TAO1, a Representative of the Molybdenum Cofactor Containing Hydroxylases from Tomato*

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Aldehyde oxidase and xanthine dehydrogenase are a group of ubiquitous hydroxylases, containing a molybdenum cofactor (MoCo) and two iron-sulfur groups. Plant aldehyde oxidase and xanthine dehydrogenase activities are involved in nitrogen metabolism and hormone biosynthesis, and their corresponding genes have not yet been isolated. Here we describe a new gene from tomato, which shows the characteristics of a MoCo containing hydroxylase. It shares sequence homology with xanthine dehydrogenases and aldehyde oxidases from various organisms, and similarly contains binding sites for two iron-sulfur centers and a molybdenum-binding region. However, it does not contain the xanthine dehydrogenase conserved sequences thought to be involved in NAD binding and in substrate specificity, and is likely to encode an aldehyde oxidase-type activity. This gene was designated tomato aldehyde oxidase 1 (TAO1). TAO1 belongs to a multigene family, whose members are shown to map to clusters on chromosomes 1 and 11. MoCo hydroxylase activity is shown to be recognized by antibodies raised against recombinant TAO1 polypeptides. Immunoblots reveal that TAO1 cross-reacting material is ubiquitously expressed in various organisms, and in plants it is mostly abundant in fruits and rapidly dividing tissues.

Molybdenum cofactor (MoCo) binding is common to a group of ubiquitous enzymes, including nitrate reductase, xanthine dehydrogenase, sulfite oxidase, and aldehyde oxidase (1, 2). These enzymes are involved in various types of oxidative metabolism, and show broad and occasionally overlapping specificities (1). A subset of this enzyme class, MoCo hydroxylases, includes the structurally similar enzymes xanthine dehydrogenase (XD) and aldehyde oxidase (AO). These enzymes contain, in addition to the molybdenum cofactor, FAD and two types of iron-sulfur centers (3). They have been shown to be related to the AO from Desulfovibrio gigas (MOP) for which a crystal structure has recently been described (4).

AO (aldehyde-oxygen oxidoreductases) are widely distributed among various organisms. They are characterized as dimers of two 150-kDa subunits and catalyze the oxidation of N-heterocyclic compounds in the presence of O₂ but appear to display a broad range of substrate specificities (5). In humans, AO has been implicated in familial amyotrophic lateral sclerosis (6) and hepatotoxicity of alcohol (7). In plants, AO activities are implicated in the biosynthesis of two plant hormones, abscisic acid (ABA) and indole acetic acid (IAA). The plant hormone ABA is involved in various processes, including the reaction of plants to environmental stresses such as wounding, water stress, seed development, and plant development (8). In the ABA biosynthesis pathway, AO is thought to catalyze the conversion of ABA aldehyde to ABA, which is considered to be the last step in the biosynthesis of ABA. Indeed, MoCo defective tobacco and barley mutants were found to be deficient in ABA synthesis (9, 10). IAA is involved in many aspects of plant growth and development, however, its biosynthetic pathway remains to be elucidated. Recently, an AO activity from maize coleoptiles was shown to efficiently oxidize indole-3-acetaldehyde, a putative precursor of IAA (11). It is unknown whether the broad substrate specificities attributed to AO originate from a single enzyme or from a family of closely related enzymes.

XD is very similar to aldehyde oxidase in cofactor content, molecular weight, and sequence (1, 3, 12). It is a ubiquitous enzyme which is involved in purine metabolism. In plants, XD plays a central role in nitrogen assimilation, and was found to be highly enriched in nitrogen-fixing nodules of legumes of the ureide class (13). Genes encoding XD have been identified in mammals, chicken, flies, and Aspergillus (14–20).

The distribution and substrate specificities of human AO and XD activities were shown to overlap but to be distinct (5, 21). Comparison of the sequences of recently described AO genes with those of various genes reveals high homology between eukaryotic AO and XD genes, particularly in the sequences involved in the binding of the different cofactors. However, several sequences thought to be involved in NAD binding and substrate specificity are absent from AO, and can be used to differentiate between the two enzymes. The absence of the NAD-binding site is in agreement with the fact that AO does not require NAD for its action, and the lack of substrate binding sequences corresponds to the different specificity range of the two enzymes. Strikingly, a high level of homology was found between eukaryotic XD and AO genes and the bacterial MOP in the domains which participate in the iron-sulfur centers and MoCo binding (4).

The genes which encode either XD or AO have not been described in plants. Their isolation will shed light on the origin of the different attributed activities, and will aid in the elucidation of the biosynthetic pathways in which these enzymes
are involved. Here we describe a novel gene family from tomato, tomato aldehyde oxidase (TAO), which is highly homologous to XD and AO genes. Detailed sequence comparisons in the different functional domains suggest that this gene family belongs to the AO rather than the XD type of MoCo containing the different functional domains. The region between these two parts is indicated with a solid black line. TAO1–10, TAO1–1, and TAO1–5 are cDNA clones in the Agt10 vector. TAO1-G7 is a genomic subclone, originating from YAC 340-63. The insert of the cDNA clone TAO1–10 was used as a probe for Southern blot hybridizations. The fragments ab 1 (amino acids 950-1128) and ab 15 (amino acids 1129–1315), from the partial cDNA clone TAO1–10, were used for the preparation of antibody 1 and 15, respectively, as described under "Experimental Procedures." Symbols are: E, EcoRI site originating from the TAO1 open reading frame; E', EcoRI sites originating from the vector sequence. B, an immunoblot of total protein extract from tomato ovaries. Protein (35 μg) was fractionated by SDS-PAGE and immunoblotted. Identical blots were incubated with antibodies 1 (ab1) and antibodies 15 (ab15), as indicated (see "Experimental Procedures" for description of the antibodies), and developed using the ECL method (Amerham). Left panel, 20-s and 1-min exposure of ab 1 and ab 15, respectively; right panel, 20-min overexposure.

Experimental Procedures

Genetic and Physical Mapping—The Lycopersicon pennellii introgression lines population (ILs), described by Eshed and Zamir (22) was used to genetically map the different copies of TAO1. RFLP analysis of Southern blots was performed as described previously (23). Physical distance of TAOs from TG105 was established by partial digestion of YAC 340-63. The digests were fractionated on counter-clamped homogeneous electric field gels (Bio-Rad), blotted, and hybridized with probes. The maximal distance between a pair of markers was estimated according to the smallest partial band that contained both markers.

Clones and Sequence Analysis—YAC 340-63, which contains the RFLP marker TG105A, was generated from the tomato line Rio Grande-PtO, and cloned in the vector pYAC 4 (24). The sequence of TAO1 was obtained from four overlapping clones, as shown in Fig. 1. Clones TAO1–1 and TAO1–10 were isolated by using YAC 340-63 for screening of a cDNA library from roots of Lycopersicon esculentum c.v. Monegros. Since these cDNA clones were not complete, the 5’ end of TAO1–1 was used to screen again the same cDNA library, and a longer but still partial cDNA clone was isolated, TAO1–5. The 5’ end of the sequence was obtained from a genomic clone, TAO1-G7. Overlapping sequence confirmed that the different clones originated from the same gene. Sequence analysis was performed using the sequence analysis software package of the University Wisconsin, Genetics Computer Group (25). The insert of cDNA clone TAO1–10 was used as a probe for Southern blot analysis.

Production of Anti-IAO1 Antibodies and Immunoblotting—Two segments of TAO1 were overexpressed in bacteria, using two different types of expression vectors (Fig. 1). For antibody 1, a 500-base pair long EcoRI fragment from the partial cDNA clone TAO1–10 (containing one internal EcoRI (E) site and one EcoRI site from the vector (E’), was fused to the glutathione S-transferase protein from Schistosoma japonicum, using the pGEX expression vector (Pharmacia). The resulting fusion protein contained amino acids 950–1128 of the TAO1 protein (Fig. 1A, ab 1). For antibody 15, the construct contained the flanking 800-base pair EcoRI segment from the same cDNA clone (again containing one internal EcoRI site and one site from the vector), which was fused to a stretch of histidine residues, using the pSET expression vector (Invitrogen). The resulting fusion protein contained amino acids 1129–1315 of the TAO1 protein (Fig. 1A, ab 15). The polypeptides produced in these clones were purified by glutathione-agarose for the glutathione S-transferase fusion protein and by bound nickel columns for the histidine-fusion proteins. The purified peptides were injected into guinea pigs to obtain antibodies ab 1 and ab 15. Both antibodies recognized on immunoblots polypeptides of apparent 78 kDa (Fig. 1B), which were not detected by control antibodies (not shown).

Protein Gels, Immunoblotting, and Activity Assays—SDS-PAGE and immunoblotting were performed as described by Raz and Fluhr (26), using the ECL system (Amersham). Young green fruits were ground in 20 ml of extraction buffer containing 50 mM Tris-HCl (pH 2.5), 1 mM EDTA, 1 mM sodium molybdate, 2 mM 2-mercaptoethanol, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 μM FAD, and 0.5 g of polyvinylpyrrolidone, at 4 °C. The extracts were filtered through 4 layers of gauze, and centrifuged at 10,000 × g for 20 min. The supernatant was applied to a 2-ml Q-Sepharose column (Pharmacia). The column was washed with 3 volumes of extraction buffer without polyvinylpyrrolidone, and proteins were eluted by 1 volume of extraction buffer containing 0.5 M NaCl. The eluate was either dialyzed overnight or desalted on a P6 (Bio-Rad) minidialyzing column. Samples (50 μg) were fractionated on a native PAGE and activity was assayed as described (10).

Results

Sequence Analysis of the TAO Gene Family—During a high resolution mapping study of the I2 Fusarium wilt resistance gene from tomato, we used a 350-kilobase YAC clone, YAC 340-63, to screen a root cDNA library. One of the partial cDNAs isolated was sequenced and found to be closely homologous to the mammalian AO, not yet described in plants. The gene has been designated tomato aldehyde oxidase 1 (TAO1). The sequence of TAO1 was obtained by a combination of overlapping sequence fragments from 3 different clones (see "Experimental Procedures"). The deduced amino acid sequence, of 1315 amino acids, is compared in Fig. 2 with the deduced amino acid sequences of a representative aldehyde oxidase, the human xdh2 (20), and a representative xanthine dehydrogenase, the rat xdh (14), which are the most similar to TAO1. The human xdh2 was originally defined as a xanthine dehydrogenase-type (15), but was shown to actually encode an aldehyde oxidase activity (19, 27). Regions that contain identical amino acids are indicated with boxes. The homology resides mainly in the NH2 terminus of the protein, which contains the iron-sulfur centers, and in the COOH-terminal half that contains the MoCo-binding domain (see below). The region between these two parts is conserved to bear the FAD-binding region and remains less conserved. We wished to narrow this region by finding TAO1 to either the XD or AO groups of MoCo hydrolases. In Fig. 3, we compare conserved functional regions of TAO1 to the consensus sequence of XD-type genes, and to 3 AO-type genes. Xanthine dehydrogenases contain two iron-sulfur centers, an FAD-binding domain, an NAD-binding site, a MoCo complexing domain, and a substrate binding domain (Fig. 3, top; Ref. 19). The locations of the two iron-sulfur domains have now been pin-
pointed by the recently described crystal structure of MOP (4), and shown to be highly conserved among XD and AO. The consensus sequence of both iron-sulfur domains is also conserved in TAO1 (Fig. 3, iron sulfur 1 and iron sulfur 2), except for the substitution of the first cysteine for methionine in the first iron-sulfur center of TAO1. The cysteines are considered crucial for the iron-sulfur domain and we are not aware of their substitutions in other cases. As the sequence of the TAO1 gene was obtained from a genomic clone (see “Experimental Procedures”), the deduced sequence at that point may reflect an intron junction rather than the actual first methionine residue. However, sequencing of an additional 1020-base pair upstream region showed no evidence for the presence of an additional cysteine-containing exon, nor do the relevant surrounding nucleotides give evidence for a clear splicing consensus sequence. TAO1, as well as MOP, BAO, and HXD2 do not contain the sequence Gly394-Tyr-Arg (underlined in Fig. 2), which in the XD-type enzymes is thought to be involved in NAD cofactor interaction (28). The precise domain involved in the flavin adenine dinucleotide (FAD) binding is not fully established.

Fig. 2. Comparison of the deduced amino acid sequence of TAO1 with XD and AO-type sequences. Humanxdh2 (20) represents an aldehyde oxidase (19, 27) and Ratad (14) represents a xanthine dehydrogenase. Boxes show identical residues. The alignment was obtained with the PILEUP function of the Wisconsin sequence analysis software package (Genetics Computer Group, Madison, WI). Identical amino acids were grouped by the ShadyBox software (Camson Huynh, Australian National Genomic Information Service). The putative NAD binding site, and a sequence suggested to be involved in substrate specificity of XD, are underlined in the Ratadh sequence. Degrees of similarity and identity with TAO1 are, respectively, 55 and 31% for Humanxdh2, and 56 and 35% for Ratadh.
and cannot be deduced from the crystal structure of MOP as this region is completely absent from MOP. Indeed in this region a relatively low level of homology exists among the different genes (Fig. 2).

The three-dimensional structure of MOP suggests a funnel-like structure which leads from the surface of the protein to a substrate binding pocket, in close proximity to the site that binds MoCo (4). The domains which participate in MoCo complexing and substrate binding are highly conserved among XDs, AO, and TAO1 as shown in Fig. 3, regions 1–5 (4). However, in several positions amino acid substitutions differentiate TAO1 from XDs, and in those positions TAO1 is more similar to MOP, BAO, and HXD2. Thus, glutamate 807 of rat XD, which is completely conserved among all XDs, is substituted with hydrophobic amino acids in BAO, HXD2, MOP, and TAO1, respectively (Fig. 3, region 1). The equivalent position in MOP is occupied by Phe425, which is situated in the substrate binding pocket, and may participate in determining the substrate specificity that differentiates XD and AO activities within the MoCo binding site. Region 4 of the MoCo-binding domain, which was shown by mutational analysis to play a role in substrate specificity of XD (2), is highly conserved among XDs, but is not conserved between XDs, AOs, and TAO1. In addition, the consensus sequence ERXXXH (underlined in Fig. 2) conserved between all XD-type genes, is completely absent from TAO1, as well as from BAO, HXD2/AO, and MOP. This sequence was suggested by mutational analysis to also be involved in determining substrate specificity of XD (19). Thus, the main apparent differences between TAO1 and XD-type enzymes lie in the proposed NAD and substrate-binding domains. The similarity of TAO1 to MOP, BAO, and HXD2 in these regions suggests that TAO1 encodes a MoCo hydroxylase of the AO-type.

TAO1 Cross-reacting Material Is Highly Abundant in Tomato Fruits—In order to examine the expression pattern of TAO1, two non-overlapping segments from its 3′ end were expressed as fusion proteins in bacteria, and antibodies were raised against the recombinant fusion proteins (see “Experimental Procedures”). In immunoblots of tomato tissue a 78-kDa size protein band was detected (Figs. 1B and 4). Occasionally two protein bands of similar size can be resolved, which may represent several related but distinct proteins of the TAO1 family (see below), or additional successive degradation products. The 78-kDa size is considerably less than the calculated 144.5-kDa molecular mass for TAO1. However, as both antibodies, but not control antibody, reacted on immunoblots with polypeptides of the same apparent 78-kDa molecular mass, the possibility that the polypeptide detected is irrelevant is unlikely (Fig. 1B). Thus, the observed size is probably a physiological or extraction-based degradation product. Similarly, a degradation of XD proteins to 80-kDa products was detected in SDS-PAGE analysis of Drosophila proteins (29), and an aldehyde oxidase from maize was shown to degrade upon SDS-PAGE, giving a product of 85 kDa (11).

We wished to examine the expression pattern and tissue distribution of proteins from the TAO family in tomatoes. An immunoblot of various tomato organs is shown in Fig. 4. TAO1 cross-reacting material (CRM) is present in all tomato organs. This material is abundant in ovary, in developing fruits, and in dividing tissues, such as the shoot tips, which contain apical meristematic tissue. TAO1 CRM was found to be present at high levels in all fruit tissues examined at various developmental stages (not shown). An additional, lower molecular weight band is apparent in some tissues. This is probably an

FIG. 3. Comparative analysis of the functional domains of TAO1. The schematic structure of select MoCo hydroxylases is shown at the panel top; ASPXD, an XD-type gene from Aspergillus nidulans (19); HXD2/AO, a humanxdh2, an AO-type gene (19, 20, 27); BAO, bovine aldehyde oxidase (12); and MOP, D. gigas aldehyde oxidoreductase (4). Sequence comparisons of the iron-sulfur centers and the MoCo and substrate binding sites are shown below (4). CON, consensus sequence of the iron-sulfur domains; XDCON, consensus sequences of the motifs found in 5 XD-type genes from: Drosophila melanogaster (16, 17); rat (14); human (15); chicken (18); and A. nidulans (19). Residue numbers of the consensus XD-type domains refer to Drosophila melanogaster XD. The arrow indicates an amino acid in the substrate binding pocket highly conserved among the XD-type genes, but different in AO-type genes (4).
activities which cross-react with anti-TAO1 antibodies—The relatively high expression of TAO1 CRM in tomato fruits prompted us to examine directly aldehyde oxidase and xanthine dehydrogenase activities in these tissues by a native activity gel assay (Fig. 5). The upper band in Fig. 5A (asterisk) was substrate independent. In leaf tissue, a band of xanthine dehydrogenase activity was detected when hypoxanthine was used as the substrate (Fig. 5, arrow). In fruits, overlapping bands could be detected, resulting in a continuous region of stain (bar in Fig. 5A, panel HX). When the xanthine dehydrogenase-specific inhibitor allopurinol was added to the reaction, the single XD activity band from leaves disappeared, as well as the most rapidly migrating band from the fruits. Thus, most activity bands in the fruits were not inhibited by allopurinol the most rapidly migrating band from the fruits. Thus, most single XD activity band from leaves disappeared, as well as genase-specific inhibitor allopurinol was added to the reaction, tions. To this end, leaf and fruit proteins comigrating with the observed and the TAO1 CRM, detected in denaturing conditions. The proteins were eluted be separated to smaller fragments upon SDS-PAGE, as has been documented for XD during ischemia (14).

Tomato Fruits Are Enriched in Several Aldehyde Oxidase Activities Which Cross-react with Anti-TAO1 Antibodies—The relatively high expression of TAO1 CRM in tomato fruits prompted us to examine directly aldehyde oxidase and xanthine dehydrogenase activities in these tissues by a native activity gel assay (Fig. 5). The upper band in Fig. 5A (asterisk) was substrate independent. In leaf tissue, a band of xanthine dehydrogenase activity was detected when hypoxanthine was used as the substrate (Fig. 5, arrow). In fruits, overlapping bands could be detected, resulting in a continuous region of stain (bar in Fig. 5A, panel HX). When the xanthine dehydrogenase-specific inhibitor allopurinol was added to the reaction, the single XD activity band from leaves disappeared, as well as the most rapidly migrating band from the fruits. Thus, most activity bands in the fruits were not inhibited by allopurinol (Fig. 5A, panel HX+AP), moreover allopurinol itself served as a substrate for these activities (not shown). The allopurinol-in-sensitive fruit activities were also detected when the AO-specific substrate 6-methylpurine was used, indicating that this tissue is rich in AO activity (5). This correlates with the high abundance of TAO1 CRM in fruit tissue.

We wished to establish a correlation between the activities observed and the TAO1 CRM, detected in denaturing conditions. To this end, leaf and fruit proteins comigrating with the activity bands shown in Fig. 5A were excised from lanes adjacent to those stained for activity. The proteins were eluted electrophoretically and fractionated by a SDS-PAGE denaturing gel. As shown in Fig. 5B, anti-TAO1 antibodies detected a major band, of apparent molecular mass of 78 kDa. Minor bands are probably degradation products of the 78-kDa band. This result suggests that the smaller than expected 78-kDa CRM originates from enzymatically active proteins. Specific cleavage may be a result of denaturation during SDS-PAGE fractionation.

Additional degradation product and is occasionally observed.

Fig. 4. Immunoblot analysis of TAO1 cross-reacting proteins in various tomato organs. Proteins (35 μg) from the indicated tomato tissues were fractionated by denaturing SDS-PAGE and immunoblots were developed with anti-TAO1 antibodies (ab 15). The blot was exposed for 3 min. Longer exposures did not reveal additional bands. The different parts of the tomato fruits from which samples were taken are shown below. Apical meristem is the top 5 mm of shoot tips, which include apical meristematic tissue.

Fig. 5. MoCo hydroxylase activities in leaf and fruit tissue. A, total proteins (50 μg) from leaves and fruits of L. esculentum were fractionated on an 8% native PAGE, as indicated. The gels were stained with hypoxanthine (HX); hypoxanthine and allopurinol (HX+AP); or 6 methylpurine (6-MP). Asterisk indicates position of a substrate-independent activity band. Arrow indicates specific hypoxanthine-dependent activity detected in leaf. The bar indicates fruit specific activity. B, immunoblot of the activity bands excised from the native gel and subsequently fractionated by denaturing SDS-PAGE. The blot was incubated with anti-TAO1 antibodies (ab 15) and exposed for 5 min.

Fig. 6. Immunoblot analysis of TAO1 cross-reacting proteins from different organisms. Proteins (40 μg) were fractionated by denaturing SDS-PAGE and immunoblots were developed with anti-TAO1 antibodies (ab 15). Plant protein extracts are from young developing fruits of tomato, pepper, petunia, and from a developing ear of wheat, as indicated. Animal protein extracts are from highly differentiated human hepatoma cells (HUMHEPG2 and HUMHEPG3) and from wild-type (wt) and the XD null mutant ab 15 (506) of Drosophila (DROS). The blot was exposed for 5 min.

Analysis. Alternatively, the native protein may be processed in vivo but still retain intactness and activity in native gels, and be separated to smaller fragments upon SDS-PAGE, as has been documented for XD during ischemia (14).

TAO1 Cross-reactive Material Is Ubiquitous in Plants and Animals—TAO1 antibodies were derived from highly conserved regions in the MoCo binding area (see Figs. 1–3). This prompted us to examine the antibodies for cross-species reactivity in a “zoo-garden-type” immunoblot (Fig. 6). Different levels of TAO1 cross-reactive material of apparent 78-kDa migration size could be detected in young fruits of all plants assayed, dicots and monocots. In petunia seed pods a considerable amount of 140-kDa apparent molecular mass fragment was also detected. Cross-reacting material was also detected in liver cells and in Drosophila. In Drosophila, mutants which do not express XD are well known (30). We examined one of these mutants, r7 506 null for XD, for the presence of TAO1 cross-reacting material (Fig. 6, compare lanes DROS wt and DROS 506). A significant level of cross-reacting material could be detected in the mutant. The findings are consistent with the observation that the antibodies recognize a broad family of MoCo hydroxylases, in which TAO1 is related to the AO type.

Genetic Mapping of TAO1—Southern blot analysis revealed that TAO1 was part of a multigene family (Fig. 7). To position
the members of the TAO family on the tomato genetic map we used a near isogenic lines (IL) mapping population, in which single chromosome segments from *L. pennellii* were introgressed in 50 ordered lines into a *L. esculentum* background (31). The absence of an *L. esculentum*-type polymorphic fragment or the presence of a *L. pennellii*-type polymorphic fragment in a Southern blot of a specific IL, indicates that the origin of the particular fragment is from the introgressed chromosomal segment. The ILs which proved relevant for the mapping of the TAO family are illustrated in Fig. 7. A high level of polymorphism was detected between the original parental lines on a *TagI* digest Southern blot, probed with a segment of the TAO1 gene (Fig. 7, right panel, compare *L. pennellii* and *L. esculentum*). Nearly all the ILs, as exemplified by IL 8-1, displayed the *L. esculentum*-type fragment pattern (Fig. 7, right panel). However, specific ILs lacked a subset of *L. esculentum*-type bands, indicating that the introgressed region contained a TAO copy. A TAO gene cluster was localized in this way to the region of overlap between lines IL 11-3 and IL 11-4, as several *L. esculentum*-type fragments were absent from both of these ILs (fragments are indicated with arrows and designated “a” in the right panel of Fig. 7). These fragments, as well as other nonpolymorphic fragments, were also present in YAC 340-63, generated from *L. esculentum*, which was previously mapped to this region of chromosome 11 (32). This result confirms the mapping of the “a” fragments to chromosome 11, and also delineates the additional non-polymorphic bands, mutual to *L. pennellii* and *L. esculentum*, which map to the same region of chromosome 11. Physical pulse field electrophoretic mapping of YAC 340-63 has revealed that TAOa is distal of marker TG105, with a distance of approximately 50 kilobase between TG105 and TAOa (data not shown). Other TAO copies were similarly mapped to a second loci, TAOb, on chromosome 1 in the region of overlap between IL 1-1 and IL 1-2 (Fig. 7, left panel). Although screening the rest of the 50 introgression lines did not reveal additional mapping loci, some TAO fragments were not polymorphic between the parental lines. Many of these could be assigned to YAC 340-63 positioned at the TAOa locus. However, the possibility exists that the few remaining nonpolymorphic TAO genes map to loci other than TAOa and TAOb. TAO1, the representative TAO gene sequenced above, was assigned unambiguously to the TAOa locus based on direct sequencing of YAC 340-63 subclones.

**DISCUSSION**

We describe here the isolation and characterization of a new gene from tomato, TAO1. Highest homology to TAO1 was found among xanthine dehydrogenase (XD) and aldehyde oxidase (AO) genes from several organisms (12, 14–20). It contains the consensus sequences of the two iron-sulfur domains and the MoCo-binding domains of XD and AO. We classify TAO1 as an AO-type structure, as it lacks sequences indicative of the NAD-binding domain and sequences suggested to be involved in XD substrate specificity. The lack of these particular features is reminiscent of the aldehyde oxidoreductase structure from *D. gigas*, (4), the bovine AO (12), and HXD2, a human MoCo containing hydroxylase gene (20) which was recently suggested to encode AO-type activity rather than XD-type (19, 27). Isolation of the cognate TAO1 polypeptide and direct examination of its activity will be necessary to verify these observations.

TAO1 belongs to a multigene family. The family members detected may code for other XD- or AO-type activities with variable substrate specificities and expression patterns. This may suggest that broad substrate range of AO activities originates from different, closely related, enzymes, rather than a single enzyme. The TAO family members display an unusually high level of genetic polymorphism detected by RFLP. This is reminiscent of the high level of polymorphism, detected in both RNA and protein, of XD enzymes from *Drosophila* ecotypes (33, 34). The polymorphism enabled facile mapping of TAO members to two gene clusters, on chromosomes 1 and 11. The clustering of closely related genes from the same gene family is akin to the clustering found in the recently isolated plant resistance genes (35–37). This phenomenon was also observed by us for the *Fusarium* wilt disease resistance gene candidate *I2C*, which maps in close genetic and physical proximity to TAO1. Resistance genes are also characterized by their highly polymorphic nature which are thought to be part of the plants adaptability to the changing pathogenic environment. Interestingly, AO activities in the liver carry out biochemical detoxification (12). If a similar detoxification function is played by the plant AO activities, then environmental adaptability may dictate pressure toward maintaining the observed genetic polymorphism in plant AO genes.

Two types of MoCo containing hydroxylase activities have been detected in plant extracts. One activity is inhibited by the XD specific inhibitor allopurinol and is thus classified as XD-type activity. The second type of activity is highly enriched in tomato fruits, is allopurinol insensitive, and can utilize both typical AO substrates such as 6-methylpurine and hypoxan-

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3 N. Ori, Y. Eshed, I. Paran, G. Presting, D. Aviv, S. Tanksley, D. Zamir, and R. Fluhr, unpublished observation.
thine, and the XD inhibitor allopurinol. Anti-TAO1 antibodies detect polypeptides in leaf and fruit. Due to the polyclonal nature of the anti-TAO1 antibodies we cannot rule out crosstalk between AO- and XD-type polypeptides, however, the antibody reactivity appears well correlated with the prominent AO activity detected in fruits. In addition, we have noted that the spectral stress induces accumulation of AO activity in leaves which was also correlated with an increase in cross-reacting polypeptide accumulation as detected by TAO1 antibodies.4

TAO1 CRM indicated immunoreactive polypeptides of approximately 78 kDa, which were detected by 2 different antibodies prepared from fusion peptides originating from sequential nonoverlapping carboxyl-terminal regions. In the cases of Drosophila and fruit pods from petunia plants an additional approximately 140-kDa polypeptide was detected, consistent with the predicted molecular mass of AO. In tomato, the 78-kDa molecular mass was detected when enzymatically active proteins were eluted from native gels and refractionated on SDS-PAGE. Based on the deduced amino acid sequence, the terminal 78-kDa part would begin roughly at amino acid 590, with the predicted molecular mass of AO. In tomato, the 78-kDa polypeptide accumulation as detected by TAO1 antibodies.4

Here we show that TAO1 immunoreactive polypeptides are abundant in ovaries and fruits of tomato and other plant species. The presence of immunoreactive polypeptide in tomato fruits correlates with the high level of AO-type activity detected in tomato fruits. A biological role for the presence of TAO in apical meristematic tissue and fruit is not known, and it may be directly related to the biosynthetic capacities required for typical plant metabolic “sink” tissues. Alternatively, the expression of AO in those tissues may fulfill the specific needs for the final steps in biosynthesis of two plant hormones, ABA and IAA. Recently, several AO activities were shown to be involved in the first step of ABA biosynthesis (38). An AO-type activity has been implicated by mutational analysis to be essential for the last step of ABA biosynthesis which is the conversion of ABA aldehyde to the active carboxylic form of the plant hormone ABA (9, 10). Interestingly, stitens, one of the tomato ABA-deficient mutants putatively lacking AO activity, maps to the same chromosomal region as TAO9 (39). With the help of a high resolution mapping population of that region we are pursuing the possibility that a member of TAO9 is involved.5 In maize an AO activity, enriched in the coleoptile apical region, was shown to oxidize indole-3-acetaldehyde into IAA (11, 40). The abundant tissue-specific expression detected by TAO1 antibody in fruit may reflect the role ABA plays in seed maturation and dormancy, while the elevated expression detected in apical meristems is consistent with the role this tissue plays as a known source of auxin biosynthesis. The isolation of a member of the novel TAO gene family from tomato, highly homologous to the AO group of MoCo containing hydrolases offers a useful starting point for the analysis of this pivotal gene family in plants.

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