTCCCGATCTGTTCAGACGAC-3′ for CCCCS-Hahb-10 and CCCSS-Hahb-10) using either native or CCCSC-Hahb-10 sequences as templates. Mutants with Cys to Ser changes within the leucine zipper were constructed using complementary oligonucleotides 5′-TAAATACTCAGAGTCCACCT-3′ and 5′-AGGTGGACTCTGAGTATTTA-3′ (for SCCCC-Hahb-10) or 5′-TAATGTGTTGGAGGATCTCTTTAA-3′ and 5′-TTAAAGAGATCCTCCAACACATTA-3′ (for CSSCC-Hahb-10) together with primers pGEX1 and CC10 to amplify partially overlapping N-terminal and C-terminal Hahb-10 fragments. The resulting products were mixed in buffer containing 50 mM Tris-HCl (pH 7.2), 10 mM $MgSO_4$, and 0.1 mM dithiothreitol (DTT), incubated at 95 °C during 5 min, and annealed by allowing the solution to cool to 24 °C in ~1 h. After this, 0.5 mM each dNTP and 5 units of the Klenow fragment of *Escherichia coli* DNA polymerase I were added, and incubation was followed for 1 h at 37 °C. A portion of this reaction was directly used to amplify the annealed fragments using primers pGEX1 and CC10. Mutants SCCSS-Hahb-10 and CSSSS-Hahb-10 were obtained in a similar way but using primer CS10 instead of CC10 and DNA from CCCSC-Hahb-10 as template. Mutant SSSSS-Hahb-10 was obtained by mutating CSSSS-Hahb-10. All constructions were checked by DNA sequence analysis.

For expression, *E. coli* JM109 cells bearing the corresponding plasmids were grown and induced as described previously (35). Purification and cleavage of the fusion products were carried out essentially as described by Smith and Johnson (37), with modifications described by Palena *et al.* (35). 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 (38).

His-tagged *E. coli* thioredoxin encoded in plasmid pET-32a(+) (Novagen, Inc.) was expressed from this plasmid and purified by nickel affinity chromatography. *E. coli* thioredoxin reductase was expressed from plasmid pTrR301 and purified as described by Mulrooney (39).

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 for 1 h at room temperature. Reagents were dissolved in the same buffer.

DNA Binding Assays—For electrophoretic mobility shift assays (EMSAs), aliquots of purified proteins were incubated with double-stranded DNA generated by hybridization of the complementary oligonucleotides 5′-AATTCAGATCTCAATGATTGAGAG-3′ and 5′-GATCCTCTCAATCATTGAGATCTG-3′ (for Hahb-10) or 5′-AATTCAGATCTCATTAAATGAGAG-3′ and 5′-GATCCTCTCATTTAATGAGATCTG-3′ (for HAHR1) and labeled with [α-^{32}P]dATP by filling in the 3′-ends using the Klenow fragment of *E. coli* DNA polymerase I. Binding reactions (20 μl) containing 20 mM HEPES (pH 7.5), 50 mM KCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 0.5% Triton X-100, 1 μg of poly(dI-dC), 10% glycerol, 0.6 ng (30,000 cpm) of labeled oligonucleotide and 50 ng of protein were incubated for 20 min at room temperature, supplemented with 2.5% Ficoll, and immediately loaded onto a running gel (5% acrylamide, 0.08% bisacrylamide in 0.5× TBE plus 2.5% glycerol (1× TBE is 90 mM Tris borate (pH 8.3), 2 mM EDTA). The gel was run in 0.5× TBE at 20 mA for 2 h and dried before autoradiography. Control experiments indicated that after 20 min of incubation, binding equilibrium was attained. For quantitative analyses of binding affinity, poly(dI-dC) was omitted, and radioactive bands were cut from exposed gels and measured by scintillation counting. Data handling and curve fitting were performed using Sigma plot software. Overall dissociation constants (K_{12}) of the dimer-DNA complexes into monomers and free DNA were calculated using the equation $K_{12} = P_2 \times D/P_2D$, according to the binding sequence $2P \rightarrow P_2$, and $P_2 + D \rightarrow P_2D$, were P, P_2, D, and P_2D represent protein monomers and dimers and free and bound DNA, respectively (17).

Electrophoresis of Proteins—Non-reducing SDS-polyacrylamide gels were performed essentially as described by Laemmli (40), 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 12% (w/v) polyacrylamide gel. After electrophoresis, gels were stained with Coomassie Brilliant Blue.

Preparation of Nuclei and Western Blots—Sunflower nuclei and nuclear extracts were prepared from 4-day-old seedlings according to the technique described in Maliga *et al.* (41). Protein patterns were analyzed by SDS-polyacrylamide gel electrophoresis, and total protein concentration was measured as described (38). For Western blots, aliquots of extracts (5 μg of protein) previously dialyzed in buffer without reductants were incubated under different conditions and loaded onto non-reducing SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose and developed using anti-HAHR1 polyclonal antibodies (35) and chemiluminescent peroxidase reagents (Amersham Biosciences) using standard protocols.

RESULTS

Redox Agents Modulate HARH1 Binding to DNA—We have previously demonstrated that the sunflower HD protein HAHR1 binds a pseudopalindromic 9-bp DNA sequence as a dimer (20, 42). Amino acid sequence comparisons of HAHR1 with other proteins from the glabra2 family showed a high conservation along the HD and dimerization domain, with the sole exception of the disordered loop that separates helix I and II of the dimerization domain (Fig. 1*A*). This loop is characterized by the presence of Gly, Pro, and Ser but is variable in length, and defined positions are only poorly conserved, suggesting that its main role is as a flexible linker between the two helices. Within this context, it is noteworthy that a block that contains two cysteines (C*XX*CG) is present within the loop of all glabra2-like proteins described up to now (Fig. 1*A*). This conservation is likely an indicator of an essential function of this segment and, particularly, of the conserved cysteines within it.

To test the role of the oxidation state of the conserved cysteines on the properties of HAHR1, we have incubated the recombinant protein in the presence of the oxidant diamide and the sulfhydryl reductant DTT and analyzed the extent of DNA binding using EMSA (Fig. 2). Incubation in the presence of DTT produced a marked increase in DNA binding with respect to samples incubated with diamide, suggesting that cysteines in the reduced state are required for efficient binding. A similar observation was made when the binding reactions were subjected to UV photocross-linking and analyzed by denaturing polyacrylamide gels (not shown). This indicates that the observed changes in DNA binding are not due to differential dissociation of the protein-DNA complex during electrophoresis. Similar differences in binding activity were obtained in the absence and presence of the nonspecific competitor poly(dI-dC). Determination of the overall dissociation constant of bound dimers into free monomers (K_{12}) yielded values of 3.10 ($\pm$0.01) $\times$ 10^{-14} M^2 and 1.88 ($\pm$0.09) $\times$ 10^{-13} M^2 for the proteins incubated in the presence of DTT or diamide, respectively, indicating that the reducing agent produces an increase in the affinity of the protein for DNA. Kinetic analysis showed that the rate of association of the complex is increased by incubation with DTT, whereas the rate of complex dissociation is not significantly affected (not shown).

When both cysteines present within the dimerization motif were mutated to serine a protein active under both redox conditions was obtained (Fig. 2). To further define the requirements for the redox conversion of HAHR1, we have tested the effect of the redox pair oxidized glutathione (GSSG)/reduced glutathione (GSH). As shown in Fig. 3 (*left panel*), a significant activation was obtained with GSH, indicating that this compound, which has a lower reduction potential than DTT, is able

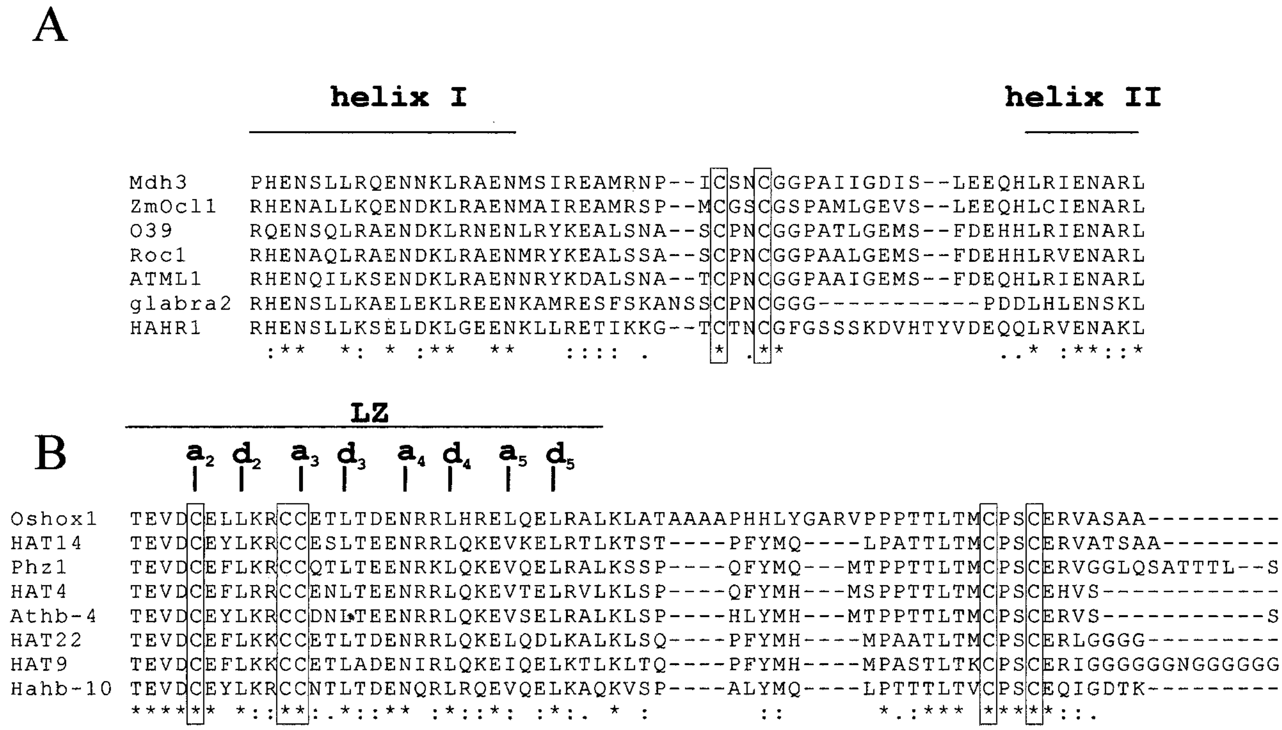

FIG. 1. **Conserved cysteines at or near the dimerization domain of plant HD proteins.** Full-length protein sequences were aligned using ClustalW (52). Conserved cysteines are *boxed*. Identical amino acids at a defined position are denoted by *asterisks*. *Double dots* indicate similar amino acids. *A*, amino acid sequence alignment of a region of the dimerization motif of seven glabra2-like proteins: *Malus x domestica* Mdh3 (accession number AAC79430), *Zea mays* ZmOcl1 (accession number CAB51059), *Phalaenopsis* sp. O39 (accession number AAB37230), *Oryza sativa* Roc1 (accession number BAB85750), *Arabidopsis thaliana* ATML1 and glabra2 (accession numbers T05850 and P46607), and *Helianthus annuus* HAHR1 (accession number AAC37514). A detail of the alignment, comprising helix I, the loop, and helix II, is shown. *B*, the leucine zipper (*LZ*) and adjacent regions of eight Hd-ZipII proteins: *O. sativa* Oshox1 (accession number AAF19980), *A. thaliana* HAT14, HAT4, Athb-4, HAT22, and HAT9 (accession numbers CAD24012, CAA79670, AAC31833, T06026, and P46603), *Pimpinella brachycarpa* Phz1 (accession number CAA64491), and *H. annuus* Hahb-10 (accession number AAA79778); *a* and *d* indicate the first and fourth residue of each heptad, respectively.

to reduce HAHR1 cysteines, although less efficiently than DTT. Interestingly, a sample incubated without any oxidant or reductant showed similar binding activity as those incubated with oxidants (Fig. 3, *left panel*), suggesting that the protein is spontaneously oxidized during dialysis or incubation without reducing agents.

The individual role of each of the two cysteines present in the loop of the dimerization motif was analyzed by producing proteins with single mutations to serine. Both proteins were sensitive to oxidation although to a different extent. CS-HAHR1 was partially active under oxidizing conditions and was activated by GSH even if DTT was required for full activation (Fig. 3, *right panel*). SC-HAHR1, on the contrary, showed significant binding only in the presence of DTT. The different behavior of the single mutants may reflect differences in redox potential of the respective cysteines.

Oxidized HAHR1 Forms Intermolecular Disulfide Bonds—The fact that HAHR1 forms dimers in solution suggests that the cysteines present within the loop of the dimerization motif 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. 4 (*upper panel*), proteins with either one or both cysteines formed species with significantly reduced mobility corresponding to dimers in the absence of reducing agents. Proteins treated with DTT behaved as monomers, suggesting that they are non-covalent dimers. Incubation in the presence of oxidizing agents did not enhance the amount of species with reduced mobility, whereas GSH was as efficient as DTT as reducing agent (Fig. 4, *middle* and *lower panel*). It should be noted that there is not an exact correlation

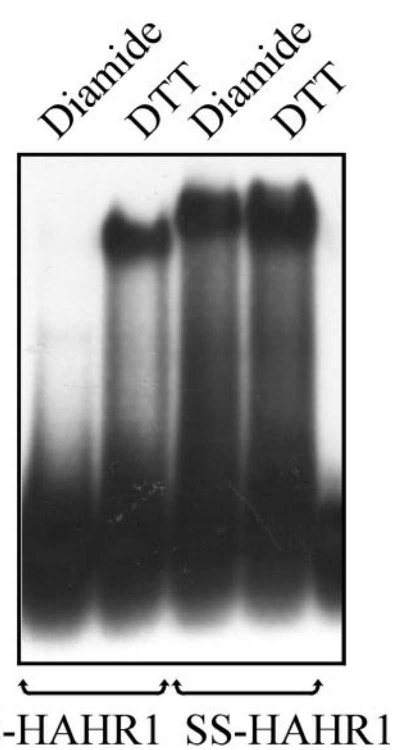

FIG. 2. **Redox changes modulate HAHR1 DNA binding activity.** EMSA of wild type and its double Cys-to-Ser mutant (*CC-HAHR1* and *SS-HAHR1*, respectively). Proteins were incubated in the presence of either 10 mM diamide or 25 mM DTT for 1 h at room temperature before performing the DNA binding assay.

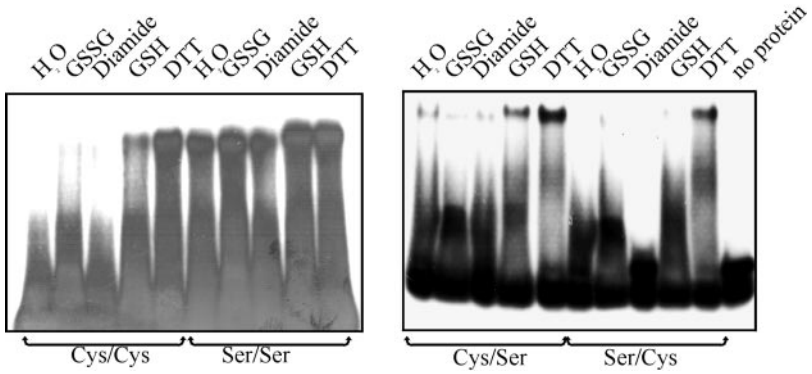

FIG. 3. **Effect of different redox agents on the DNA binding activity of HAHR1 and its cysteine substitution mutants.** EMSA of wild type HAHR1 (*Cys/Cys*) and its single and double Cys-to-Ser mutants (*Cys/Ser*, Cys-188 → Ser; *Ser/Cys*, Cys-185 → Ser; *Ser/Ser*, double mutant) previously incubated in the presence of 25 mM GSSG, 10 mM diamide, 25 mM GSH, 25 mM DTT, or in the absence of redox agents (H_2O) as indicated in Fig. 2.

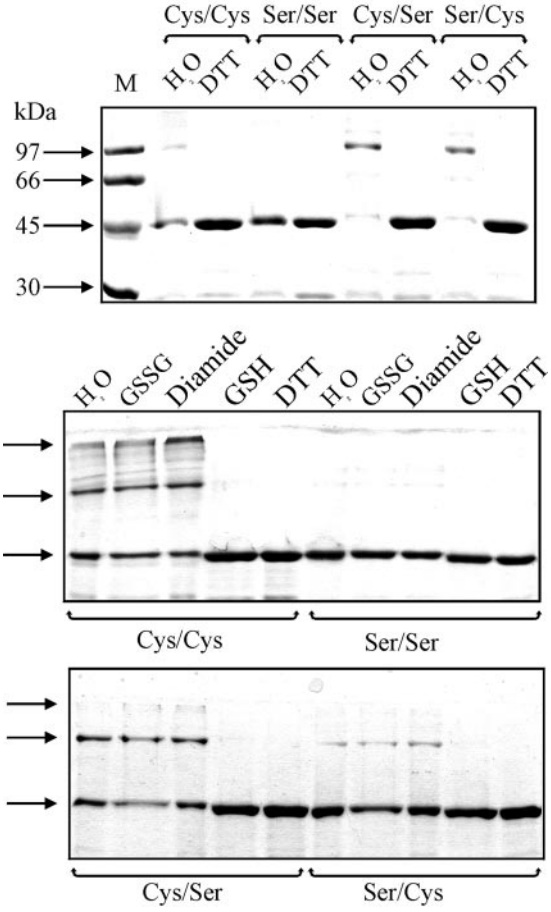

FIG. 4. **Non-reducing polyacrylamide gel of HAHR1 and its cysteine mutants incubated in the presence of various redox agents.** The different proteins were incubated under the conditions described in Fig. 3 before the addition of sample buffer and electrophoretic separation. *Arrows* indicate the position of monomers, dimers, and higher order structures according to standard molecular weight markers shown in *lane M* of the *upper panel*.

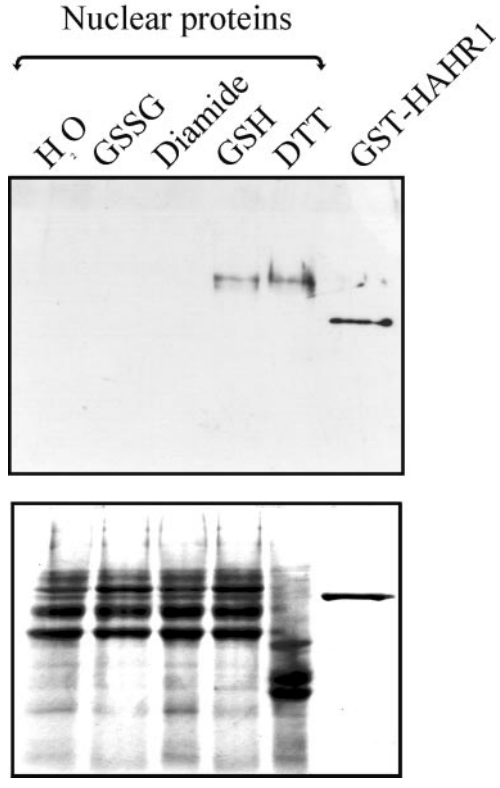

FIG. 5. **Native HAHR1 in nuclear extracts undergoes redox-dependent changes in electrophoretic migration.** Aliquots of a sunflower nuclear protein extract were incubated in the presence of redox agents as described in Fig. 3, subjected to non-reducing polyacrylamide gel electrophoresis, and either analyzed with anti-HAHR1 antibodies by Western blot (*upper panel*) or stained with Coomassie Brilliant Blue (*lower panel*). A sample of recombinant HAHR1 (*GST-HAHR1*), incubated in the presence of DTT, was included as control.

of the amount of monomers and DNA binding activity. This may be due to the different pre-treatment of samples (*i.e.* samples to be analyzed in SDS-polyacrylamide gels were boiled before loading to completely disrupt non-covalent interactions, whereas samples to be analyzed by mobility shift assays were incubated at room temperature). Nevertheless, the formation of interchain disulfide bonds between adjacent monomers seems to be related with the decrease in DNA binding.

The native protein present in sunflower nuclei showed a similar behavior. Western blots of nuclear extracts incubated in the presence of reductants developed with anti-HAHR1 antibodies showed a distinct band of about 70 kDa, corresponding to the monomer (Fig. 5, *upper panel*). This band was not observed when extracts were incubated under oxidizing conditions, most likely because of the formation of larger species, which barely entered the gel. In fact, reactive bands in the region corresponding to the stacking gel were evident under these conditions (not shown). The response of HAHR1 differed from that of other proteins from the nuclear extract, as judged from Coomassie Blue-stained gels (Fig. 5, *lower panel*). Although a major group of proteins changed their mobility only in the presence of DTT, several minor bands remained unchanged under all conditions.

Hahb-10 Is Activated by Treatment with DTT—Analysis of conserved cysteines in other plant HD proteins revealed that in all proteins from the Hd-ZipII subclass described up to now there is a CPSCE motif adjacent to the leucine zipper (Fig. 1*B*). This motif contains two conserved cysteines and resembles the C*XX*CG motif present in glabra2 proteins. To determine

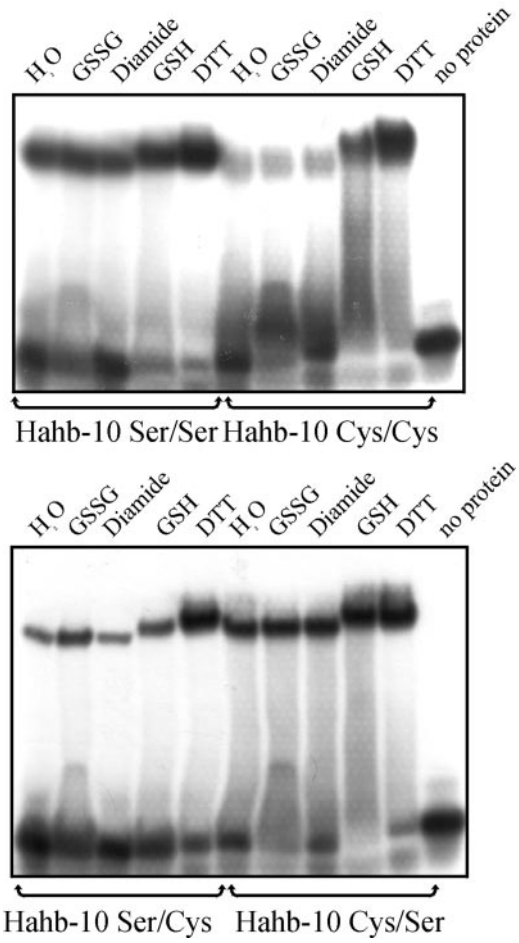

FIG. 6. **Effect of different redox agents on the DNA binding activity of Hahb-10 and its cysteine mutants within the CPSCE motif.** EMSA of wild-type Hahb-10 (*Cys*/*Cys*) and single and double Cys-to-Ser mutants (*Ser*/*Cys*, Cys-201 → Ser; *Cys*/*Ser*, Cys-204 → Ser; *Ser*/*Ser*, double mutant) previously incubated in the presence of redox agents as indicated in Fig. 3.

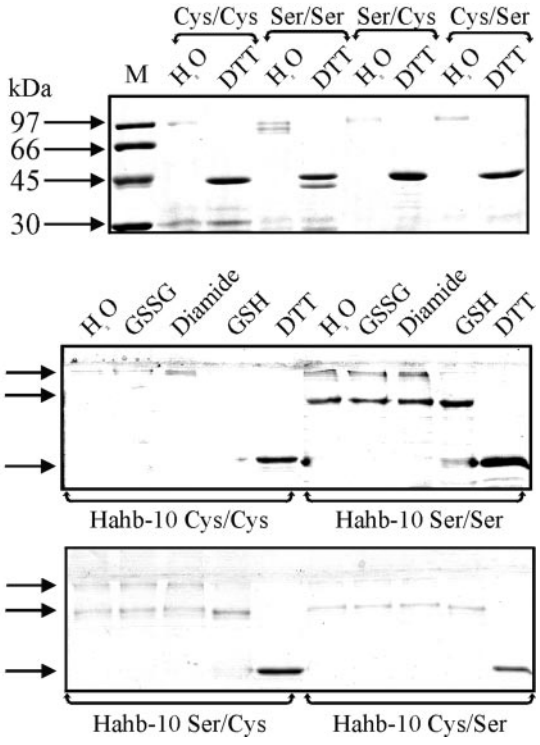

FIG. 7. **Non-reducing polyacrylamide gel of Hahb-10 and its cysteine mutants incubated in the presence of various redox agents.** The different proteins were incubated under the conditions described in Fig. 3 before the addition of sample buffer and electrophoretic separation. *Arrows* indicate the position of monomers, dimers, and higher order structures according to standard molecular weight markers shown in *lane M* of the *upper panel*. See the legend of Fig. 6 for the designation of the mutants.

whether Hd-ZipII proteins also undergo redox-dependent changes, we have expressed the sunflower Hd-ZipII protein Hahb-10 (36) in *E. coli* and performed similar studies as those described above for HAHR1. Just as HAHR1, Hahb-10 binds DNA poorly under oxidizing conditions (Fig. 6, *upper panel*). Treatment with DTT produced a significant increase in DNA binding, indicating that the reduction of cysteines is required for its activation. GSH also produced an increase in binding although lower than that produced by DTT. Measurement of the dissociation constants under oxidizing (K_{12} = 2.1 (±0.1) × 10^{-13} M^2) and reducing (K_{12} = 1.3(±0.1) × 10^{-14} M^2) conditions indicated that DTT produces an increase in binding affinity. Kinetic analysis showed that both the association and dissociation rates of the complex were changed (*i.e.* the association rate was increased, and the dissociation rate was decreased in the presence of DTT). Mutation of both cysteines to serine significantly reduced the oxidation-dependent decrease in DNA binding (Fig. 6, *upper panel*). However, the mutated protein still retained partial sensitivity to oxidizing agents, which is more evident when DNA binding is analyzed at lower protein concentrations (see below). This may be caused by the presence of additional cysteines within the leucine zipper of Hahb-10.

Role of Individual Cysteines in Hahb-10 Redox-mediated Activation—Single mutations of cysteines in the CPSCE motif of Hahb-10 produced proteins with intermediate DNA binding activity under oxidizing conditions (Fig. 6, *lower panel*). SC-Hahb-10 was less active that CS-Hahb-10 in the presence of oxidants and was significantly activated only in the presence of DTT. This indicates that the behavior of the single cysteine mutants within the C*XXCX* motif is very similar for HAHR1 and Hahb-10.

Non-reducing gels indicated the presence of covalently bound species under oxidizing conditions for all the proteins under study (Fig. 7). Native Hahb-10 formed species that migrated considerably slower than dimers, which in turn were predominant in cysteine mutants under oxidizing conditions. Full conversion into monomers was only attained in the presence of DTT, whereas GSH was only partially active (Fig. 7). This shows that there is a close correlation between the formation of intermolecular covalent bonds and the decrease in DNA binding. On comparing these parameters for the different proteins under oxidizing conditions, however, it becomes evident that at least part of the proteins that participate in covalent bond formation are active in DNA binding. This suggests that the formation of certain disulfide bonds influences binding activity in a different way than the formation of others. Accordingly, the role of cysteines present in the leucine zipper of Hahb-10 was also investigated.

The portion of Hahb-10 used for these studies has three additional cysteines within the leucine zipper (Fig. 1*B*). Two of them are present at *a* positions (that is, facing the dimer interface) of the second and third heptads (a_2 and a_3, respectively), whereas the other is at the *g* position of the second heptad (g_2), adjacent to a_3 and also near the interface according to known leucine zipper structures (43). Cysteines a_2 and a_3 are conserved in all Hd-ZipII proteins, whereas cysteine g_2 is present in most of them. The role of these cysteines was studied by the analysis of the properties of proteins with different combinations of mutated cysteines. The nomenclature used for the

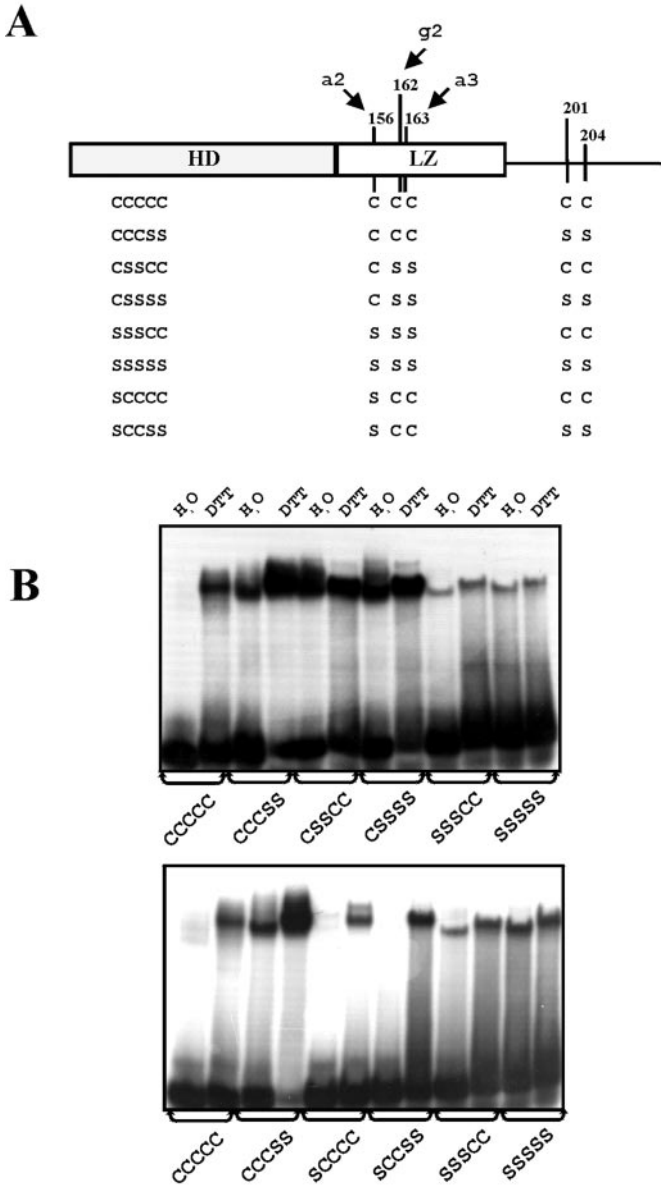

FIG. 8. **Role of cysteines at different positions in the redox-dependent activation of Hahb-10.** *A*, scheme showing the relative positions of the cysteines in Hahb-10 and the nomenclature of the different mutants analyzed in this study. The amino acid positions of each cysteine relative to the initial methionine and the position within the leucine zipper (a_2, g_2, or a_2) are shown. Regions corresponding to the HD and the leucine zipper (*LZ*) are boxed. *Below*, the name and amino acids present at each position are shown. *B*, effect of incubation with DTT on the DNA binding activity of Hahb-10 and its mutants. The different proteins were incubated in the presence or absence of 25 mM DTT as described before analyzing DNA binding by EMSA. See *A* for the nomenclature used for the different proteins.

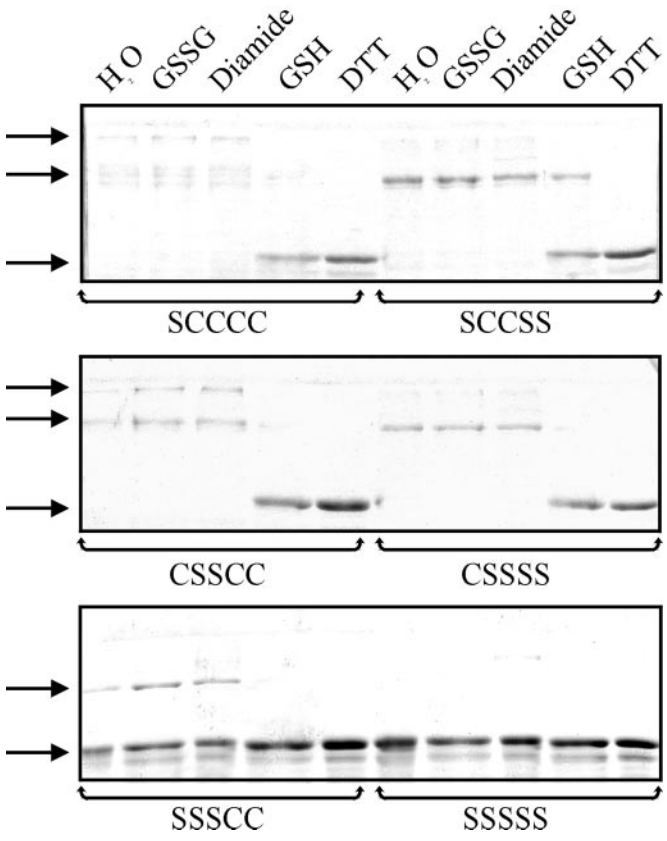

FIG. 9. **Formation of intermolecular disulfide bonds by Hahb-10 cysteine mutants under different redox conditions.** The different proteins were incubated under the conditions described in Fig. 3 before the addition of sample buffer and electrophoretic separation on a non-reducing polyacrylamide gel. *Arrows* indicate the position of monomers, dimers, and higher order structures according to standard molecular weight markers. See Fig. 8*A* for nomenclature of the proteins.

different proteins is presented in Fig. 8*A*. From the analysis of the DNA binding properties of mutants (Fig. 8*B*), the following observations were made. 1) Proteins in which cysteines g_2 and a_3 are mutated to serine are largely insensitive to redox conditions in terms of binding activity (Fig. 8*B*, *upper panel*); 2) proteins with cysteine at positions g_2 and a_3 are highly sensitive to oxidation (Fig. 8*B*, *lower panel*); 3) proteins in which cysteine a_2 is mutated to serine show a significant decrease in binding under all conditions. These results indicate that adjacent cysteines g_2 and a_3 are main determinants of the sensitivity of DNA binding to oxidation and that cysteines within the CPSCE motif also influence this behavior. Oxidation of cysteine a_2 does not influence DNA binding.

The formation of intermolecular disulfide bonds was analyzed by non-reducing polyacrylamide gel electrophoresis. As expected, all proteins, with the sole exception of the all-serine mutant, produced cross-linked species under oxidizing conditions (Fig. 9). SSSCC-Hahb-10 also showed a significant proportion of monomers (*lower panel*), which were not observed for the other proteins. Cysteines within the leucine zipper are then probably responsible for the formation of cross-linked species. CSSSS-Hahb-10 forms dimers under oxidizing conditions (Fig. 9, *middle panel*), suggesting that cysteines a_2 from two adjacent monomers form cross-linked active dimers. Conversely, covalent dimers formed by SCCSS-Hahb-10 (*upper panel*) seem to be inactive. This confirms that disulfide bond formation may lead to either active or inactive proteins, depending on the nature of the cysteines that are involved.

Reduced Thioredoxin Catalyzes the Activation of HAHR1 and Hahb-10—The fact that the reduction of disulfide bonds promotes the activation of HAHR1 and Hahb-10 raises the possibility that a similar reaction is used under physiological conditions to regulate the properties of these transcription factors. Redox changes in protein cysteines are usually catalyzed *in vivo* by thioredoxin, a small protein that is in turn reduced by NADPH in the presence of thioredoxin reductase (28, 44). We have then analyzed the thioredoxin-dependent activation of HAHR1 and Hahb-10 and their respective mutants using recombinant thioredoxin and thioredoxin reductase purified from *E. coli*. As shown in Fig. 10, this system was as efficient as DTT in the activation of HAHR1 and Hahb-10. This is consistent with a role of thioredoxin in catalyzing thiol/disulfide exchanges in these transcription factors *in vivo*.

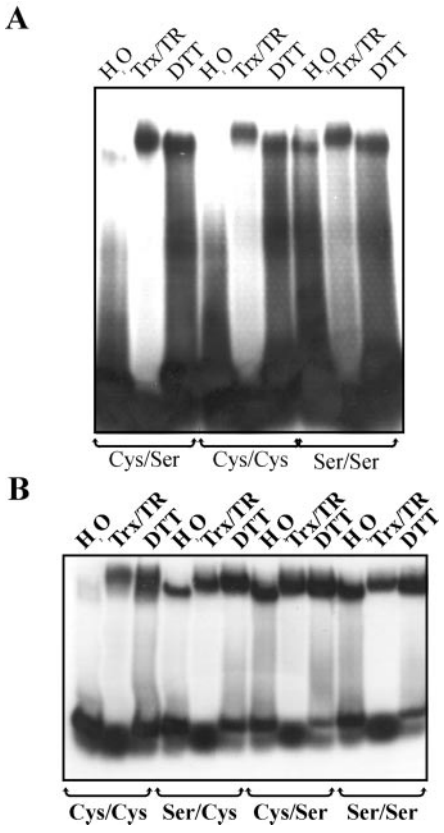

FIG. 10. **Thioredoxin can replace DTT in the redox activation of HAHR1 and Hahb-10.** *A*, EMSA of wild-type HAHR1 (*Cys*/*Cys*) and its single and double Cys-to-Ser mutants (*Cys*/*Ser*, Cys-188 → Ser; *Ser*/*Ser*, Cys-185/188 → Ser double mutant) previously incubated in the presence of 0.013 μg/μl each thioredoxin and thioredoxin reductase plus 0.55 mM NADPH (*Trx*/*TR*), 25 mM DTT, or in the absence of redox agents (H_2O) as indicated in Fig. 2. *B*, same as in *A* for wild type (Cys/Cys) and mutants of Hahb-10 within the CPSCE motif (*Ser*/*Cys*, Cys-201 → Ser; *Cys*/*Ser*, Cys-204 → Ser; *Ser*/*Ser*, double mutant).

DISCUSSION

Post-translational modifications are extensively used by cells to modulate the activity of proteins. Among these modifications, dithiol/disulfide exchanges are key components of regulatory systems that respond to changes in redox conditions (28, 44–46). In the present work, we show that two plant HD transcription factors undergo dithiol/disulfide exchanges that produce changes in their affinity for their DNA target sequences. One of these factors, HAHR1, belongs to the glabra2 family whose members are thought to participate in epidermal cell development both at the embryo and adult plant level (22–26). Because the cysteines involved in redox modulation (*i.e.* those present in the loop of the dimerization motif) are conserved in all members of the glabra2 family, we propose that all glabra2-like plant HD proteins undergo similar changes. This also applies to Hahb-10 and Hd-ZipII proteins, which possess a set of conserved cysteines in and outside the leucine zipper motif.

Although both proteins tested are activated in the presence of reductants, they show a different response to the cellular redox agent GSH than to DTT. The first of these compounds produces only a partial activation of HAHR1 and Hahb-10, probably because only part of the disulfide bonds are reduced. Full activation requires the action of more potent reductants. *In vivo* GSH itself participates in the reduction of proteins, sometimes with the aid of a protein called glutaredoxin (45). A different system, with higher reducing potential, is composed by thioredoxin, a protein that is in turn reduced by NADPH in a reaction catalyzed by NADPH-thioredoxin reductase (28, 44–46). Our results using purified proteins *in vitro* indicate that this system is able to promote full activation of HAHR1 and Hahb-10. We propose, then, that the intracellular levels of GSH and reduced thioredoxin operate in concert to influence the activation state of the proteins under study.

Regarding the physiological significance of our observations, it should be mentioned that redox agents are known to influence several aspects of development in plants. Root growth,

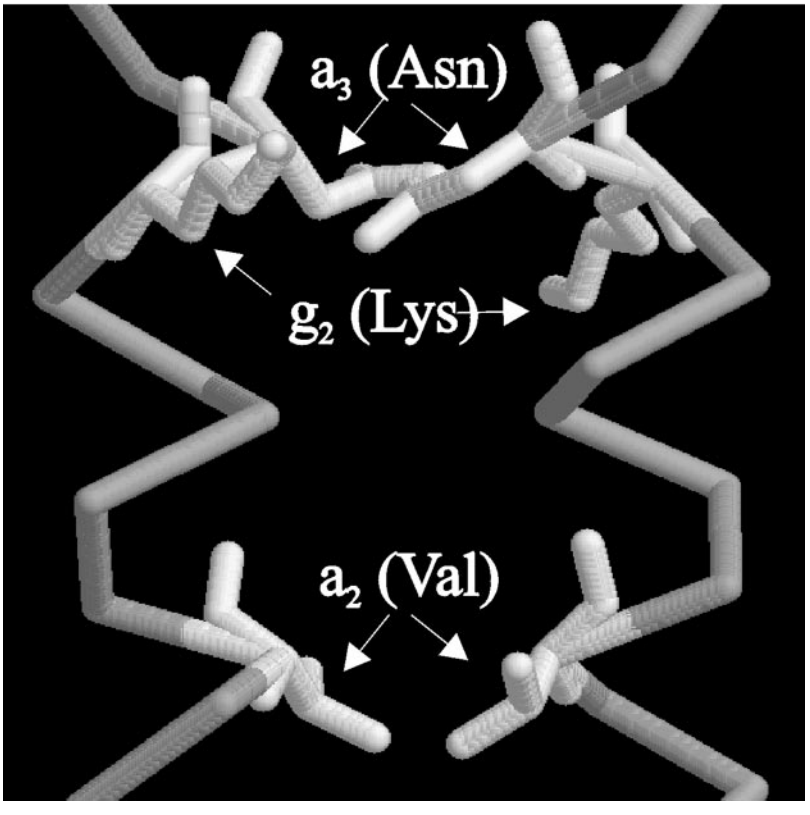

FIG. 11. **Proximity of amino acids at *a* and *g* positions in the GCN4 leucine zipper.** The picture, based on the crystal structure of the GCN4 leucine zipper (43) and generated using the program RasMol, shows the backbone of the two strands of the leucine zipper coiled coil together with the side chains of residues at positions a_2, a_3, and g_2. These positions are occupied by cysteines in the Hahb-10 leucine zipper.

root hair number, and root hair length respond to the inclusion of redox agents in the growth medium (47, 48), and mutants in the enzyme γ-glutamylcysteine synthetase, involved in the synthesis of GSH, show altered root development and reduced cell division rates (49). Root hair development establishes a link between GSH and glabra2 proteins. Mutations in *glabra2* produce an increase in root hair number, suggesting that the encoded protein is involved in repressing root hair formation (21, 23). If GSH acts through activation of glabra2, then the inclusion of this redox agent should promote a decrease in root hair number. Previous studies indicate, however, that GSH has the opposite effect (48). Although these results are difficult to reconcile, it should be mentioned that a recent report indicates that the overexpression of glabra2 produces plants similar in phenotype to glabra2 mutants (50). An increase in glabra2 activity over a certain threshold may then produce an increase in root hair number.

Considering Hd-ZipII proteins, current knowledge indicates that some of these proteins are involved in certain developmental responses of plants to illumination. The genes for two *Arabidopsis* Hd-ZipII proteins, Athb-2 and -4, are induced by far red-rich light (18), and changes in the expression levels of HAT4 or Athb-2 (which are the same protein) influence hypocotyl elongation and leaf expansion, mimicking certain effects of illumination (19, 51). Then, also in this case there is a link between environmental conditions and the modification of plant development. We propose that part of these modifications is brought about by environmentally induced changes in cellular redox agents that in turn modulate the activity of transcription factors of the type reported in this study.

Regarding the nature of the structural modifications that render these proteins inactive under oxidation conditions, it is noteworthy that the residues that participate in this process are located outside the DNA binding motif. For Hahb-10, it can be envisaged that intermolecular cross-linking of residues within the leucine zipper may affect the correct positioning of monomers for DNA binding. In the crystal structure of the GCN4 leucine zipper, the side chains of residues at position *a* lie in close proximity (Fig. 11). In addition, contacts between residue *g* from one chain and residue *a* from the following heptad of the other chain have been shown to occur (43). From our results, it is evident that a_2 residues form intermolecular cross-links that do not affect DNA binding by Hahb-10. Cross-links involving g_2 and a_3, on the contrary, seem to produce inactive dimers, probably because they cause a distortion in the leucine zipper structure. Our results also suggest that cysteines located within more disordered regions of HAHR1 and Hahb-10 also influence DNA binding. Knowledge of the changes brought about by redox transitions within these regions will require detailed structural studies of these proteins.

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