Functional analyses of the Dof domain, a zinc finger DNA-binding domain, in a pumpkin DNA-binding protein AOBP

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Received 6 April 1998; revised version received 28 May 1998

Abstract AOBP, a DNA-binding protein in pumpkin, contains a Dof domain that is composed of 52 amino acid residues and is highly conserved in several DNA-binding proteins of higher plants. The Dof domain has a significant resemblance to Cys2/Cys2 zinc finger DNA-binding domains of steroid hormone receptors and GATA1, but has a longer putative loop where an extra Cys residue is conserved. We show that the Dof domain in AOBP functions as a zinc finger DNA-binding domain and suggest that the Cys residue uniquely conserved in the putative loop might negatively regulate the binding to DNA.

Key words: Ascorbate oxidase; Zinc finger; DNA binding domain; DNA binding protein; Pumpkin

1. Introduction

Ascorbate oxidase (EC 1.10.3.3) catalyzes the oxidation of ascorbic acid to dehydroascorbic acid. Ascorbate oxidase is highly expressed in cucurbitaceous plants such as pumpkin and cucumber [1–3]. Its definitive biological function is unclear, although the enzyme is considered to be localized in the cell wall [4]. Ascorbate oxidase is highly expressed in young and growing tissues and markedly induced by auxin, suggesting that the enzyme may be involved in cell growth [5,6]. We have studied the transcriptional regulatory mechanism of the ascorbate oxidase gene.

The unique element AGTA repeat (AAAAAGTAAAAAGTAAAAAAGTAAAAAG), which has three AGTA sequences surrounded by A tracts, is found in the silencer region of the pumpkin ascorbate oxidase gene [7]. A cDNA for DNA-binding protein was isolated by screening a pumpkin cDNA expression library with the AGTA repeat as a probe [7]. The cDNA encodes a polypeptide of 380 amino acid residues. The protein, named AOBP, has been shown to be a novel DNA-binding protein [7]. AOBP has been suggested to function as a suppressor for ascorbate oxidase gene expression (Kisu et al., submitted).

The Dof domain, which is composed of 52 amino acid residues, was first identified in maize MNB1a [8]. AOBP also contains a Dof domain in the N-terminal region (aa 40–91) [9]. Furthermore, the Dof domain has been found in DNA-binding proteins such as *Arabidopsis* OBP1 [10], tobacco NtBBF1 [11] and maize PBF1 [12]. A recent survey of databases shows that the Dof domain occurs in at least nine proteins of *Arabidopsis*. Thus, the Dof domain is highly conserved in DNA-binding proteins of higher plants. In a previous report [9], we proposed that the N-terminal region of the Dof domain may function as a Cys2/Cys2 zinc finger with four conserved Cys residues (Fig. 1), since the sequence feature has a significant resemblance to zinc fingers in steroid hormone receptors and GATA1. Four Cys residues may be used for coordinating to zinc ion and therefore may be essential for DNA binding. Indeed, Yanagisawa [8] experimentally showed that the first two Cys residues of the domain were required for the DNA binding of MNB1a and that the metal chelators 1,10-phenanthroline and EDTA inhibited the DNA binding of MNB1a. Two aromatic residues found C-terminal to the Cys residues may be positioned on the opposite side of the DNA and fix the structure so that the putative recognition helix may interact with DNA, as suggested in zinc fingers of steroid hormone receptors and GATA1 (Fig. 1). On the other hand, the putative loop region of the Dof domain is much longer than those of steroid hormone receptors and GATA1 and has one more Cys residue near the center of the loop (Fig. 1), although the extra Cys residue has been suggested to take no part in DNA binding of MNB1a [8]. Furthermore, the Dof domain exists in plant proteins as a single unit although steroid hormone receptors and GATA1 have two zinc finger units. Thus, it is proposed that the Dof domain may be a unique zinc finger that is widely utilized for DNA binding in the plant kingdom [9,13]. In addition, some conserved basic amino acids were found in the C-terminal region of the Dof domain. There is a possibility that these basic amino acids play important roles in DNA binding.

In the present study, we show that the Dof domain in AOBP truly functions as a zinc finger DNA-binding domain and that the C-terminal region of the Dof domain is also involved in DNA binding. Furthermore, we suggest that the Cys residue uniquely conserved in the putative loop of the Dof domain may negatively control DNA binding.

2. Materials and methods

2.1. Site-directed mutagenesis

AOBP cDNA was digested by *Eco*RI and *Hin*cII, and subcloned into pBluescript SK$^-$ (Stratagene) to yield the plasmid pWT for the expression of the region from Met^1 to Val^{119}. The plasmid pWT was used for site-directed mutagenesis. The mutagenesis was performed with the QuikChange site-directed mutagenesis system (Stratagene), using mutagenic oligonucleotides. The plasmid pC42A, for substitution of Cys^{42} by Ala, was constructed using the mutagenic oligonucleotide AGATAAGATACTTCCAGCTCCCCGCTGCAATAGC. In the same way, the other plasmids were constructed using the following oligonucleotides: pC53A, for substitution of Cys^{53} by Ala, GGAAACCAAGTTTGCTTATTATAATAATTATAATGTCAATCAACC; pC70A, for substitution of Cys^{70} by Ala, CGCCATTTTTGCAAAGCCGCTCAAAGATATTGGACTGA; pY73A, for substitution of Tyr^{73} by Ala, CAAAGCCTGTCAAAGAGCTTGGACTGAAGGCG; pW74A, for substitution of Trp^{74} by Ala, GCCTGTCAAAGATATGCGACTGAAGGCGGTAC; pR81A, for substitution of Arg^{81} by Ala, GGCGGTACCATCGCGAATGTCCCTG-

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PII: S0014-5793(98)00670-X

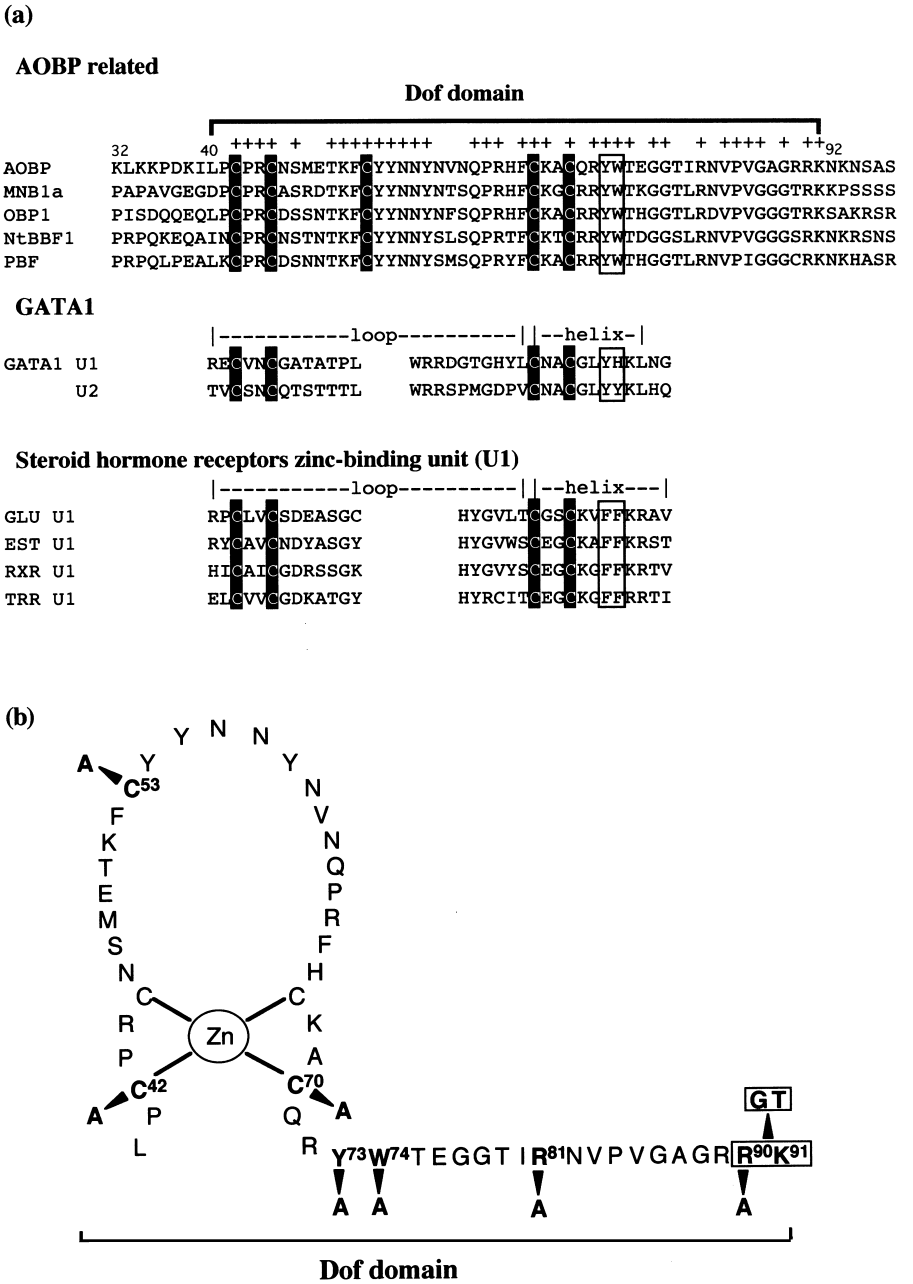

Fig. 1. Structure of the Dof domain. a: Comparison of the amino acid sequences of the Dof domain, the GATA1 DNA-binding domain and the first zinc-binding unit (U1) of the steroid hormone receptor DNA-binding domain. The Dof domains of pumpkin AOBP, maize MNB1a [8], *Arabidopsis* OBP1 [10], tobacco NtBBF1 [11] and maize PBF [12] were used for the comparison. Plus signs above the sequences indicate that the positions are occupied by the same amino acid residues in all the protein sequences. Two zinc-binding domains (referred to as U1 and U2) of GATA1 [20] were compared. DNA-binding domains of steroid hormone receptors also have two zinc-binding units, U1 and U2. The U1 units of the glucocorticoid receptor (GLU) [21,22], estrogen receptor (EST) [23], retinoid X receptor (RXR) [24] and thyroid receptor (TRR) [25] were compared. Four Cys residues conserved in all the groups and the Cys residue uniquely conserved in the Dof domain are inverted. The two positions occupied by aromatic residues in all the groups are boxed; Tyr and Trp in Dof/MOA domains, Tyr and His (or Tyr) in GATA1, and two Phe residues in the steroid hormone receptors. Amino acids in AOBP are numbered from the amino-terminal residue and indicated at the top of the alignment. b: Proposed structure of the Dof domain in AOBP and amino acid residues replaced in this study. Four Cys residues coordinate to zinc ion (Zn). The amino acid residues (C42, C53, C70, Y73, W74, R81 and R90) replaced by Ala (A) are shown in bold. Two amino acid residues (R90 and K91) simultaneously replaced by Gly (G) and Thr (T) are boxed. The superscript figures of amino acids indicate numbers from the amino-terminal residue.

TTG; pR90A, for substitution of Arg^{90} by Ala, GTTGGAGC-TGGCCGCGCAAAAAACAAGAACTC; and pR90G/K91T, for substitutions of Arg^{90} and Lys^{91} by Gly and Thr, GGAGCT-GGCCGCGAACAAACAAGAACTC). Mutations were confirmed by DNA sequence analyses using a DNA sequencer (373A DNA Sequencing System; Applied Biosystem).

2.2. Expression and purification of the recombinant proteins in transformed Escherichia coli

Plasmids pWT, pC42A, pC53A, pC70A, pY73A, pW74A, pR81A, pR90A and pR90G/K91T were digested by *Eco*RI and *Hin*cII and subcloned into the pGEX-4T-1 (Pharmacia) expression vector between the *Eco*RI and *Sma*I sites. *Escherichia coli* strain BL21(DE3)

Fig. 2. DNA binding assays of mutant proteins with substitution of Cys residues of the Dof domain in AOBP. The deleted AOBP protein with the N-terminal region from Met^{1} to Val^{119} was used as a control protein (WT) for the DNA-binding assay. The mutant proteins C42A, C53A and C70A, in which the Cys^{42}, Cys^{53} and Cys^{70} residues of the Dof domain in AOBP were substituted by Ala residues, were expressed from recombinant *E. coli* and used for the binding assays to the AGTA repeat (AAAAAGTAAAAAG-TAAAAAAGTAAAAAG). a: Immunological blot analysis. 3 μg of WT (lane 1), C42A (lane 2), C53A (lane 3) and C70A (lane 4) proteins were subjected to SDS-PAGE and subsequent immunoblotting with anti-peptide antibody against pumpkin AOBP. b: Southwestern blot analysis. 30 μg of WT (lane 4), C42A (lane 1), C53A (lane 2) and C70A (lane 3) proteins were subjected to SDS-PAGE and subsequent Southwestern blotting with a ^{32}P-labeled AGTA repeat. c: Gel retardation analysis. 0 μg (lane 1), 1 μg (lanes 2, 4, 6 and 8) or 3 μg (lanes 3, 5, 7 and 9) of WT (lanes 2 and 3), C42A (lanes 4 and 5), C53A (lanes 6 and 7) and C70A (lanes 8 and 9) proteins were incubated with a ^{32}P-labeled AGTA repeat and analyzed by gel retardation.

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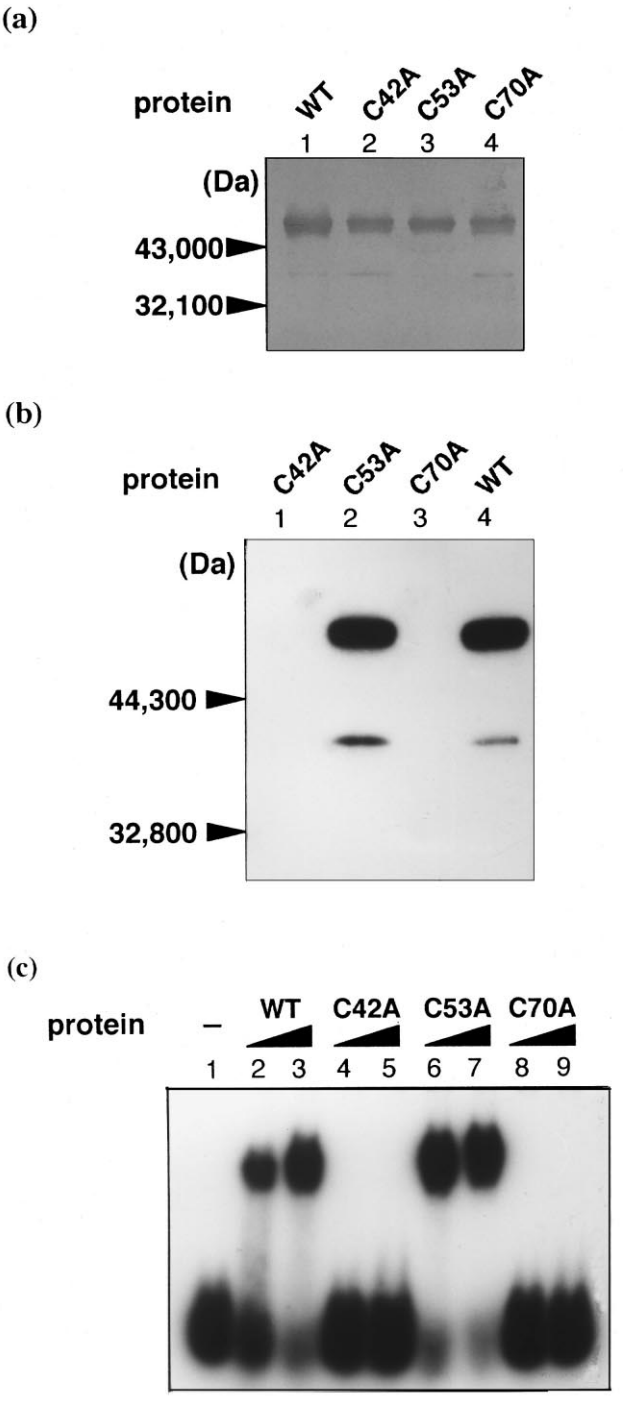

(Pharmacia) cells were transformed with the recombinant plasmids. The recombinant proteins were induced in *E. coli* by 0.1 mM IPTG for 4 h. The proteins were extracted by sonicating the cells in the PBS buffer (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4) on ice, and by incubating them in the PBS buffer containing 1.0% (v/v) Triton X-100 on ice for 30 min. The cell suspension was centrifuged at $12\,000\times g$ for 10 min at 4°C. From the supernatant, recombinant protein was purified by glutathione Sepharose 4B column (Pharmacia) chromatography. The amount of protein was measured with a protein assay kit (Bio-Rad) [14].

2.3. Immunological blot analysis

SDS-PAGE on a 12% (w/v) polyacrylamide gel was carried out by the method of Laemmli [15]. The anti-peptide antibody against AOBP was prepared according to the method of Van Regenmortel et al. [16]. The peptide (CTEGGTIRNVPVGAGRRK) was synthesized by the Fmoc (9-fluorenylmethyloxycarbonyl) method. The anti-peptide antibody was produced from a rabbit by injecting it with the synthetic peptide conjugated to Imject maleimide activated keyhole limpet hemocyanin (Pierce). Immunological blot analysis was performed as described by Towbin et al. [17].

2.4. Southwestern blot analysis

The gene products (30 μg) were separated by SDS-PAGE on a 12% (w/v) polyacrylamide gel, and blotted on nitrocellulose filter (BA85, Schleicher&Schuell). A double-stranded oligonucleotide (AAAAAG-TAAAAAGTAAAAAAGTAAAAAG), named AGTA repeat, was synthesized. The AGTA repeat was labeled with [γ-^{32}P]ATP (3000 Ci/mmol, Amersham) using T4 polynucleotide kinase and was used as a probe. The filter was incubated with the ^{32}P-labeled probe (AGTA repeat), according to the method of Vinson et al. [18].

2.5. Gel retardation analysis

Gel retardation analysis was performed according to the method of Ausubel et al. [19]. All DNA-binding reactions were carried out in 17 mM HEPES-KOH (pH 7.9), 60 mM KCl, 7.5 mM $MgCl_2$, 0.12 mM EDTA, 12% (v/v) glycerol, 1.2 mM DTT and 10 μg/ml poly-dIdC. A double-stranded oligonucleotide (AAAAAGTAAAAAG-TAAAAAAGTAAAAAG), named AGTA repeat, was synthesized. The AGTA repeat was labeled with [γ-^{32}P]ATP (3000 Ci/mmol, Amersham) using T4 polynucleotide kinase and was used as a probe. Gel retardation analysis was performed with the ^{32}P-labeled probe (AGTA repeat) and 1 μg or 3 μg of translation product. After incubation for 30 min at 25°C, the mixtures were subjected to electrophoresis in 4% (w/v) polyacrylamide gel.

3. Results

3.1. Involvement of Cys residues of the Dof domain in DNA binding of AOBP

AOBP is composed of 380 amino acid residues. The DNA-binding analysis of a deleted protein that lacks the C-terminal 260 amino acids of AOBP proved that the DNA-binding domain of AOBP was located in the N-terminal region from Met^{1} to Val^{119}, containing the Dof domain (data not shown). The binding affinity of the deleted protein to the AGTA repeat was almost identical to that of the complete protein. In the present study, this deleted protein was used as a control protein (WT) for the DNA-binding assay. The Dof domain has been suggested to function as a zinc finger DNA-binding domain [8,9]. Indeed, AOBP needs zinc ion for DNA binding. Thus, we investigated whether the Dof domain in AOBP functions as a zinc finger DNA-binding domain. Namely, the im-

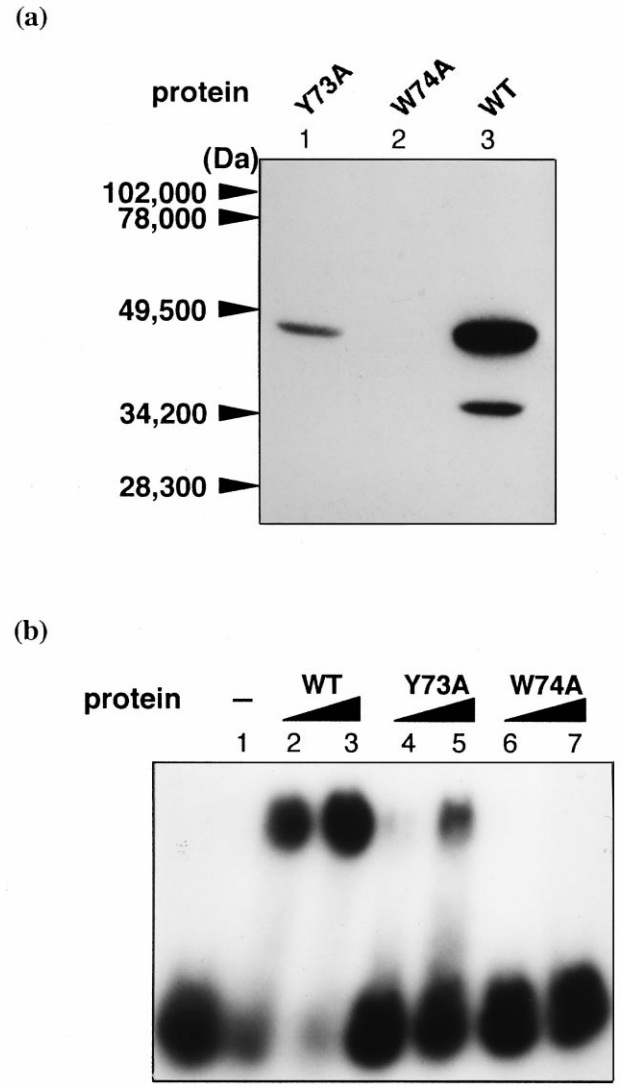

Fig. 3. DNA-binding assays of mutant proteins with substitution of two aromatic residues of the Dof domain in AOBP. The deleted AOBP protein with the N-terminal region from Met^{1} to Val^{119} was used as a control protein (WT) for the DNA-binding assay. The mutant proteins named Y73A and W74A, in which the Tyr^{73} and Trp^{74} residues of the Dof domain in AOBP were substituted by Ala residues, were expressed from recombinant *E. coli* and used for the binding assays to the AGTA repeat (AAAAAGTAAAAAG-TAAAAAAGTAAAAAG). a: Southwestern blot analysis. 30 μg of WT (lane 3), Y73A (lane 1) and W74A (lane 2) proteins were subjected to SDS-PAGE and analyzed by Southwestern blotting with a ^{32}P-labeled AGTA repeat. b: Gel retardation analysis. 0 μg (lane 1), 1 μg (lanes 2, 4 and 6) or 3 μg (lanes 3, 5 and 7) of WT (lanes 2 and 3), Y73A (lanes 4 and 5) and W74A (lanes 6 and 7) proteins were incubated with a ^{32}P-labeled AGTA repeat and analyzed by gel retardation.

portance of each amino acid was assessed by amino acid substitution with site-directed mutagenesis. First, the Cys^{42} and Cys^{70} residues of the Dof domain in AOBP, which may coordinate to zinc ion, were substituted by Ala residues. These mutant proteins were named C42A and C70A, respectively. Also, the Cys^{53} residue, uniquely conserved only in the putative loop of the Dof domain, was replaced with an Ala residue (named C53A). These mutant proteins (C42A, C70A and C53A) and wild-type (WT) protein were produced in *E. coli*, purified by glutathione Sepharose 4B column (Pharmacia) chromatography and used for the binding analyses to the AGTA repeat (AAAAAGTAAAAAGTAAAAAAGTAAA-AAG) which can bind to AOBP. When 3 μg of the wild-type protein and these mutant proteins were subjected to SDS-PAGE and subsequent immunoblotting with anti-peptide antibody against pumpkin AOBP, the signals of these immunoreactive bands were almost the same (Fig. 2a). Thus, the immunological blot analysis suggested that the wild-type protein and these mutant proteins were stably produced in *E. coli* at almost equal levels and purified with almost the same recovery. Southwestern blot and gel retardation analyses showed that C42A and C70A proteins had no ability to bind to the AGTA repeat (Fig. 2b,c). Thus, it is clear that both Cys^{42} and Cys^{70} residues are essential to DNA binding and that the Dof domain of AOBP functions as a zinc finger which coordinates to zinc ion through these Cys residues. Interestingly, the binding affinity of C53A to the AGTA repeat was shown to be much higher than that of WT protein by gel retardation analysis. Namely, the shift band in 1 μg of C53A mutant protein (lane 6) was more abundant than that in 3 μg of WT protein (lane 3) (Fig. 2c). Thus, there is a possibility that the Cys^{53} residue conserved in the putative loop may negatively regulate DNA binding.

3.2. Involvement of two aromatic residues of the Dof domain in DNA binding of AOBP

In the putative recognition helix of the Dof domain, two aromatic residues, Tyr and Trp, are found at the equivalent positions of two Phe residues in the zinc finger of steroid hormone receptors, and Tyr and His in GATA1 (Fig. 1). Thus, it is possible that Tyr^{73} and Trp^{74} in AOBP stabilize the structure of the domain as suggested in these reference proteins [9]. The involvement of Tyr^{73} and Trp^{74} in DNA binding was investigated by Southwestern blot and gel retardation analyses. The Tyr^{73} and Trp^{74} residues of the Dof domain in AOBP were substituted by Ala residues. These mutant proteins (Y73A and W74A) and WT protein were produced in *E. coli* and were used for binding analyses to the AGTA repeat. The immunological blot analysis showed that the WT protein and these mutant proteins were produced at almost equal levels in *E. coli* and were all stable (data not shown). The substitution of Trp^{74} with Ala (W74A) completely inhibited the binding to the AGTA repeat (Fig. 3), suggesting that Trp^{74} is essential to DNA binding. In the substitution of Tyr^{73} with Ala (Y73A), on the other hand, the binding was greatly reduced but not completely inhibited (Fig. 3). These results suggest that these two aromatic residues, particularly Trp^{74}, may contribute to the structural stabilization of the Dof domain for DNA binding.

3.3. Involvement of basic amino acid residues in the C-terminal region of the Dof domain in DNA binding of AOBP

The Dof domain consists of a putative zinc finger and the conserved C-terminal region. It is interesting whether the conserved C-terminal region in the Dof domain is important for DNA binding. The abundance of basic amino acids conserved in the C-terminal region leads one to suppose that these basic amino acids are involved in the direct interaction with DNA. Arg^{81} and Arg^{90} were substituted by Ala residues. These mutant proteins (R81A and R90A) were produced in *E. coli* at almost the same levels as WT protein (data not shown). DNA-binding activities of these mutant proteins (R81A and

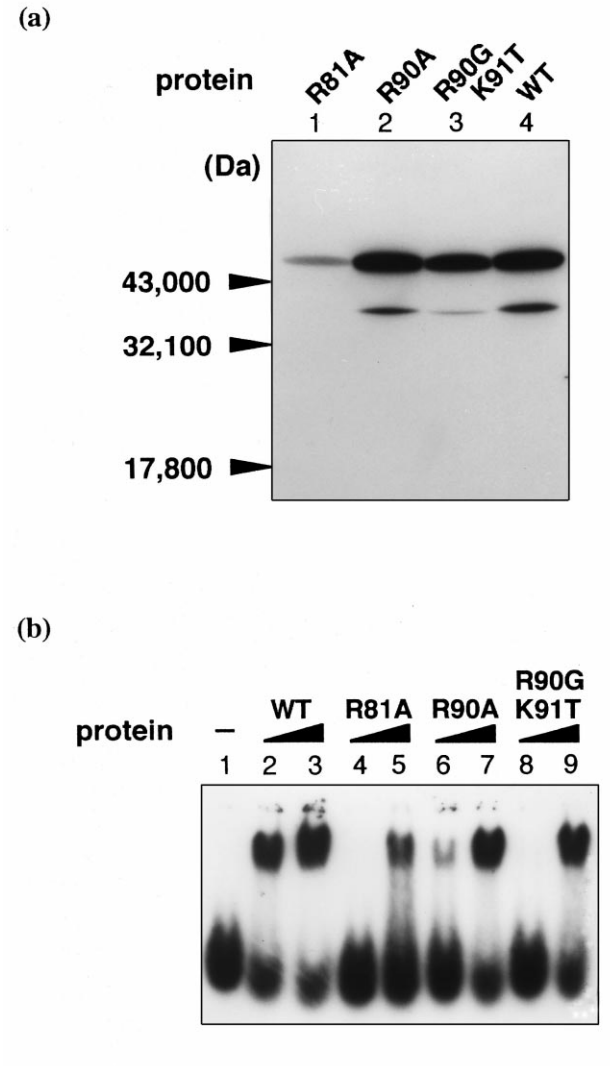

Fig. 4. DNA-binding assays of mutant proteins with substitution of basic amino acid residues in the C-terminal region of the Dof domain in AOBP. The deleted AOBP protein with the N-terminal region from Met^{1} to Val^{119} was used as a control protein (WT) for the DNA-binding assay. The mutant proteins named R81A and R90A, in which the Arg^{81} and Arg^{90} residues of the Dof domain in AOBP were substituted by Ala residues, and R90G/K91T, in which the two amino acid residues Arg^{90} and Lys^{91} of the Dof domain in AOBP were simultaneously substituted by Gly and Thr residues, were expressed from recombinant *E. coli* and used for the binding assays to the AGTA repeat (AAAAAGTAAAAAGTAAAAAAG-TAAAAAG). a: Southwestern blot analysis. 30 μg of WT (lane 4), R81A (lane 1), R90A (lane 2) and R90G/K91T (lane 3) proteins were subjected to SDS-PAGE and analyzed by Southwestern blotting with a ^{32}P-labeled AGTA repeat. b: Gel retardation analysis. 0 μg (lane 1), 1 μg (lanes 2, 4, 6 and 8) or 3 μg (lanes 3, 5, 7 and 9) of WT (lanes 2 and 3), R81A (lanes 4 and 5), R90A (lanes 6 and 7) and R90G/K91T (lanes 8 and 9) proteins were incubated with a ^{32}P-labeled AGTA repeat and analyzed by gel retardation.

R90A) significantly decreased (Fig. 4). In particular, the substitution of Arg^{81} almost lost DNA-binding activity, suggesting that Arg^{81} plays an important role in DNA binding. Furthermore, the DNA-binding activity was dramatically reduced when both Arg^{90} and Lys^{91} were simultaneously replaced with Gly and Thr (R90G/K91T; Fig. 4), suggesting that Lys^{91} may also contribute to DNA binding. These data indicate that the conserved C-terminal region in the Dof domain containing some basic amino acids is closely related to DNA binding of the Dof domain.

4. Discussion

The Cys2/Cys2-type zinc fingers found in GATA1 and steroid hormone receptors are composed of a loop and a recognition α-helix (Fig. 1). These fingers are primarily characterized by two Cys residues in a loop region and two Cys residues in a recognition α-helix which coordinate to a zinc ion. Furthermore, two aromatic residues found C-terminal to the Cys residues stabilize the recognition α-helix [9]. The involvement of these amino acids in DNA binding is evident from NMR and X-ray crystallographic studies [20–25]. GATA1, which is of animal origin, has two zinc finger units (U1 and U2), and it is believed that two units do not fold into a single domain but function independently [20]. Steroid hormone receptors also have two zinc finger units. However, the N-terminal unit (U1) plays a more important role in discriminating among DNA sequences and binding to the DNA major groove through its α-helix, while the C-terminal unit (U2) binds the recognition helix of U1 on the opposite side of the DNA [21–25]. On the other hand, the Dof domain exists as a single unit in plant proteins and has a longer putative loop that contains an extra conserved Cys residue (Fig. 1). Thus, the Dof domain is different from the other two types. Yanagisawa [8] showed that the Dof domain in MNB1a was involved in the interaction with DNA and that the DNA-binding activity required Zn^{2+}. Furthermore, he demonstrated by Southwestern blot analysis that two Cys residues (Cys^{49} and Cys^{52} of MNB1a) in the putative loop of the Dof domain in MNB1a were essential to DNA binding while an extra Cys residue (Cys^{60} of MNB1a) did not contribute to DNA binding. In the present study, by both Southwestern blot and gel retardation analyses of the Dof domain of AOBP, we proved that not only the Cys residues in the putative loop region but also the Cys residues in the putative α-helical region are required for DNA binding. Furthermore, we attempted to clarify the function of the Cys residue uniquely conserved only in the putative loop region of the Dof domain. Interestingly, DNA-binding activity was significantly enhanced when this Cys residue (Cys^{53}) was replaced with an Ala residue. Thus, there is a possibility that this Cys residue weakens the binding of the Dof domain to DNA. However, further detailed quantitative analyses and the replacement of Cys^{53} by amino acids other than Ala will be required to verify the possibility. Two hypotheses may be raised for the mechanism that the Cys residue weakens the binding to DNA. One hypothesis is that the longer loop with Cys^{53} results in a unique conformation of the Dof domain which interferes with DNA binding. The other is that Cys^{53} competes with four Cys residues which must coordinate to a zinc ion to inhibit zinc binding of the finger. We believe that AOBP functions as a suppressor that negatively regulates the expression of the ascorbate oxidase gene (Kisu et al., submitted). If so, it is possible that Cys^{53} in the Dof domain of AOBP weakens the binding to silencer to control the suppression of ascorbate oxidase gene expression.

In the putative α-helical region of the Dof domain, two aromatic residues, Tyr^{73} and Trp^{74}, are found C-terminal to Cys residues at the identical positions of those of steroid hor-

mone receptors and GATA1. As proposed in the reference proteins, these aromatic residues may be positioned on the opposite side of the DNA, and thus the geometry of the recognition helices relative to the DNA is fixed so that these bulky residues do not clash with the DNA [9]. Indeed, our results demonstrated that these aromatic residues of the Dof domain play an important role in DNA binding. In particular, Trp^{74} was suggested to be essential to DNA binding.

The Dof domain consists of a zinc finger region and the conserved C-terminal region. No attention has been given to clarifying the role of this conserved C-terminal region. In the present study, we showed that the basic amino acids (Arg^{81}, Arg^{90} and Lys^{91}) conserved in this region were also important for DNA binding of the Dof domain. Considering that a basic amino acid with a positive charge can electrically bind to the DNA with a negative charge, these conserved basic amino acids may stabilize and maintain the conformation of the zinc finger by direct interaction with the DNA.

Finally, it was concluded that the Dof domain of AOBP functions as a novel DNA-binding domain which includes a single zinc finger. Further work is required to clarify whether the Cys residue uniquely conserved in the putative loop of the Dof domain negatively regulates DNA binding. In addition, it is important to analyze the function of the amino acids in the putative recognition helix of the Dof domain and to understand the biological significance of the Dof domain in higher plants.

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