Primary structural information of a plant aldehyde oxidase (AO), which was purified from maize coleoptiles using indole-3-acetaldehyde as a substrate, was obtained by sequencing a series of cleavage peptides, permitting the cloning of the corresponding cDNA (zmAO-1). The complete nucleotide sequence was determined; the deduced amino acid sequence encodes a protein of 1349 amino acid residues of \( M_r 146,681 \), which is consistent with the size of the AO monomeric subunit. There is a significant similarity with AO from mammals and xanthine dehydrogenases from various sources. The maize AO polypeptide contains consensus sequences for iron-sulfur centers and a putative molybdopterin cofactor-binding domain. In addition, another cDNA (zmAO-2), highly homologous to zmAO-1 at both the nucleotide and amino acid sequence levels, was cloned. zmAO-2 would encode a protein of 1349 amino acid residues of \( M_r 145,173 \) and has molecular characteristics similar to those of zmAO-1. zmAO-1 was expressed at a high level in roots, which was closely correlated with immunoblotting results using antiserum raised against the purified maize AO protein, whereas zmAO-2 was expressed at a higher level in coleoptiles than in roots. We propose each zmAO may have a unique function during plant development.

**Experimental Procedures**

Plant Material—Seeds of maize (Z. mays L. cv Golden Cross Bantam 70) were germinated, and coleoptiles (about 1.5 cm long) were harvested from 4-day-old seedlings (18).

Purification of AO and Sequencing of Proteolytic Peptides—AO was purified from maize coleoptile tips as described previously (17) and subjected to SDS-polyacrylamide gel electrophoresis for final purification. Coomassie-stained bands of 150 kDa were excised and subjected to SDS-polyacrylamide gel electrophoresis for final purification. Coomassie-stained bands of 150 kDa were excised and treated with 0.3 \( \mu \)g of Achromobacter protease I (API; a gift from Dr. Masaki, Ibaraki University (19)) at 37 °C for 12 h in 0.1 M Tris-HCl (pH 9.0) containing 0.1% SDS. Peptides generated were extracted from the gel.
TABLE I
Amino acid sequence and mass values of selected API peptides of maize AO

| Peptide sequence   | Observed mass (H⁺) | Calculated mass (H⁺) |
|--------------------|--------------------|----------------------|
| 1. SIEELHRLFSSWFDSSVK | 2400.0             | 2398.6               |
| 2. QVNDVP1AASGDKWHPK | 1956.2             | 1955.1               |
| 3. TNSDGLVHGDTWTYK   | 1809.8             | 1807.9               |
| 4. VGAEIQGASEVYVDIPAFK | 2131.9             | 2130.3               |

**Fig. 1. Structural organization and physical map of zmAO cDNAs.** The shaded and white boxes indicate the protein coding regions and the 5′- or 3′-untranslated regions, respectively. The poly(A)尾巴 are indicated by the symbol (AAA)n. The thin lines shown in the lower part of each figure represent reverse transcription-polymerase chain reaction or RACE-polymerase chain reaction products.

and separated on columns of DEAE-5PW (2 × 20 mm; Tosoh, Tokyo) and Supersphere RP-select B (2 × 120 mm; Tosoh) connected in series with a model 1090M (Hewlett Packard) liquid chromatography system. Peptides were eluted at a flow rate of 0.2 ml min⁻¹ using a linear gradient of 0–60% solvent B, where solvents A and B were 0.1% (v/v) aqueous trifluoroacetic acid and 0.075% (v/v) trifluoroacetic acid in 80% (v/v) acetonitrile, respectively. Selected peptides were subjected to Edman degradation using a model 477A automated protein sequencer (Applied Biosystems, Inc.) connected on-line to a model 120A PTH-man degradation kit (Perkin Elmer) and to matrix-assisted laser desorption ionization time of flight mass spectrometry on a Reflex MALD-TOF Analyzer (Perkin Elmer) and to matrix-assisted laser desorption ionization time of flight mass spectrometry on a Reflex MALD-TOF Analyzer (Perkin Elmer) and to matrix-assisted laser desorption ionization time of flight mass spectrometry on a Reflex MALD-TOF Analyzer (Perkin Elmer). The DNA was digested with EcoRI, PvuII, and XbaI and fractionated on a 3% agarose gel, denatured, and transferred to a GeneScreen nylon membrane. Hybridization was carried out according to standard conditions (21), using 32P-radiolabeled full length zmAO-1 and zmAO-2 cDNA as probes. Northern blot analysis was also performed with the Genexy software package (Software Development Co., Tokyo).

**Genomic Southern and Northern Blot Analysis—Genomic DNA was prepared from maize seedlings according to the method of Shure et al. (20).** The DNA was digested with EcoRI or HindIII, fractionated on a 0.7% (w/v) agarose gel, denatured, and transferred to a Hybond-N⁺ (Amersham) nylon membrane. Hybridization was carried out according to standard conditions (21), using 32P-radiolabeled full length zmAO-1 or zmAO-2 cDNA as probes. Northern blot analysis was also performed (21), using same probes as Southern hybridization.

**Molecular Cloning and Sequencing of the cDNA Encoding AO and the Homologous cDNA**—The amino acid sequence information from API peptide 4 was used for synthesis of the sense oligonucleotide primer (5′-GGIGA(A/G)GCIGTITA(T/C)GTIGA(T/C)GA-3′ corresponding to amino acids 9–16). The antisense primer (5′-GTCCAIGTICC(A/G)TC(A/G)CTGTAAC(T/C)GTGTAAC(T/C)GCC-3′ corresponding to amino acids 1–8) was synthesized on the basis of the sequence of zmAO-1 and zmAO-2 cDNA as probes. Northern blot analysis was also performed with the Genexy software package (Software Development Co., Tokyo).

**Structural organization and physical map of zmAO cDNAs.** The shaded and white boxes indicate the protein coding regions and the 5′- or 3′-untranslated regions, respectively. The poly(A)尾巴 are indicated by the symbol (AAA)n. The thin lines shown in the lower part of each figure represent reverse transcription-polymerase chain reaction or RACE-polymerase chain reaction products.
Native Polyacrylamide Gel Electrophoresis and Immunoblot Analysis—Freshly isolated maize organs were homogenized in 2 volumes of 50 mM Tris-HCl (pH 7.5) buffer containing 1 mM EDTA, 2 mM dithiothreitol, 10 mM FAD, 1 mM sodium molybdate, and 5 mM leupeptin, and the homogenates were centrifuged at 12,000 g for 20 min. The supernatants were collected and subjected to native polyacrylamide gel electrophoresis followed by immunoblotting. Native polyacrylamide gel electrophoresis was performed with a 7.5% acrylamide gel in Laemmli buffers (22) in the absence of SDS at 4 °C. Immunoblotting was performed using anti-AO mouse serum (17).

RESULTS

Partial Amino Acid Sequences of Purified AO and cDNA Cloning—Amino-terminal sequencing of the maize AO protein failed. This might be due to blocking as a result of unknown post-translational modifications, as in the case of bovine AO (7). Partial sequence information for maize AO was obtained from protein subjected to cleavage with API. Of many peptides separated by reversed phase chromatography, the four most prominent fragments were sequenced, allowing the cloning of cDNA fragments corresponding to maize AO mRNA. Polymerase chain reaction amplification with a combination of two degenerate oligonucleotides as primers produced two kinds of 1959-bp cDNA fragments (Fig. 1). Comparison of the deduced amino acid sequences of these cDNAs to the amino acid sequences of the corresponding peptides indicated that both cDNA fragments might encode part of AO subunits. The sequences of the remaining 5'- and 3'-stretches were established by the RACE-polymerase chain reaction technique using nucleotide sequence information from the respective fragments (Fig. 1). The complete sequence of a cDNA was determined from the sequence of several independent clones obtained by 5'- and 3'-RACE procedures with oligonucleotide primers to complete the sequences as required.

One nearly full-length cDNA (zmAO-1) exhibits a 45-bp 5'-untranslated region, followed by a 4074-nucleotide open reading frame and a 263-nucleotide 3'-untranslated sequence (Fig. 2). Three presumptive polyadenylation site sequences (AATAA) are observed (double underlined) in the 3'-untranslated region. The open reading frame of the cDNA predicts a protein of 1358 amino acids of Mr 146,681 and a translation product that contains the sequence of the four peptides obtained by API cleavage of the AO protein (see also Table I).

The other nearly full-length cDNA (zmAO-2) consists of a 90-bp 5'-untranslated region, followed by a 4047-nucleotide open reading frame and a 196-nucleotide 3'-untranslated sequence (data not shown). Two presumptive polyadenylation site sequences (AATAA) are observed in the 3'-untranslated region. The open reading frame of the cDNA predicts a protein of 1349 amino acids of Mr 145,173 and a translation product that contains a sequence identical to peptide 3, and similar but not identical to peptides 1, 2, and 4 obtained by API cleavage of the AO protein. These cDNAs are highly homologous in respect to both nucleotide (83.8% identity) and amino acid (83.6% identity), excepting the nucleotide sequences of their presumptive 5'- and 3'-untranslated regions.

Analysis of the Deduced Amino Acid Sequences of zmAOs—zmAO proteins have no sequences that encode presumptive hydrophobic signal peptides, indicating they are cytosolic enzymes. One of two [2Fe-2S] centers is found between amino acid residues 50 and 80 for zmAO-1 protein and between 46 and 76 for zmAO-2 protein, respectively (Fig. 3). This iron-sulfur center is of the same type observed in ferredoxin from a number of photosynthetic organisms, in bacterial fumarate reductase.
and in eukaryotic succinate dehydrogenase (23, 24). The second putative iron-sulfur center is probably located between amino acid residues 120–123 for zmAO-1 and 116–119 for zmAO-2, respectively, since they are conserved in two other classes of molybdoflavoproteins, such as bovine AO and several XDs (7, 10, 25). Sequences conforming to the consensus for the molybdopterin cofactor binding site reside between amino acids 760–853 (zmAO-1) and 752–845 (zmAO-2), respectively (Fig. 3). An FAD-binding consensus sequence of the type described by Correll et al. (26) cannot be precisely identified in either amino acid sequence. Consensus sequences for binding of NAD are observed between amino acids 49–54 and 816–821 for zmAO-1 and 45–50 and 808–813 for zmAO-2, respectively (Fig. 3). Despite their high homology overall, some regions that are difficult to align occur between amino acids 451–453, 532–549, and 897–902 for zmAO-1 and 447–454, 533–540, and 889–894 for zmAO-2, respectively (Fig. 3).

A homology search in the SwissProt and EMBL data banks determined that the two zmA0s have a significant level of similarity with animal AOs and XDs from various sources (Fig. 3). The overall level of respective identities with zmAO-1 and zmAO-2 are 30.9 and 31.2% to bovine AO (7), 29.9 and 30.1% to human AO (initially reported as human XD) (10), 31.2 and 32.1% to human XD (27), 31.5 and 30.3% to Drosophila pseudoobscura XD (28), and 30.3 and 30.8% to Aspergillus nidulans XD (25).

**DISCUSSION**

We cloned two cDNAs for plant AO. The molecular mass values of predicted proteins for these cDNAs are coincident with that of the purified protein, and one of them (zmAO-1) contains the sequence of four peptides obtained by API cleavage of the purified AO protein. We conclude that zmAO-1 encodes maize AO that had been purified previously. In the case of human AO, the predicted amino acid sequence shows a relatively lower level of homology to their XD (49.3%) than to bovine AO (85.7%). Since the deduced amino acid sequence for the other cDNA (zmAO-2) shows high similarity to zmAO-1 (83.6%), we considered that both encode maize AOs that may have related functions.

As shown in Fig. 3, predicted proteins for zmA0s have a significant level of similarity to those animal AOs and XDs and can be aligned along the whole length of the proteins. zmA0s exhibit unusual clustering of cysteines in the amino-terminal 200 amino acid residues. Nine of these 12 cysteines are conserved in both animal and plant AOs, and eight of them are

**FIG. 3. Amino acid sequence comparison between AOs and XDs.** Amino acid sequences for AOs from bovine (bAO) and from human (hAO) and for XDs from human (hXD), D. pseudoobscura (D.p.XD), and A. nidulans (A.n.XD) were aligned with the zmAO-1 and zmAO-2 amino acid sequences determined here. Identical amino acids are boxed.
conserved in both AOs and XDs. Many of them must contribute to the structure of the two Fe-S centers. Li Calzi et al. suggested that one (or both) of the two cysteines, amino acids 149 and 151 for bovine AO located in a strictly conserved stretch of amino acids, were involved in iron ligation in AO and XDs. These cysteines and the conserved region are found in both zmAOS (amino acids 165–176 for zmAO-1), supporting this possibility. In addition, zmAOS also contain sequences that conform to the consensus for the molybdopterin cofactor binding site (amino acids 760–853 for zmAO-1). These results are helpful for confirmation of the previous report concerning the biochemical characters of purified maize AO (17). As shown in Fig. 3, there are some additional regions conserved to some extent among plant AOs, mammalian AOs, and XDs (amino acids 597–608, 814–822, 1053–1064, and 1173–1230 for zmAO-1). Thus, plant AOs are similar to related enzymes from XDs. These cysteines and the conserved region are found in both zmAOS (amino acids 165–176 for zmAO-1), supporting this possibility. In addition, zmAOS also contain sequences that conform to the consensus for the molybdopterin cofactor binding site (amino acids 760–853 for zmAO-1). These results are helpful for confirmation of the previous report concerning the biochemical characters of purified maize AO (17). As shown in Fig. 3, there are some additional regions conserved to some extent among plant AOs, mammalian AOs, and XDs (amino acids 597–608, 814–822, 1053–1064, and 1173–1230 for zmAO-1). Thus, plant AOs are similar to related enzymes from phylogenetically different organisms, indicating they evolved from a common ancestral gene. The most primitive animal species in which AO activity has been found is the coelenterate *Semania luciae* (29).

Two consensus sequences for NAD binding are found in both zmAOS; however, AO does not require the cofactor for its catalytic activity and in fact purified maize AO is devoid of dehydrogenase activity. It is thus unlikely that these structural elements have functional significance. This is also the case for bovine AO (7).

Glatigny and Scazzocchio suggested that the ERXXXH motif (amino acid residues 910–915 of *A. nidulans* XD) is involved in determining substrate specificity. These regions are not conserved in animal AO proteins and plant AOs. In addition, no significant similarities are observed in those corresponding regions among animal AO proteins and zmAO-1 and zmAO-2 proteins. This may reflect the different substrate specificities of plant and animal AOs, since the former have affinity for aromatic aldehydes while the latter do not. To analyze the substrate specificity of the two zmAOS, we tried to produce recombinant proteins, but they were almost completely degraded when the cDNA were expressed in *Escherichia coli*. This problem also occurs with another molybdopterin cofactor-containing protein, nitrate reductase. Further research will be required for elucidation of the different substrate specificities of the zmAOS.

In plants, AOs are thought to be involved in plant hormone biosynthesis. Molybdopterin cofactor-deficient mutants of barley and tobacco are deficient in AO and XD activities and have impaired ABA production (15, 16). This indicates that ABAlD oxidase is a molybdenum-containing enzyme which is indispensable for ABA biosynthesis. The ABAlD oxidase oxidizes ABAlD to ABA, but such an enzyme has not been purified. In the case of IAA biosynthesis, the pathway has been extensively investigated (18, 30–32), but is still poorly understood. IAAlD oxidase could catalyze the final step in the pathway where IAA is produced from tryptophan (11, 14, 17). However, this has only been studied in a few plants. Recently, a *sur* mutant that forms an abnormal number of roots has been isolated from *Arabidopsis thaliana* (33). It was thought that the phenotype was caused by overproduction of IAA, and, in fact, IAA levels are much higher in seedlings of the mutant. We have checked AO activity in the wild-type and mutant seedlings after native polyacrylamide gel electrophoresis, revealing that at least three AO activity bands. One band, showing a substrate preference for indole-3-aldehyde, is much more intense in the mutant seedlings. Three independent cDNA clones have also been isolated from *A. thaliana* using degenerate primers, and one of these is highly expressed in the *sur* mutant. 

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Addendum—While this article was being submitted and reviewed, we found a paper describing a gene for a putative molybdenum cofactor-containing plant hydroxylase (Ori, N., Ested, Y., Pinto, P., Paran, I, Zamir, D., and Fluhr, R. (1997) J. Biol. Chem. 272, 1019–1025).

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