Characterization and Expression of a New Class of Zinc Finger Protein that Binds to Silencer Region of Ascorbate Oxidase Gene

Yasutomo Kisu 1,3, Tomoyuki Ono 1, Naoko Shimofurutani 1, Masashi Suzuki 2 and Muneharu Esaka 1,4

1 Faculty of Applied Biological Science, Hiroshima University, Kagamiyama 1-4-4, Higashi-Hiroshima, 739 Japan
2 AIST-NIBHT Structural Biology Centre, Higashi-1-1, Tsukuba, 305 Japan

A unique A/T-rich sequence (5'-AAAAAGTAAAAAGTAAAAAG-3'), referred to as the AGTA repeat, was isolated from pumpkin by the southwestern method. The AOBP protein has a new class of zinc/DNA-binding domain named Dof/MA domain that is highly conserved in many plant proteins and is significantly related to those of steroid hormone receptors and GATA1. Gel retardation analysis indicated that AOBP bound to the AGTA repeat through the Dof/MA domain. Metal chelators, 1,10-phenanthroline and EDTA, specifically inhibited the DNA binding of AOBP, indicating that metal coordination plays an important role in DNA binding of AOBP. Thus, the Dof/MA domain acts as a zinc/DNA-binding domain in AOBP. Gel retardation analysis with mutated oligonucleotides suggested that the Dof/MA domain recognized the AGTA core sequence. AOBP mRNA was expressed in mature tissues of pumpkin, but was expressed only in small amounts or was not expressed in growing tissues. Furthermore, the expression was auxin-independent. The expression pattern of AOBP and that of ascorbate oxidase did not show a positive correlation.

Key words: Ascorbate oxidase (EC 1.10.3.3) — DNA-binding protein — Dof/MA domain — Pumpkin (Cucurbita sp.) — Zinc finger.

In eucaryotes, many genes are transcriptionally regulated during cell growth, differentiation and development, and by environmental stimuli. Transcriptional regulation ultimately depends on transcription factors that bind to regulatory cis-elements in the promoter of the corresponding genes (Mitchell and Tjian 1989). In plants, many DNA sequence-specific transcription factors involved in specific gene expression have been identified (Meshi and Iwabuchi 1995). These transcription factors often form families of structurally related proteins with similar DNA-binding specificities. These factors often have conserved DNA-binding domains, including zinc fingers (Takatsuji et al. 1992), basic-region leucine-zipper (bZIP) (Schmidt et al. 1987), basic-region helix-loop-helix (bHLH) (Ludwig et al. 1989), homeodomain (Vollbrecht et al. 1991), MYB (Paz-Ares et al. 1987), MADS box (Yanofsky et al. 1990), HMG-box (Shinozaki and Shinozaki 1992) and AP2/EREBP (Jofuku et al. 1994, Ohme-Takagi and Shinshi 1995). Some motifs are also found in yeast or animal transcription factors while others are unique to plant transcription factors. In spite of many studies, however, the detailed mechanism for gene regulation through transcription factors remains unclear.

Ascorbate oxidase (EC 1.10.3.3) catalyzes the oxidation of ascorbic acid to dehydroascorbic acid and is used widely for clinical and food analyses. The enzyme belongs to a family of blue multicopper oxidases and occurs in higher plants such as pumpkin and cucumber (Esaka et al. 1988, Lee and Dawson 1973, Nakamura et al. 1968). The structure of ascorbate oxidase is of interest for a multicopper enzyme containing three spectroscopically distinct copper centers (Mayerschmidt et al. 1989). The cDNAs for ascorbate oxidase have been isolated from cucumber (Ohkawa et al. 1989), pumpkin (Esaka et al. 1990) and tobacco (Kato and Esaka 1996). Genomic DNAs have also been isolated from cucumber (Ohkawa et al. 1994) and pumpkin (Kisu et al. 1997). The in vivo function of ascorbate oxidase remains unclear although it may be localized in cell walls (Mertz 1961). Weis (1975) has suggested that the enzyme functions as part of the redox system. Lin and Varner (1991) and Esaka et al. (1992) have suggested that the enzyme plays an important role in reorganization of cell walls during cell growth or division. We have shown that the enzyme is markedly induced by auxin (Esaka et al. 1992). Interestingly, a gene family which shows sequence similarity to ascorbate oxidase has been reported to be specifically expressed in developing pollen (Albani et al. 1992). Recently, ascorbate oxidase has been reported to be specifically expressed in the quiescent center of maize root and involved in organization of root meristems through auxin-dependent expression (Kerk and Feldman 1995). Further-
more, ascorbate oxidase has been suggested to be induced during Rhizobium-induced nodule development in the legume Medicago (Gamas et al. 1996). Thus, ascorbate oxidase is most likely involved in cell growth or division of higher plants.

We have identified an auxin-responsive cis-region of the pumpkin ascorbate oxidase gene using a transient assay method (Kisu et al. 1997). Interestingly, a silencer region is most likely involved in cell growth or division of legume Medicago (Gamas et al. 1996). Thus, ascorbate oxidase has been suggested to be induced during the Dof/MOA domain. The Dof/MOA domain is found in DNA-binding domain, composed of 52 amino acid residues, was named Dof domain by Yanagisawa (1995) and MOA domain by Zhang et al. (1995). Thus, we refer to it as the Dof/MOA domain. The Dof/MOA domain is found in DNA binding proteins such as maize MNBlA (Yanagisawa 1995), Arabidopsis OBP1 (Zhang et al. 1995), tobacco NtBBFl (De Paolis et al. 1996) and maize PBF (Vicente-Carbajosa et al. 1997) and in proteins encoded by many mammalian cDNA clones (EST clones) of Arabidopsis and maize.

In this paper, we report the primary structure, DNA-binding properties and gene expression of pumpkin Dof/MOA domain protein AOBP.

**Materials and Methods**

Preparation of pumpkin tissues—Seeds of pumpkin (Cucurbita sp.; Ebisu nankin) were soaked in water for 2 h, and germinated on a piece of moist paper at 28°C for 4 d. The seedlings were then grown on moist vermiculite at 28°C under illumination. After being harvested at appropriate stages, they were frozen in liquid nitrogen and stored at −80°C. For isolating DNA or RNA they were ground into powder in liquid nitrogen using a mortar and pestle.

Pumpkin fruits were cut into disc-shaped pieces and were incubated on a 0.8% (w/v) agarose plate containing Murashige and Skoog’s basal medium (Murashige and Skoog 1962) and 3% (w/v) sucrose at 28°C for appropriate numbers of days. When indicated, 1.0 mg liter⁻¹ 2,4-D (2,4-dichlorophenoxyacetic acid) was added to the plate.

Preparation of mRNA—Total RNA was isolated from powdered fruit discs and various tissues by the method of Ausubel et al. (1997). The poly(A)+ RNA was purified from total RNA using Oligo(dT)₃₀ (Takara Shuzo).

**Northern blot analysis**—Poly(A)+ RNA was loaded on a 1.5% (w/v) agarose gel containing 15% (v/v) formaldehyde, 20 mM MOPS (pH 7.0), 8 mM sodium acetate and 1 mM EDTA for electrophoresis. After electrophoresis, the RNA was transferred to a membrane (Hybond-N, Amersham) and hybridized with a 32P-labeled AOBP cDNA probe (the 1.2 kb PvuII/HindIII fragment) or an ascorbate oxidase cDNA probe (the 1.8 kb EcoRI fragment) (Esaka et al. 1990) according to the method of Maniatis et al. (1982). A 32P-labeled probe was prepared by random hexamer priming using [α-32P]dCTP (3,000 Ci mmol⁻¹, Amersham) and a kit (Random Primer DNA Labeling Kit, Takara Shuzo). The filters were exposed on Kodak XAR-5 X-ray film.

**Southwestern blot analysis of AOBP-DNA interaction**—Double-stranded oligonucleotide probes A and 1–5, were synthesized, (Fig. 1, 3). Probe A was tandemly ligated and labeled with [α-32P]dCTP (3,000 Ci mmol⁻¹, Amersham) using a Random Primer DNA Labeling Kit (Takara Shuzo), while probes 1–5 were labeled with [α-32P]ATP (3,000 Ci mmol⁻¹, Amersham) using T4 polynucleotide kinase.

The recombinant genes were expressed in an E. coli-phage system using an inducer, 10 mM isopropyl-β-D-thiogalacto-

pyranoside (IPTG). The gene products were blotted on nitrocellulose filters (BA85, Schleicher & Schuell), denatured in the 6 M guanidine chloride solution, and renatured by decreasing the guanidine chloride concentration stepwise. The filters were incubated with a 32P-labeled DNA probe according to the method of Vinson et al. (1988).

**Cloning of AOBP cDNA**—A cDNA expression library was constructed using poly(A)+ RNA which was prepared from fruit discs incubated on Murashige and Skoog’s basal medium containing 0.8% (w/v) agarose, 3% (w/v) sucrose and 1.0 mg liter⁻¹ 2,4-D, at 28°C for 1 and 2 d, by a cloning kit (Agt11 DNA Synthesis System Plus, Amersham). The library was screened by the southwestern method (Vinson et al. 1988) using 32P-labeled probe A. A clone which contained the AOBP cDNA was identified. The cDNA was excised by EcoRI, and subcloned into a vector (pBluescript SK+ vector, Stratagene).

A series of DNA fragments was made from the vector by the method of Yanisch-Perron et al. (1985) and sequenced by the dideoxy nucleotide chain-termination method (Sanger et al. 1977) using a modified T7 DNA polymerase (Sequenase II, U.S. Biochemical).

**Expression of recombinant AOBP in E. coli**—AOBP cDNA was prepared from the recombinant Agt11 phage by digesting the DNA with EcoRI, with HindIII, or with EcoRI and KpnI, and subcloned into plasmid vector, pGEX-4T-1 (Pharmacia). The gene product of the recombinant plasmid contained the cDNA. The transformed E. coli cells (BL21(DE3) strain) were transformed with the recombinant plasmid that contained the cDNA. The transformed E. coli cells were induced by 0.1 mM IPTG for 3 h, and then harvested. Crude proteins were extracted by sonicating the cell in ice in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ (pH 7.3)) containing 1% (v/v) Triton X-100. The cell suspension was centrifuged at 12,000 × g for 15 min at 4°C, and the supernatant was used as the crude protein extract.

To purify the GST fusion protein, the supernatant was applied to glutathione Sepharose 4B column, washed with PBS buffer, and eluted with 50 mM Tris-HCl (pH 8.0) containing 10 mM glutathione. The amount of protein was determined by the method of Bradford (1976).

The crude protein extracts were loaded on a 12% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS, electrophoresed, and transferred to a nitrocellulose filter (BA85, Schleicher & Schuell). The proteins on the filter were analyzed using 32P-labeled probe A or probe B by the southern blot method as described above.

**Gel retardation assays**—Gel retardation assays were performed according to Ausubel et al. (1997). The double-stranded oligonucleotide probes 1–8 and 35S AAGG were synthesized (see Fig. 7, 8). The protein-DNA-binding mixture contained, in a total volume of 10 μl, 1 pmol probe DNA, 1 μg poly-dIdC and 3.3 μg of purified protein in binding buffer (17 mM Hepes-KOH (pH 7.5), 100 mM KCl, 0.1 mM EDTA and 10% (v/v) glycerol).
cDNA cloning of a DNA-binding protein by the southwestern method—A silencer region has been found upstream of the pumpkin ascorbate oxidase gene (Kisu et al. 1997). The silencer region has a unique sequence, 5'-AAAAAGTAAAAAGTAAAAAAGTAAAAAGTAAAAAAG-3' (-310 to -283), here referred to as the AGTA repeat.

Oligonucleotide probe A, containing the AGTA repeat, was designed (Fig. 1a). A cDNA library was constructed from sliced fruit tissues of pumpkin, which was pre-incubated on culture medium, and the library was screened by the southwestern method with probe A.

A single positive clone was obtained from over 360,000 examined clones, which expressed a protein that bound to probe A (Fig. 1b). The protein is named AOBP (DDBJ accession number; D45066).

The positive cDNA clone had an insert of 1,557 bp nucleotides, in which a poly (A) tail of 72 bp was found at the 3' terminus (Fig. 2). A putative polyadenylation signal, AATAA, was found 67 bp upstream of the poly (A) tail. The identified clone appears to have the entire AOBP cDNA, or almost all of it, since the size (1,557 bp) coincides with that of the AOBP transcript found in pumpkin tissues (about 1.5 kb) (Fig. 10a). The largest open reading frame (ORF) in the insertion, which is in frame with the λgt11 β-galactosidase gene, spans 1,140 bp, and encodes a polypeptide of 380 amino acid residues. The predicted molecular mass of the protein is about 42 kDa.

To characterize the DNA-binding properties of the protein, probes 1-4 were synthesized, spanning the AGTA repeat in different lengths (Fig. 3). Probe 5 was also synthesized, having AATA instead of AGTA in the first and third AGTA sequences (Fig. 3). The protein bound only to probe 1 and probe A, not to probes 2-5 (Fig. 3). Thus, AOBP specifically binds to the AGTA repeat.

Fig. 1  Screening of the cDNA which encodes a protein (AOBP) that binds to the ascorbate oxidase gene promoter. (a) Promoter of the pumpkin ascorbate oxidase gene. Position of the AGTA repeat (used as probe A in this study) is shown. The positions of the TATA box (TATAAA) and the CAAT box are also indicated. (b) Binding of the AOBP protein to probe A. λ phage expressing the AOBP cDNA and those expressing other cDNAs (negative control) were cultured on, respectively, left and right halves of the same agar plate. The expressed proteins were blotted on nitrocellulose filters, denatured, and renatured, and binding of these proteins to 32P-labeled probe A was examined.
DNA-binding protein for ascorbate oxidase gene

Fig. 2 Nucleotide sequence and the deduced amino acid sequence of AOBP cDNA. The Dof/MA domain in the N-terminus, and the C-terminal domain which is homologous to those in two anonymous Arabidopsis cDNA products are boxed. Part of the amino acid sequence in the C-terminus, which resembles the helix-turn-helix (HTH) motif is further underlined. The Pro-rich region is marked with a dotted line. The putative polyadenylation signal, AATAA, is doubly underlined. The Pvull-Hindill fragment was used as a probe in the Northern blot analysis. The cleavage sites of restriction endonucleases are marked with arrowheads.
The AGTA repeat is characteristically different from other A/T-rich plant promoters (Lam et al. 1990, Takahashi et al. 1990, Tjaden and Coruzzi 1994), in that it is composed of alternate runs of A tracts, An (n=5-6), and GT sequences. In this sense, the AGTA repeat more closely resembles the repeat found in the *Crithidia fasciculata* kinetochore repeat (Marini et al. 1982, Wu and Crothers 1984). The kinetochore repeat is composed of alteration of An (n=4-5) and other types of bases (Fig. 3), and shows some unusual characteristics in terms of bendability. A bent DNA can be identified by its anomalous migration in polyacrylamide gel electrophoresis. A 463 bp fragment (−703 to −241) excised from the ascorbate oxidase promoter with HindIII/HincII, which contain the AGTA repeat sequence, was analyzed by polyacrylamide gel electrophoresis. The mobility of the 463 bp fragment in polyacrylamide gel electrophoresis was lower than that expected from the actual fragment size, and its anomalous behavior in polyacrylamide gel electrophoresis was more obvious at a lower temperature (data not shown). This anomalous behavior in polyacrylamide gel electrophoresis is a hallmark of bent DNA (Koo et al. 1986, Marini et al. 1982), and suggests that the ascorbate oxidase promoter has bendability, perhaps in the AGTA repeat. The DNA bending has been suggested to play important roles in DNA replication, transcription and packaging. Indeed, some regulatory proteins have been shown to bind to regions of DNA bending and to increase in response to protein binding (Suzuki and Yagi 1995, Wu and Crothers 1984). Thus, there is a possibility that the binding of AOBP to AGTA repeat stimulates the bending to regulate ascorbate oxidase gene expression.

**Primary structure of AOBP**—In an earlier survey of databases, we found that only part of the AOBP protein, composed of 52 amino acid residues, is homologous to that of maize MNB1a which can bind to cauliflower mosaic virus 35S promoter (Yanagisawa 1995) (Fig. 4), and have shown that the structure of the homologous region, which has 5 conserved cysteines: Cys-X2-Cys-X7-Cys-X13-Cys-X2-Cys, seems to be closely related to those of the zinc/DNA-binding domains of steroid hormone receptors and GATA1 as described in a previous report (Kisu et al. 1995). The homologous region, referred to as Dof/MOA domain, has been found in many plant proteins (Yanagisawa 1996). Zhang et al. (1995) have also isolated a cDNA for the Dof/MOA domain containing protein, OBP1 (OBF binding protein), which can bind to the ocs element binding factor (OBF), from *Arabidopsis*. OBP1 specifically increased the binding of the OBF proteins to ocs element sequences, although it can also bind to the cauliflower mosaic virus 35S...
promoter as well as maize MNBl1. De Paolis et al. (1996) isolated a tobacco cDNA for a Dof/MOA domain containing protein, NiBBF1, which binds to regulatory domain B necessary for expression of the plant once gene rolB in root meristems. More recently, Vicente-Carbajosa et al. (1997) have reported that the Dof/MOA domain is found in maize PBF which binds to the prolamin box in zein gene promoters. Moreover, the Dof/MOA domain is found in proteins encoded by anonymous maize cDNA clones (acccession number: T88559, T14116, T23343) and genomic clones (AC002341 and AC002343) which have been isolated in random cloning projects. Five cysteine residues conserved in the Dof/MOA domain are highlighted. Two positions occupying amino acid residues in all the protein sequences. The asterisk at the C-terminus of T88559 indicates that the sequence ends there. X characters in T88559, T14116, and F14111 indicate that the amino acid residues which occupy these positions have not been identified.

Fig. 4 Comparison with helix-turn-helix (HTH) sequences in DNA-binding protein for ascorbate oxidase gene 1059

Fig. 5 Comparison with helix-turn-helix (HTH) sequences in DNA-binding protein for ascorbate oxidase gene 1059

AOBP has many Ser residues which might be used for phosphorylation. Pro residues are found near the Ser residues (Fig. 2). However, a particular combination of the two residues, Ser-Pro, which is used for phosphorylation in some other DNA-binding proteins (Suzuki 1989, 1990) is not frequent in the AOBP sequence.

The C-terminus of AOBP is homologous to the partial amino acid sequences predicted from anonymous Arabidopsis cDNA clones (T42233 and F14082) (data not shown). The latter, however, does not have a Dof/MOA domain.

DNA-binding domain and DNA-binding properties of AOBP—The AOBP cDNA was subcloned into pGEX-4T-1 expression vector and transfected into E. coli. When the AOBP sequence (Lys-322 to Leu-341) has some but not all the characteristics of the HTH motif (Fig. 5).
expressed GST-AOBP fusion protein was analyzed by southwestern blotting using probe 1 (AGTA repeat), two positive bands were detected (Fig. 6b). A minor band at the corresponding position of about 80 kDa may be that for a complete GST-AOBP fusion protein, and a major band at 45 kDa may be that for an incomplete protein by the incomplete transcription or translation, or proteolysis. In the expression of the AOBP recombinant protein with pET expression vector (Novagen), an incomplete product was likewise observed (data not shown). It is unclear why an incomplete fusion protein was produced in the E. coli expression system.

To assure that the Dof/MOA domain of AOBP has DNA-binding ability, the partial cDNA encoding only the N-terminal part from Met-1 to Val-119 of AOBP, containing the Dof/MOA domain (Leu-40 to Lys-91), was obtained by HindII digestion. The partial cDNA (N79) encoding only N-terminal part from Met-1 to Thr-79 of AOBP, which lacks the C-terminal region (ile-80 to Lys-91) of the Dof/MOA domain, was obtained by KpnI digestion. Shaded boxes represent the Dof/MOA domain. Vertical lines represent five cysteine residues, and black boxes represent two aromatic residues, Tyr and Trp, conserved in the Dof/MOA domain. (b) The AOBP and two mutant (N119 and N79) cDNAs were subcloned into the pGEX-4T-1 expression vector and transfected into E. coli. The expressed GST-AOBP, GST-N119, and GST-N79 fusion proteins were analyzed by Southwestern blotting using probe 1 (AGTA repeat). The positions of marker proteins are shown by arrowheads on the left.
the C-terminal region (Ile-80 to Lys-91) of the Dof/MOA domain, was obtained by KpnI digestion (Fig. 6a), and the expressed protein (N79) did not bind to the AGTA repeat (Fig. 6b), indicating that the C-terminal region (Ile-80 to Lys-91) of the Dof/MOA domain is required for the binding.

The DNA-binding specificity of AOBP protein was investigated by gel retardation analysis. As shown in Figure 7, a clear retarded band was observed after incubation of the GST-AOBP fusion protein expressed in E. coli with probe 1 (AGTA repeat, shown in Fig. 3), indicating efficient formation of the AOBP-probe 1 complex. The intensity of this retarded band was reduced by incubation with an excess of unlabeled probe 1 as a competitor, but not an excess of unlabeled probe 2, 3 or 4 as a competitor (Fig. 7). These results suggest that AOBP binds specifically to probe 1 (AGTA repeat), but not or only slightly to probes 2, 3 and 4, which corresponds to the results of the filter binding assays for the AOBP protein expressed in the E. coli-lgt11 phage system (Fig. 3). Furthermore, the binding of AOBP to mutant probe 5, 6, 7 or 8 in which the AGTA was mutated to AATA was investigated. AOBP bound with high affinity to probes 6 and 8, but with low affinity to probes 5 and 7 (Fig. 7). Thus, two tandem juxtaposed AGTA sequences may be required for DNA binding of AOBP, raising the possibility that a dimer of AOBP binds to two tandem AGTA sequences.

Two other Dof/MOA domain proteins, MNB1a and OBP1, bind to AAGG motif (referred to as 35SAAGG):
TTTATCAAAAGGACAGTAGAAAAGG) in the cauliflower mosaic virus 35S promoter (Yanagisawa and Izui 1993, Zhang et al. 1995). Thus, we investigated the binding of AOBP to 35SAAGG by gel retardation analysis (Fig. 8). AOBP binds to 35SAAGG in the cauliflower mosaic virus 35S promoter, but, the binding activity of AOBP to 35SAAGG was much lower than that to the AGTA repeat (Fig. 8).

AOBP may bind to DNA through the Dof/MAO domain, a putative zinc/DNA-binding domain. Thus, it is required to investigate whether zinc coordination is involved in the DNA-binding of this protein. We tested the inhibition of complex formation by the metal chelators, 1,10-phenanthroline and EDTA. As shown in Figure 9, the formation of AOBP-probe 1 complex was inhibited by adding the metal chelators, 1,10-phenanthroline and EDTA. The inhibition by adding 1,10-phenanthroline was more effective than that by EDTA. This more effective inhibition of the DNA-protein complex by 1,10-phenanthroline has also been shown for another zinc/DNA-binding protein, 3AF1 (Lam et al. 1990). Thus, these results strongly suggest that the Dof/MAO domain of AOBP acts as a zinc/DNA-binding domain.

The Dof/MAO domain has five conserved Cys residues. Interestingly, the Dof/MAO domain may be related to DNA-binding domains of steroid hormone receptors and GATA1 as suggested in a previous report (Kisui et al. 1995). AOBP binds to the AGTA repeat in the ascorbate oxidase promoter. Using the similarity to DNA-binding domains of GATA1 and steroid hormone receptors, the recognition helices of Dof/MAO domains and their contacts to the target DNA bases can be predicted; i.e., 5'-AGTA-3' is predicted to be the direct contact site of AOBP. In the present study, we have shown that probe 1 is sufficient for recognition by AOBP but neither the upstream half (probe 2), nor the downstream half (probe 4) is sufficient (Fig. 3, 8). Probe 3 is shorter than probe 1 and is insufficient for the recognition (Fig. 3, 8). Thus, the missing part, the A tract upstream of the first AGTA and/or another A tract downstream of the third AGTA, seems to be necessary. Probe 5, in which two AGTA in sites 1 and 3 are mutated to AATA, and probe 7, in which AGTA in site 2 is mutated to AATA, are not fully recognized by AOBP. On the other hand, probe 6, in which AGTA in site 1 is mutated to AATA, and probe 8, in which AGTA in site 3 is mutated to AATA, are recognized by AOBP (Fig. 7). The simplest interpretation appears to be that AOBP recognizes two AAAAGTAAAA sequences and that two molecules of AOBP might bind to the promoter in tandem. MNBl has been reported to bind to the AAGG core sequence found in the cauliflower mosaic virus 35S promoter (Yanagisawa and Izui 1993). NtBBFI has been suggested to recognize
the ACTTTA target sequence (De Paolis et al. 1996). Neither AAGG nor ACTTTA sequences are contained in the AGTA repeat sequence. On the other hand, the Arabidopsis OBP1 (Zhang et al. 1995) and maize PBF Dof/MAO domain proteins (Vicente-Carbajosa et al. 1997) have been suggested to interact primarily with the AAAG sequence, found in the cauliflower mosaic virus 35S promoter and in the prolamin box in zein gene promoters. In the present study, we showed that AOBP also binds to the cauliflower mosaic virus 35S promoter (35S AAGG) which contains one AGTA sequence, but the binding activity was much lower than that to the AGTA repeat (Fig. 8). We propose that the AGTA sequence may be the core of the interaction with AOBP, but further studies of the DNA binding styles and recognition mechanisms of the unique zinc/DNA-binding domain, Dof/MAO domain, are required.

Expression of AOBP and ascorbate oxidase transcripts—Poly(A)+ RNA was obtained from pumpkin fruit tissues incubated either in the presence or absence of 2,4-D and was hybridized with the ascorbate oxidase or AOBP cDNA (Fig. 10a). The transcript of ascorbate oxidase was increased in the presence of 2,4-D, but rapidly decreased in its absence (Fig. 10a). In contrast, the 1.5 kb transcript of AOBP was detected after incubation for 2 d with or without 2,4-D (Fig. 10a), suggesting that AOBP expression is less dependent on auxin. Thus, the auxin-dependent expression of ascorbate oxidase gene may not be controlled through the transcription level of AOBP.

The transcription level of the AOBP and ascorbate oxidase genes in various pumpkin tissues was investigated (Fig. 10b). The ascorbate oxidase transcript was found in larger amounts in young leaves and the epicotyl, but only in trace amounts in the 4-day-old seedlings, mature leaves, hypocotyl, and roots, and was not detected in the seeds, mature cotyledons or etiolated cotyledons (Fig. 10b). In contrast, the AOBP transcript was found in larger amounts in mature tissues (mature cotyledons, roots, mature leaves, and hypocotyl), but only trace amounts in the epicotyl and etiolated cotyledons. No or little transcript was detected in growing tissues (4-day-old seedlings and young leaves), and seeds (Fig. 10b). Thus, the tissue-dependent expression pattern of the AOBP gene did not appear to be positively correlated with that of the ascorbate oxidase gene.

In this study, we have cloned a protein, named AOBP, which binds to the ascorbate oxidase gene promoter. AOBP binds to DNA with the Dof/MAO domain which is a repressor of the ascorbate oxidase gene. Since auxin is an inducer of ascorbate oxidase gene expression and since expression of the AOBP gene is less dependent on auxin, a possible hypothesis is that AOBP binds to the ATGA repeat, thereby repressing expression of the ascorbate oxidase gene and auxin removes, directly or indirectly, AOBP from the repeat to turn on the expression. Further studies will be required to investigate whether AOBP acts as a repressor for ascorbate oxidase gene or not.

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