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Production of Homo- and Hetero-Dimeric Isozymes from Two Aldehyde Oxidase Genes of *Arabidopsis thaliana*[1]

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Polyclonal antibodies were raised against synthetic peptides or recombinant polypeptides encoded by *Arabidopsis* atAO-1 and atAO-2 cDNAs, which have sequences similar to maize and animal aldehyde oxidase (AO) cDNAs. Anti-atAO-1 antibodies recognized AOα and AOβ among the three isoforms, AOα, AOβ, and AOγ, detected in *Arabidopsis* seedlings after native PAGE, while anti-atAO-2 antibodies reacted with AOβ and AOγ. The polypeptide specifically recognized by each antibody was collected as the Protein-A/IgG/antigen complex. The 150- and 145-kDa polypeptides were purified by SDS-PAGE and digested with *Achromobacter* Protease I. From the amino acid sequences and molecular masses of the derivative peptides, it was revealed that the 150- and 145-kDa polypeptides were the products of atAO-1 and atAO-2, respectively. Molecular masses of the native forms of AOα, AOβ, and AOγ were estimated as approximately 290–300 kDa. These results suggest that AOα and AOγ are homodimers consisting of atAO-1 and atAO-2 products, respectively, and that AOβ is a heterodimer of the atAO-1 and atAO-2 products.

Key words: aldehyde oxidase, *Arabidopsis thaliana*, indole-3-acetic acid (IAA), isozyme, molybdenum cofactor.

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Aldehyde oxidase (AO; aldehyde-oxygen oxidoreductase, EC 1.2.3.1) has been extensively investigated in animals and microorganisms. The enzyme catalyzes the oxidation of a variety of aldehydes and nitrogen-containing heterocycles. It is known that AO is a homodimer consisting of two 150-kDa subunits and contains a molybdenum cofactor (MoCo), nonheme iron, and FAD as prosthetic groups (*1-3*). In plants, studies on AO have focused on its possible involvement in the biosynthesis of plant hormones, such as indole-3-acetic acid (IAA) (*4-9*) and abscisic acid (ABA) (*10-14*). Although its physiological importance is attracting increasing attention, the purification and molecular cloning of plant AO has only been achieved in maize (*7, 15*) and the molecular analysis of plant AO has just begun.

Recently we cloned three AO cDNAs (atAO-1, atAO-2, and atAO-3) from *Arabidopsis thaliana*. A fourth AO gene (tentatively named atAO-4) was reported from the *Arabidopsis* Genome Project (accession number, AC002376). These four genes map at different loci on the *Arabidopsis* genome and are expressed in an organ- and/or stage-specific manner (*16* and Seo, M., Akaba, S., Koiwai, H., and Koshiba, T., unpublished data). On the other hand, in *Arabidopsis* seedlings, three AO activity bands are detected in an organ-specific manner after native PAGE (AOα, AOβ, and AOγ, formerly called AO1, AO2, and AO3, respectively) (*8*). AOα is abundant in roots and AOγ is relatively rich in the cotyledon and young leaf parts. Furthermore, they show different substrate preferences; AOα has a strong preference for indole-3-aldehyde (IAld), while AOγ oxidizes 1-naphthaldehyde efficiently. AOβ has properties intermediate between AOα and AOγ, such as its mobility in native PAGE and substrate preferences. These findings indicate that the AOs have different physiological functions in plant growth and that their expression is regulated during growth in a stage- and/or organ-specific manner. Among, the three isoforms, AOα has been suggested possibly to be involved in IAA biosynthesis, because it has a high affinity for indole-3-acetaldehyde (IAAld), an important intermediate in IAA biosynthesis, and is highly expressed in the IAA overproducing *sur1* mutant (*8*). The atAO-1 mRNA shows a distribution pattern similar to that of AOα activity in wild type and *superroot1* (*sur1*) seedlings of *Arabidopsis*, suggesting that it encodes AOα (*16*). However, we have no direct evidence as to whether the putative AO genes actually encode AO proteins.

Antibodies against recombinant AO (TAO1) polypep-

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Abbreviations: aa, amino acid; ABA, abscisic acid; ABAld, abscisic aldehyde; AO, aldehyde oxidase; API, *Achromobacter* Protease I; IAA, indole-3-acetic acid; IAAld, indole-3-acetaldehyde; IAld, indole-3-aldehyde; LDH, lactate dehydrogenase; MoCo, molybdenum cofactor; TBS, 20 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl.

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tides were raised in tomato, but the immuno-reacting proteins were not definitely determined to be AO (17). In the present work, we prepared polyclonal antibodies specific for atAO-1 and atAO-2 products by two strategies, against chemically synthesized peptides (20 and 18 amino acids), and C-terminal 30-kDa segments expressed in *Escherichia coli*. Using these antibodies, we could conclude that AOα is a homodimer of the atAO-1 product, AOβ is a heterodimer of the atAO-1 and atAO-2 products, and AOγ is a homodimer of the atAO-2 product.

MATERIALS AND METHODS

Plant Material and Enzyme Extraction—*A. thaliana* (Columbia ecotype) seeds were aseptically sown on agar plates and germinated under 16 h of light and 8 h of dark at 20°C. Whole seedlings were sampled, washed with water, frozen in liquid N_2 and stored at −80°C until use. A crude enzyme solution was prepared and fractionated with ammonium sulfate as described previously (8). The precipitate was solubilized in 50 mM Tris-HCl buffer (pH 7.5) containing 10 μM Mo, 5 μM leupeptin, 1 mM EDTA, and 2 mM dithiothreitol, and dialyzed for 4 h against the buffer. The supernatant was heat-treated (60°C, 4 min) to remove contaminated proteins, since *Arabidopsis* AOs have been shown to be heat-stable as in the case of maize AO (6). After centrifugation (20,000 × g, 30 min, 4°C), the supernatant was used for further studies.

Antigen Design for the Preparation of Anti-atAO-1 and Anti-atAO-2 Antibodies—A 20-amino-acid atAO-1 peptide (aa number 551-570; KNAKTTNGWLNGGCKEIGFD) and an 18-amino-acid atAO-2 peptide (518-535; HPTTDKPSNGYHLDPPKP) were chemically synthesized (see Fig. 1). The C-terminal amino acid of each peptide was coupled to a carrier protein (KLH-MB; keyhole limpet hemocyanin modified with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester) *via* cysteine and the conjugate was used for antibody production in rabbits.

The 3′-terminal DNA fragments (864 bp for atAO-1 and 852 bp for atAO-2; see Fig. 1) were amplified by a PCR using primers containing an *Nde*I site and a *Bam*HI site for the sense and antisense primers, respectively. The fragments were then cloned into an expression vector (pET16b) and overexpressed in *E. coli* (18). *E. coli* BL21 (DE3) was used as a host for T7 promoter-mediated gene expression and expression of the transgene was induced by adding isopropyl-β-D(−)-thiogalactopyranoside (IPTG). The *E. coli* were sonicated and the extracts were centrifuged. The precipitates were solubilized in 20 mM Tris-HCl buffer (pH 8.0) containing 10 mM dithiothreitol and 8 M urea. The solubilized proteins were applied to a DEAE column (DEAE-5PW, 6 mm i.d. × 50 mm; Tosoh) equilibrated with the Tris-HCl buffer containing urea. The proteins were eluted with a linear gradient of KCl from 0 to 0.5 M in the same buffer at a flow rate of 0.4 ml·min^{-1}. The peak fractions containing the recombinant polypeptides were pooled and dialyzed against 20 mM Tris-HCl buffer (pH 8.0) to remove the urea. The polypeptides were used for raising rabbit antiserum.

Preparation of Antibodies—Antibodies against the synthetic peptide conjugate and recombinant polypeptides were raised by injecting the peptides, emulsified with Freund's complete adjuvant, subcutaneously into a rabbit. A total of four injections, each containing about 200 μg of synthetic peptide conjugates or 300 μg of recombinant polypeptides, was given at 10-day intervals. The serum was fractionated by ammonium sulfate precipitation (0-40% saturation) and dialyzed against TBS.

Immuno-Precipitation—Immuno-precipitation using Protein A Sepharose was performed to test the reactivity and specificity against AOs of *Arabidopsis* seedlings. The ammonium sulfate fractionated enzyme sample (about 50 μg of protein) was incubated with serum (about 50 μg of protein) at 30°C for 1 h and then at 4°C overnight. A 10 μl bed volume of Protein A Sepharose equilibrated with TBS was added and the mixture was incubated at room temperature for 1 h, then at 4°C for 1 h with occasional mixing. After centrifugation (10,000 × g, for 5 min at 4°C), the AO activity in the supernatant was determined as described below.

Purification of Antibodies—The antibodies were purified by antigen-conjugated affinity chromatography using ECH Sepharose 4B (Pharmacia Biotech) according to the standard method. Briefly, a 3 ml bed volume of activated ECH Sepharose 4B was mixed with about 30 mg of recombinant polypeptides or 3 mg of synthetic peptide. After the addition of carbodiimide (0.1 M), the mixture was incubated at room temperature for 4.5 h. The Sepharose particles were thoroughly washed with 0.1 M acetate buffer (pH 4.0) and 0.1 M Tris-HCl (pH 8.3), each containing 0.5 M NaCl, and distilled water and TBS, successively. Then, 4.5 ml of the crude antiserum was passed through the column and the column was washed with TBS. The antibodies bound to the column were eluted with 0.1 M glycine-HCl buffer (pH 2.5) and the eluate was immediately neutralized by the addition of 1 M Tris-HCl buffer (pH 7.5). The purified antibodies were tested for their ability to collect the specific immunoreacting proteins from *Arabidopsis* extracts, and the purified anti-atAO-1-30-kDa and anti-atAO-2-18aa antibodies were chosen as good antibody preparations for this purpose.

Preparation and Analysis of Polypeptides Recognized by Specific Antibodies—A crude AO solution containing about 2 mg of proteins (extracted from about 2,000 seedlings) was incubated with 100 μg of purified anti-atAO-1 and anti-atAO-2-peptide antibodies at 30°C for 1 h and then at 4°C overnight. A 100 μl bed volume of Protein A Sepharose equilibrated with TBS was added and the mixture was incubated at room temperature with occasional mixing for 2 h. Sepharose particles were trapped by passing the mixture through a small column. After successive washes (three times with 1 ml of TBS containing 0.05% Tween-20), the immunocomplexes (IgG + immunoreacting protein) were solubilized with 50 μl of SDS sample buffer [50 mM Tris-HCl (pH 6.8), 4% SDS, 10% 2-mercaptoethanol, 0.002% bromophenol blue, and 20% glycerol]. The samples were subjected to SDS-PAGE, and the proteins in the gel were stained with Coomassie Brilliant Blue.

The 150- or 145-kDa band was excised and treated with API at 37°C for 15 h in 0.1 M Tris-HCl (pH 9.0) containing 0.1% SDS. The generated peptides were separated by reversed-phase HPLC. Their amino acid sequences were determined by automated Edman degradation using a model 492cLC protein sequencer (PE Applied Biosystems, Foster City, CA), and molecular masses were determined by matrix-assisted laser desorption ionization time of flight

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mass spectrometry on a Reflex MALD-TOF (Bruker-Franzen Analytik, Bremen, Germany) using 2-mercapto-benzothiazole as matrix as described previously (*15*).

Determination of the Molecular Masses of Native AOs—The molecular masses of the native AOs were estimated by gel filtration chromatography. An enzyme sample prepared from *Arabidopsis* seedlings was applied to a G3000SW$_{XL}$ column (8 mm i.d.×30 cm, Tosoh). AO activities in the fractions were assayed by activity staining after native PAGE using two substrates, IAld for AOα and AOβ, and naphthaldehyde for AOβ and AOγ. The relative intensities of the activity bands were estimated using computer software (NIH Image 1.6). The molecular masses were assessed from the peak positions on a standard curve with molecular markers (*7*).

Detection of AO Activity and Immunoblotting—AO activity was detected by activity staining after electrophoresis on a 7.5% native polyacrylamide gel or by reversed-phase HPLC as described previously (*7, 8*). Immunoblotting was performed using a Vectastain Elite ABC kit (Vector, Burlingame, CA) with primary antibodies (anti-atAO serum) diluted 100–1,000-fold in TBS and secondary antibody (goat anti-rabbit-IgG) diluted 1,000-fold in TBS containing 0.05% Tween-20 according to the previous study (*7*). Peroxidase activity was visualized by staining with an immunostain HRP-1000 kit (Konica, Tokyo).

RESULTS

Polyclonal Antibodies Specific for atAO-1 and atAO-2 Polypeptides—Rabbit polyclonal antibodies were prepared by two strategies: (i) by chemically synthesizing 20- and 18-amino acid peptides and (ii) by producing approximately 30-kDa recombinant C-terminal polypeptides in *E. coli.* Both strategies were based on the deduced amino acid sequences of atAO-1 and atAO-2. For the former, we chose sequences referring to four main parameters of the computer simulation (using "Laser gene" software) of the deduced amino acid sequence of the cDNAs, antigenic index, hydrophilicity, surface probability, and turn (hinge) region, as well as sequences showing low similarity among four putative AO cDNAs (atAO-1, atAO-2, atAO-3, and atAO-4). The designs of the antigens are shown in Fig. 1.

Four antibodies were checked for their specificity to the AOα, AOβ, and AOγ isoforms by immunoprecipitation and immunoblotting. An AO sample from *Arabidopsis* seedlings was treated with the four antibodies and the AO isoforms remaining in the supernatant of the immunoreaction were analyzed by native PAGE followed by activity staining using IAld and naphthaldehyde as substrates (Fig. 2). Treating the AO sample with nonimmune serum did not affect the AOα, AOβ, and AOγ patterns: AOα and AOβ were efficiently stained with IAld and AOβ and AOγ with naphthaldehyde, as found previously (*8*). Treatment of the AO sample with anti-atAO-1-30-kDa antibodies removed the AOα and AOβ activities completely, while treatment with anti-atO-1-20aa antibodies removed the activities partially; neither antibodies removed the AOγ activity (Fig. 2A). In contrast, treatment of AO samples with anti-

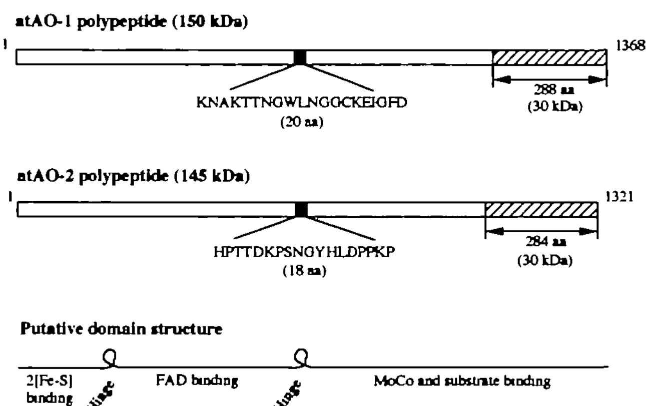

Fig. 1. **Designs of antigens for anti-atAO-1 and anti-atAO-2 antibodies.** The black boxes and shaded boxes indicate the peptide regions chemically synthesized and expressed by transformed *E. coli*, respectivily, for antigens. Putative iron-sulfur, FAD and MoCo domains are also indicated (*16*).

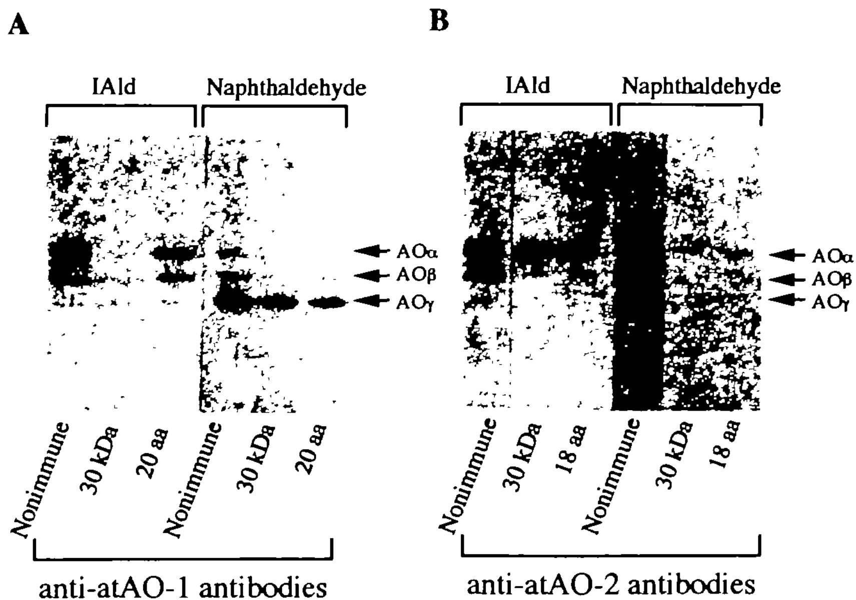

Fig. 2. **Immuno-precipitation of specific AO isoforms by various antibody preparations.** The crude extract of 6-d-old *Arabidopsis* seedlings containing the AOα, AOβ, and AOγ isoforms, was treated with four different rabbit antibodies, and the immunocomplexes formed were removed by adding Protein A Sepharose as described in "MATERIALS AND METHODS." AO isoforms remaining in the supernatant were detected after native PAGE by staining the gel using IAld or 1-naphthaldehyde as a substrate.

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atAO-2-30-kDa and anti-atAO-2-18aa antibodies removed the AOβ and AOγ activities, but not the AOα activity (Fig. 2B). These results suggest that AOα is the product of atAO-1, AOγ is the product of atAO-2, and AOβ is a mixture of the atAO-1 and atAO-2 products.

To further reveal the specificity of these antibodies, immunoblot analyses were performed after native PAGE. The anti-atAO-1-30-kDa and anti-atAO-2-30-kDa antibodies produced relatively clear band patterns compared with the anti-atAO-1-20aa and anti-atAO-2-18aa antibodies (Fig. 3). The anti-atAO-1 antibodies reacted with all three AOs, as well as one additional intense band with low mobility (indicated by an asterisk), but the low mobility band was also detected by preimmune serum. The anti-atAO-2 antibodies recognized AOγ clearly and AOβ slightly. Coincident results were obtained with anti-atAO-2 antibodies both from immunoprecipitation and immunoblotting: the antibodies recognized both AOβ and AOγ. However, anti-atAO-1 antibodies reacted only with AOα and AOβ in solution, whereas the same antibodies reacted with all three AOs by immunoblotting. This apparent discrepancy can be explained by the fact that during blotting to the nitrocellulose filter, the AOγ protein becomes unfolded and the anti-atAO-1 antibodies crossreact with AOγ at inner immunoreactive site(s) similar to AOα because the C-terminal amino acid sequences of atAO-1 and atAO-2 are very similar.

It has been shown in animals and maize that the apparent molecular mass of the native AO is about 300 kDa, and that the enzyme is composed of two 150-kDa subunits (*7*). The molecular masses of native *Arabidopsis* AOs (AOα, AOβ, and AOγ) were also estimated by molecular sieve chromatography at about 290-300 kDa (data not shown). Combining these values with the results of the immunological experiments indicates that AOα and AOγ are homodimers consisting of the atAO-1 and atAO-2 products, respectively, and that AOβ is a heterodimer of atAO-1 and atAO-2 product. This assignment is consistent with the previous observations that AOβ displays biochemical properties intermediate between AOα and AOγ in substrate specificity and gel mobility (*8*).

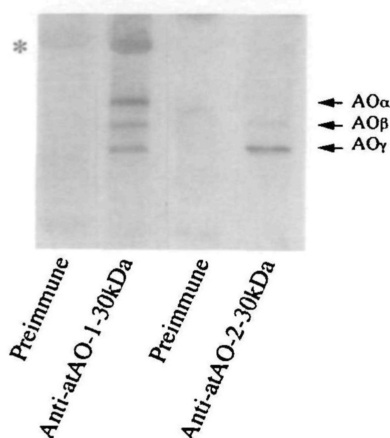

Fig. 3. **Immunoblotting with *Arabidopsis* seedling extracts after native PAGE.** Crude protein extracts (50 μg) obtained from 6-d-old seedlings were subjected to native PAGE followed by immunoblot analysis using the anti-atAO-1 and atAO-2 antibodies. Preimmune-serum was used as a control.

Evidence That the atAO-1 and atAO-2 Genes Encode Subunits of the Three Dimeric Isoforms—Since there are at least four AO genes in *Arabidopsis* and their deduced amino acid sequences are very similar to one another (*16*), it is still possible that the polyclonal antibodies recognize other AOs or related polypeptides. Therefore, it should be determined whether the polypeptides that immunoreact with the anti-atAO-1 and anti-atAO-2 antibodies are actually those encoded by atAO-1 and atAO-2, respectively. For this purpose, the atAO-1 and atAO-2 polypeptides were immunoprecipitated from the crude extract of *Arabidopsis* seedlings using anti-atAO-1 and anti-atAO-2 antibodies, and immunocomplexes were trapped by Protein A Sepharose. Polypeptides eluted from the complexes by SDS sample buffer were separated by SDS-PAGE (Fig. 4). Bands of about 150- and 145-kDa were detected in immunoprecipitates treated with anti-atAO-1 and anti-atAO-2 antibodies, respectively. These bands were excised and digested with API. The generated peptides were purified by HPLC and their amino acid sequences and molecular mass values were analyzed. The results for five selected peptides from each preparation are shown in Table I. All five peptides had molecular mass values corresponding to those calculated from the predicted sequences of the atAO-1- and atAO-2-derived peptides, and amino acid residues identified (partially or fully) were in accord with the respective sequences. These results clearly indicate that the major component of anti-atAO-1 antibody-immunocomplex is the atAO-1 product and that anti-atAO-2 antibodies specifically recognize the atAO-2 product. Because both anti-atAO-1 and anti-atAO-2 antibodies could immunoreact with AOβ, some cross-contaminating peptides derived from atAO-2 or atAO-1 polypeptides were expected in the anti-atAO-1 or anti-atAO-2 antibody-precipitated samples, respectively. In fact, several peptides derived from atAO-1 polypeptides were detected in the anti-atAO-2 antibody-precipitated fraction, although in very low amounts (data not shown).

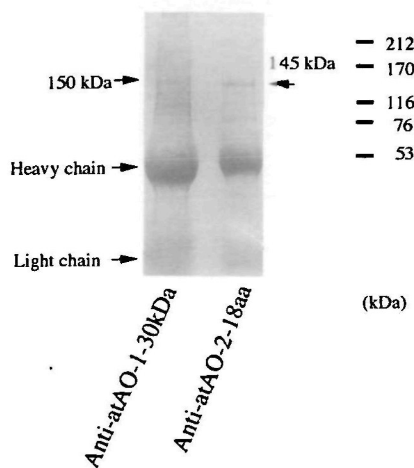

Fig. 4. **Detection of polypeptides that immunoreact with anti-atAO-1 and anti-atAO-2 antibodies.** A highly concentrated, AO enriched enzyme solution was treated with anti-atAO-1 and anti-atAO-2 antibodies, and the immunocomplexes formed were collected using Protein A Sepharose as described in "MATERIALS AND METHODS." The proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. The positions of molecular markers are indicated by numbers.

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TABLE I. **Amino acid sequences and mass values of selected API peptides derived from polypeptides that immunoreact with anti-atAO-1 antibodies (A) and anti-atAO-2 antibodies (B).**

A

Predicted amino acid sequence (atAO-1 peptide)	Determined amino acid sequence	Molecular mass (H⁺)	
		Calculated mass	Observed mass
151-SHPPPRSGFSNLTAVEAEK-169	sHPppR-	2,025.2	2,025.7
276-LVAGNTSTGYYK-287	lVAGnTSTxYYK	1,274.4	1,274.4
495-KVEEFLTGK-503	KVEEFLt-	1,051.2	1,052.3
705-HADIAANLVVIDYDTK-720	xADIAAxLVVIDY-	1,759.0	1,759.5
878-VTYSVGFK-885	VTYSV-	901.0	903.6

B

Predicted amino acid sequence (atAO-2 peptide)	Determined amino acid sequence	Molecular mass (H⁺)	
		Calculated mass	Observed mass
188-DSSSLTRFDSEK-199	xxxSLTRFDSEK	1,371.6	1,371.7
249-LVAGNTSMGYYK-260	LVAGNTSMGYYk	1,304.5	1,303.8
600-GIHFKDDLVPTGVVAVISRK-619	GIHFKDDLVPTGVVAVIsRK	2,152.5	2,153.0
948-DGAGEPHEYTLSSMWDK-964	dGAGEPHEYtLssMxxk	1,924.6	1,923.3
965-VGVSSKFEERVSVVREFNESNMWRK-989	VGVSSKFEERVSVVREFNEsNM-	3,001.4	3,000.7

The 150- and 145-kDa bands collected with atAO-1 and atAO-2 antibodies were purified by SDS-PAGE (see Fig. 4) and cleaved with API. The peptides generated were separated by reversed-phase HPLC. Five major peptides from each sample were subjected to amino acid sequencing, and their molecular masses were confirmed by matrix-assisted laser desorption ionization time of flight mass spectrometry (see "MATERIALS AND METHODS"). Lower case letters indicate weakly assigned residues. "x" gave no significant PTH-amino acid signal during sequence analysis.

TABLE II. **IAAld oxidase activity of crude AOα and AOγ preparations.**

A

AO in supernatant (Antibodies used)	IAAld oxidase activity (pmol IAA min⁻¹·mg·protein⁻¹)
AOα, AOβ, and AOγ (nonimmune)	23.7
AOα (anti-atAO-2)	27.2[a]
AOγ (anti-atAO-1)	2.07[a]

B

AO in supernatant (Antibodies used)	IAAld oxidase activity (pmol IAA min⁻¹·mg·protein⁻¹)
AOα, AOβ, and AOγ (nonimmune)	50.4[a]
AOα (anti-atAO-2)	49.4[a]
AOγ (anti-atAO-1)	4.90[a]

The enzymes extracted from 6-d-old (A) and 11-d-old (B) *Arabidopsis* seedlings were incubated separately with anti-atAO-2 and anti-atAO-1 antibodies to give crude AOα and AOγ preparations, respectively. IAAld oxidase activities were measured as the amounts of IAA formed during the reaction. [a]The values presented are the average of two assays.

IAAld Oxidase Activity of AOα—AOα has been shown to have a high substrate preference for IAld after native PAGE (*8*). Because IAAld, a direct precursor of IAA, could not be used as a substrate for activity staining after native PAGE (*7*), we do not have definite evidence about whether AOα also has a preference for IAAld. The previous study indicated that both the IAAld oxidase and IAld oxidase activities of IAA-overproducing *sur1* mutant seedlings are higher than those of wild-type by direct estimation using HPLC (*8*). To get a precise answer, crude AOα and AOγ preparations were made by using anti-atAO-2 and atAO-1 antibodies, respectively, and their IAAld oxidase activities were determined directly. The isoform purity of crude AOα and AOγ preparations was confirmed by native PAGE followed by activity staining (see Fig. 2). The IAAld oxidase activity in these samples was determined by HPLC (Table II). Crude AOα preparations from both 6-d-old and 11-d-old seedlings showed about 10 times higher IAAld oxidase activity than crude AOγ preparations. The activity was almost the same as the total IAAld oxidase activity in the non-immune control. These results indicate that AOα is able to oxidize IAAld as efficiently as IAld. Since AOα is known to be most abundant in roots (*8*), the K_m value of AOα for IAAld was measured using root extracts as a source of AOα enriched enzyme solution. The obtained K_m value was approximately 15 μM, suggesting that AOα might function in the oxidation of IAAld to IAA *in vivo*.

DISCUSSION

Plant AOs have been studied from several sources, including oat coleoptiles (*4*), potato tubers (*19*), cucumber seedlings (*5*), maize coleoptiles (*7*), *Arabidopsis* seedlings (*8*) and mature plants (*13, 20*), *Nicotiana plumbaginifolia* leaves (*14*), and barley plants (*21*). Almost all of these AOs have relatively wide substrate specificities. Several AO isoforms with different mobilities have been separated after native PAGE (*7, 8, 13, 19-21*). In many cases the expression of each form is organ specific. For example, three major AO activity bands with different substrate specificities were detected in *Arabidopsis* seedlings: one is abundant in leaves and another in roots (*8*).

A full length AO cDNA has been obtained from maize as the first plant AO cDNA (*15*). An AO cDNA (TAO1) from tomato (*17*) as well as three AO cDNAs (atAO-1, atAO-2, and atAO-3) and a putative AO gene (atAO-4) from *Arabidopsis* (*16*) have been reported in the last two years. Three cDNAs have also been cloned in *Arabidopsis* by another group (*20*), where AtAO1, AtAO2, and AtAO3 correspond to atAO-1, atAO-4, and atAO-2, respectively. The alignment of the deduced amino acid sequences of plant AO cDNAs together with those of animal AO cDNAs (*15, 16*) indicates that they share a high degree of similarity, including two iron-sulfur domains and a MoCo-binding site, but whether these cDNAs actually encode AO protein(s) remained to be clarified. The evidence in *Arabidopsis* and tomato is as follows: the expression pattern of *Arabidopsis* atAO-1 cDNA revealed by northern blot analysis is similar to the distribution pattern of AOα, which is abundant in the roots of wild-type seedlings and in the roots and hypocotyls

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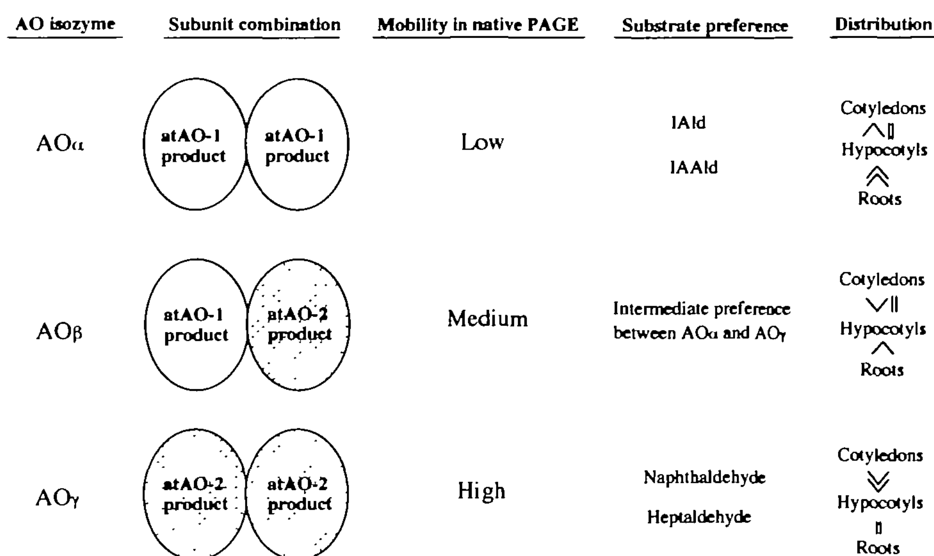

Fig. 5. **Hypothetical dimeric subunit combination of AOα, AOβ, and AOγ isozymes.** AOα and AOβ are homodimers of atAO-1 and atAO-2 products, respectively. AOβ is a heterodimer of atAO-1 and atAO-2 products. Distribution in *Arabidopsis* seedlings, mobilities in native PAGE, and substrate preferences of these isozymes (*8*) are indicated together with subunit combinations.

of *sur1* mutant seedlings (*8, 16*). Anti-TAO1 (tomato putative AO cDNA) antibodies raised against recombinant TAO1 polypeptides overexpressed in *E. coli* react with an approximately 78-kDa polypeptide from tomato tissues as well as similar with peptides from various plant and animal sources; this might be a degradation product of the 140-150-kDa AO subunit (*17*).

In the present work we succeeded in raising antibodies specific for two polypeptides encoded by two *Arabidopsis* AO cDNAs, atAO-1 and atAO-2. Using them, we showed that three AO isoforms in *Arabidopsis* seedlings are formed from the products of atAO-1 and atAO-2 as follows: AOα is a homodimer of atAO-1 products; AOβ is a heterodimer of atAO-1 and atAO-2 products; and AOγ is a homodimer of atAO-2 products (Fig. 5). The three AO isoforms exhibit different substrate preferences: AOα prefers IAld and IAAld, AOγ prefers naphthaldehyde, and AOβ shows intermediate preferences. The relative intensities of the AOα, AOβ, and AOγ activity bands are tissue specific: AOα is rich in roots while AOγ predominates in cotyledons and young leaves; AOα is more abundant in *sur1* seedlings than in the wild type (*8*). Thus it is proposed that the ratio of atAO-1 and atAO-2 mRNAs in cells determines the amounts of the three AO isoforms. This mode of isoform formation is well known for hybrid type isoforms of lactate dehydrogenase (LDH) in animals, where two distinct LDH genes are expressed differently in various organs and make five tetrameric isoforms (*22*). Each form exhibits differences in enzymatic properties such as substrate specificity, and is expressed in an organ- and/or developmental stage-specific manner. As for animal AOs, although there have been some suggestions of the presence of AO isozymes (*23, 24*), cloning of human and cow AO cDNAs (*25, 26*) indicated only one gene present in each species. In fact, recent reports have shown that the human AO is encoded by a single copy gene (*27*) and that two kinetically different AOs from male and female mice (*24*) can be explained by the expression of a single AO gene (*28*). To our knowledge, the present result that two AO genes produce three AO isozymes is the first finding with AO.

Considerable attention has been paid to plant AOs in relation to the biosynthesis of a plant hormone, IAA (*4-9*). The AOα in *Arabidopsis* has been the most likely candidate for an AO that produces IAA, because of its high affinity for IAld, an analog of IAAld, and the fact that it is rich in IAA-overproducing *sur1* mutant seedlings (*8*). In the present study we have shown that AOα efficiently oxidizes IAAld as well as IAld with a K_m value for IAAld of approximately 15 μM. This value is in the same range as the maize IAAld oxidase (*7*), supporting the possibility that AOα is involved in IAA biosynthesis *in vivo*. In addition, plant AO has also been investigated for its possible involvement in the biosynthesis of another plant hormone, ABA (*10-14*). Our preliminary experiments showed that the expression of two other *Arabidopsis* AO genes, atAO-3 and atAO-4, in rosette leaves was rapidly induced after their desiccation, suggesting that these genes encode ABAld oxidase (*29*). The final conclusions about the physiological functions of plant AOs must await the results of experiments with transgenic plants and/or with heterologous expression systems. Such work is in progress in our laboratory.

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