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Short sequence-paper

Molecular cloning and expression patterns of three putative functional aldehyde oxidase genes and isolation of two aldehyde oxidase pseudogenes in tomato[1]

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Abstract

The final steps in the biosynthesis of the plant hormones abscisic acid (ABA) and indole-3-acetic acid (IAA) have been shown to be catalyzed by aldehyde oxidases (AO). We have cloned three putative functional AO genes (*TAO1*, *TAO2* and *TAO3*) and two putative AO pseudogenes (*TAO4* and *TAO5*) in tomato. The *TAO1* cDNA described here includes the correct amino terminus of the encoded TAO1 protein and is different at the 5′-end from the *TAO1* sequence in GenBank (accession number U82558). Northern analysis shows that *TAO1* is expressed mainly in vegetative tissues and *TAO2* is expressed in both vegetative and reproductive tissues. *TAO3* expression was not detectable by Northern hybridization. These results suggest that each AO may play different roles in the regulation of tomato growth and development.

Keywords: Aldehyde oxidase; Abscisic acid; Indole-3-acetic acid; (Tomato)

The plant hormone abscisic acid (ABA) plays important roles in plant adaptation to variable environments and in seed dormancy and germination. The functions of ABA in the control of leaf stomata opening and closing and seed dormancy are clearly illustrated by ABA deficient mutants [1]. In tomato, three ABA deficient mutants, namely *notabilis* (*not*), *flacca* (*flc*), and *sitiens* (*sit*), have been known for over 40 years [2]. The recent cloning of a putative 9-*cis*-epoxy-carotenoid dioxygenase (LeNCED1) gene in tomato and analysis of the *not* mutant revealed that LeNCED1 was encoded by the *NOT* gene, and a base-pair deletion in the coding region of the *NOT* gene resulted in a frame-shift mutation in the *not* mutant [3]. Biochemical characterization of *flc* and *sit* showed that both mutants are defective in the last step of ABA biosynthesis in which abscisic aldehyde is oxidized to form ABA by a molybdenum cofactor (Moco)-containing aldehyde oxidase (EC 1.2.3.1; AO) [4]. In *flc* leaves, the activities of both AO and another Moco-containing enzyme xanthine dehydrogenase (EC 1.1.1.204; XD) were not detectable [5,6]. Thus, the *FLC* gene is thought to be involved in Moco biosynthesis while *SIT* might be the structural gene of abscisic aldehyde oxidase [5]. However, the *FLC* and *SIT* genes have not been cloned so far. Only recently, one of four known *Arabidopsis* AO genes, *AAO3*, was found to encode an abscisic aldehyde oxidase [7]. The biosynthesis of plant hormone indole-3-acetic acid (IAA) was also suggested to be catalyzed by an aldehyde oxidase [8–11]. The protein encoded by *AAO1* in *Arabidopsis*, AAOα, with a high affinity for indole-3-acetaldehyde, is highly expressed in an IAA overproducing *sur1* mutant [10]. At present, only one AO gene from tomato (*TAO1*) has been reported but it is not likely to be the *SIT* gene since *TAO1* was mapped to chromosome 11 [12] and the *SIT* gene is known to be on chromosome 1 [13]. In this paper we describe the molecular cloning and characterization of other members of the AO gene family in tomato in an attempt to isolate the *SIT* gene.

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[1] The nucleotide sequence data reported will appear in the GenBank under accession numbers: AF258808 (*TAO1* cDNA), AF258809 (*TAO2* cDNA), AF258810 (*TAO3* cDNA), AF258811 (*TAO4* cDNA), AF258812 (*TAO5* cDNA), AF259793 (*TAO1* partial genomic DNA).

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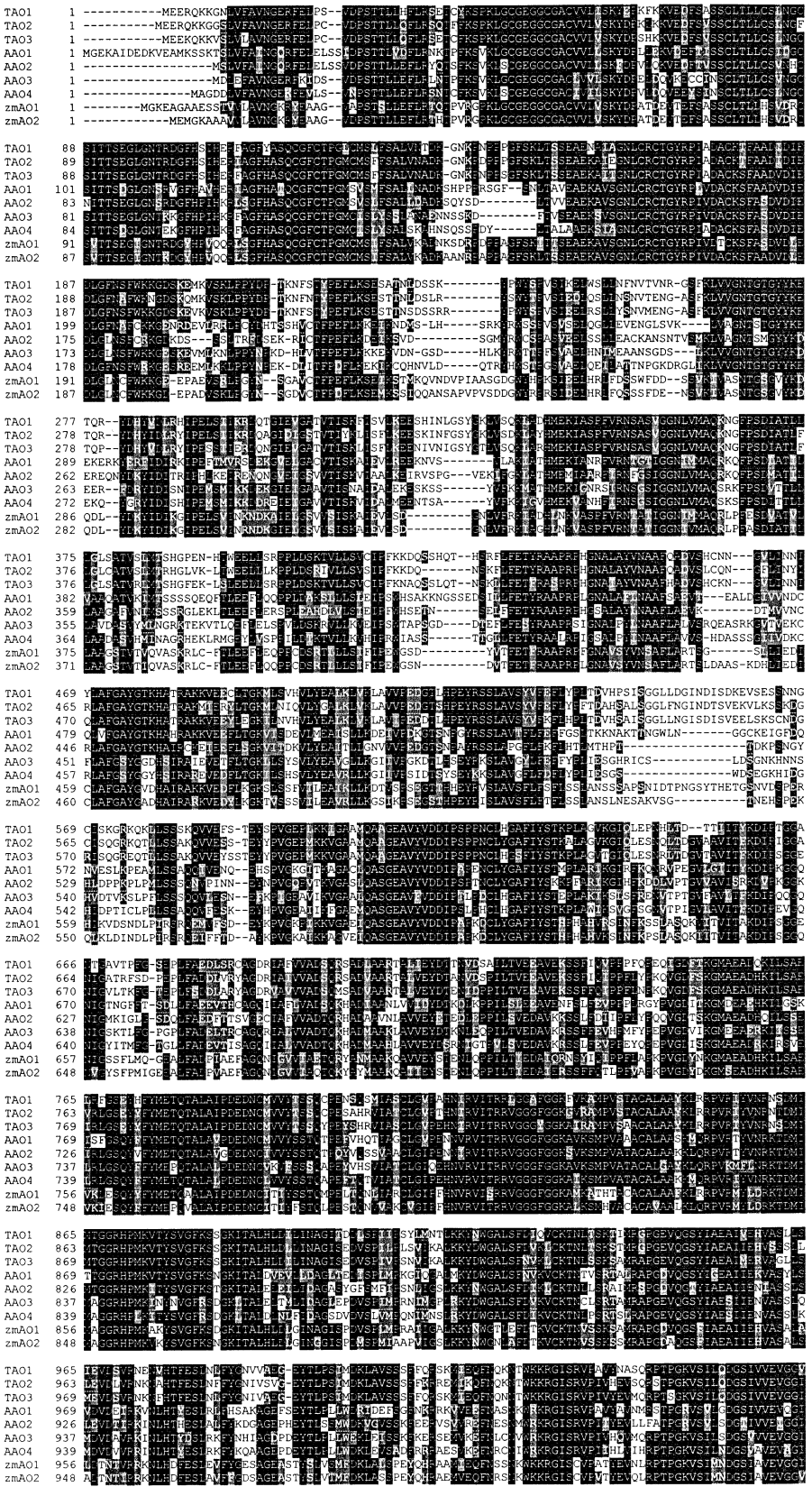

Fig. 1. Alignment of deduced amino acid sequences of three putative functional tomato aldehyde oxidases with AOs from *Arabidopsis* and maize. The sequences were aligned using the CLUSTAL program. The cDNA sequences used for amino acid translation and GenBank accession numbers are: *TAO1* (AF258808), *TAO2* (AF258809) and *TAO3* (AF258810) from tomato; *AAO1* (AB005804), *AAO2* (AB005805), *AAO3* (AB016602) and *AAO4* (AB037271) from *Arabidopsis*; *ZmAO1* (D88451) and *ZmAO2* (D88452) from maize.

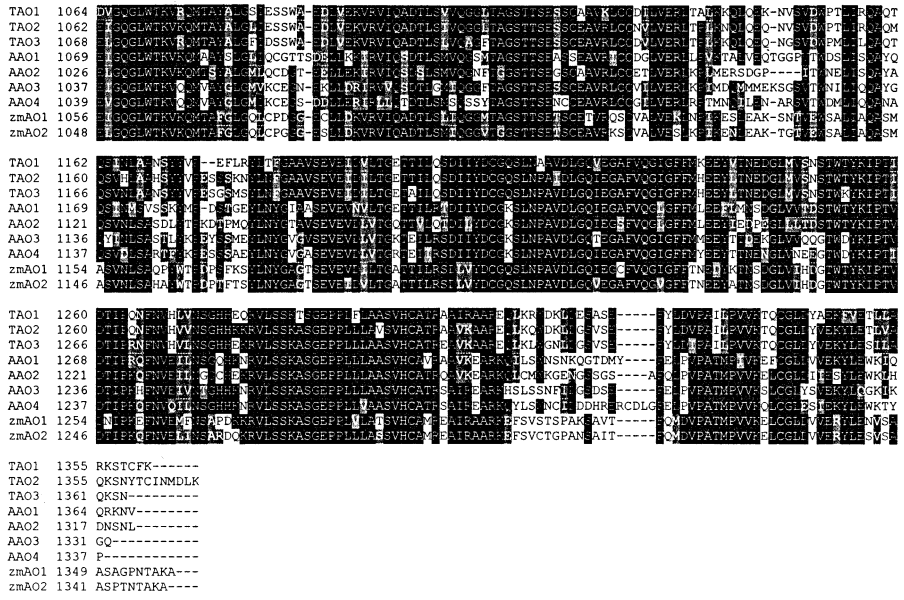

Fig. 1 (*continued*).

A tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) cDNA library was constructed with Poly(A) RNA isolated from 3-week-old wilted seedlings in the lambda ZAPII vector (Stratagene, La Jolla, CA). A 572 bp fragment near the poly(A)$^+$ end of the previously isolated *TAO1* (accession number U82559) [12] was obtained as a probe by RT–PCR (reverse transcription followed by DNA amplification by the polymerase chain reaction) with primer AO1F1 (5′-CAAGGGCTATGGACAAAGG-3′) and primer AO1R1 (5′-GGGATCTTGTATGTCCAAG-3′). Approximately 500 000 phages were screened with a fluorescein-labeled DNA probe (Vistra Fluoresence, Amersham, Buckinghamshire, UK) under low stringency (48°C, in 5×SSC 0.1% SDS; wash 2×SSC, 0.1% SDS). The fluorescence signal of the hybrids was amplified using an anti-fluorescein alkaline phosphatase conjugate and then was detected on a FluorImager (Molecular Dynamics, Sunnyvale, CA). We identified four types of TAO cDNAs and designated them as *TAO1*, *2*, *3* and *4*, respectively. The longest clones from each group were completely sequenced. The respective fragment lengths of *TAO1*, *TAO2*, *TAO3* and *TAO4* are 3261, 3991, 3264 and 4273 bp, respectively. The 5′-end portions of *TAO1* and *TAO2* were obtained using the Marathon cDNA Amplification kit (Clontech, Palo Alto, CA) with gene-specific primer (GSP) AO1GSP1 (5′-GCCATCTTCCGG-TACCACCGCTAATTTGA-3′) and nested primer AO1GSP2 (5′-GAGCAGCTCGATAGGTTTCA-3′) for *TAO1*, and AO2GSP1 (5′-GCAAAAGTCTTGCAGG-CATCAGCAATGG-3′) for *TAO2*. For cloning the 5′-end portion of *TAO3* cDNA, a forward degenerate primer, AO3DF1 [5′-GA(A/G)GG(T/C/A/G)GG(T/C/A/G)-TG(T/C)GG(T/C/A/G)GC(T/C/A/G)TG(T/C)-3′], which corresponded to the conserved peptide EGGCGAC in all known plant AOs was designed. A part of the 5′-end of TAO3 was obtained by RT–PCR using AO3DF1 and a *TAO3*-specific reverse primer (AO3GSP1, 5′-ATTTAA-TATCTTCCCCTCTAGATATTCC-3′). The rapid amplification of the *TAO3* cDNA end (5′ RACE) was carried out using primer TAO3GSP2 (5′-CTATATCAACAT-CAGCAG-3′) for template synthesis and primer AO3GSP3 (5′-ACATACACATGCCAGGAGTG-3′) for nested amplification (GibcoBRL, Life Technologies). While sequencing positive clones containing an insert that was obtained by PCR using AO3DF1 and AO3GSP1 primers, we identified a new 1.3 kb AO sequence (*TAO5*). RT–PCR was performed with a *TAO5* gene-specific sense primer (TAO5GSP1, 5′-CCAATCTAAAAAGATC-GAAG-3′) and several reverse primers derived from conserved sequences of TAOs including AO1R1 and from which a 3.6 kb *TAO5* fragment was obtained. The 3′-end of *TAO5* was obtained by using forward primer TAO5GSP2 (5′-ACAAAATGGCGCTGTTG-3′) and an abridged universal amplification primer (3′ RACE System for Rapid Amplification of cDNA Ends kit, GibcoBRL, Life Technologies). The 5′-end of *TAO5* was obtained using the 5′ RACE kit (GibcoBRL) and primer AO5GSP3 (5′-AAGCACATTCCTTAAGTCTAG-3′) for cDNA synthesis, and AO5GSP4 (5′-CAATGCCAGTCTGATCT-CTT-3′) for PCR amplification (GibcoBRL).

Comparing the cDNA and deduced amino acid sequences of TAOs (Fig. 1) with AOs from other plant species [9,11,14], we identified that *TAO1*, *2*, *3* were putative functional AO genes and *TAO4* and *TAO5* were AO pseudogenes. The sequences of *TAO1* cDNA we isolated from the Moneymaker library and its deduced amino acids were 99% identical with the partial cDNA and amino acid sequences of *TAO1* previously isolated (accession number U82559) [12]. However, the 5′-end of our *TAO1* cDNA was clearly different from the reported partial sequence in

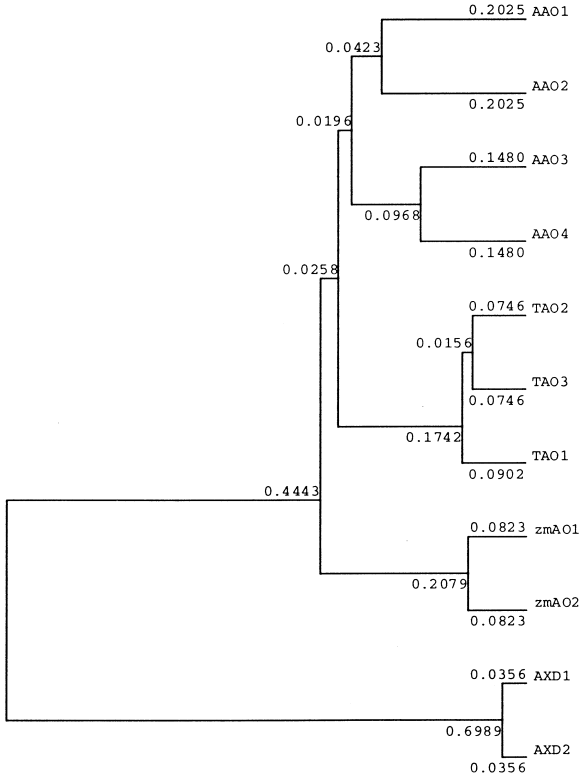

Fig. 2. Phylogenetic relationship of aldehyde oxidases and xanthine dehydrogenases from different plant species based on amino acid sequence analysis. The sequences used are the same as in Fig. 1 with the addition of *Arabidopsis* XD1 (AXD1, T10235) and XD2 (AXD2, T10236). The UPGMA program (Genetyx-Mac Ver. 8.0 software; Software Development Co., Tokyo, Japan) was used. Numbers in the figure indicate the relative phylogenetic distance.

GenBank (accession number U82558) [12]. The 4500 bp full-length *TAO1* cDNA we isolated has a coding region of 4083 bp encoding 1361 amino acids with a 180 bp 5′ untranslated region and a 237 bp 3′ untranslated region including a poly(A) tail of 18 nucleotides. The deduced amino acid sequence of TAO1 is a polypeptide of 149 kDa. The cDNA of *TAO1* reported previously [12] has an open reading frame of 1315 amino acids, 46 amino acids shorter at the N-terminus than *TAO1* we isolated. In fact, the 5′-end sequence of *TAO1* reported previously [12] was obtained from a genomic clone (accession number U82558). We confirmed that the 5′-end partial sequence of *TAO1* previously reported [12] is actually part of an intron in the *TAO1* gene by comparing our *TAO1* cDNA sequence and the genomic DNA sequence (accession number AF259793) amplified with two *TAO1* gene-specific primers. *TAO2* cDNA is 4631 bp long and consists of a 4101 bp coding region, a 359 bp 5′ untranslated region and a 171 bp 3′ untranslated region including a poly(A) tail of 21 nucleotides. *TAO2* encodes a protein of 1367 amino acids with an estimated molecular mass of 150 kDa. The full length of *TAO3* cDNA is 4683 bp, consisting of a coding region of 4092 bp, a 357 bp 5′ untranslated region and a 243 bp 3′ untranslated region including a poly(A) tail of 24 nucleotides. *TAO3* encodes a protein of 1364 amino acids with an estimated molecular mass of 149 kDa. *TAO4* shares ~80% identity with *TAO1* cDNA, but does not contain a starting codon, and contains two one-base-pair deletions resulting in a frame shift and a premature stop codon. Similar to *TAO4*, *TAO5* has a single nucleotide deletion at around 1972 bp resulting in an early termination of translation that could encode a peptide with only 599 amino acids. Presumably, such a peptide lacking Moco binding regions [14] should not have Moco-containing AO activity. Hence we assume that *TAO4* and *TAO5* are two tomato AO pseudogenes.

Alignment of the deduced amino acid sequences of *TAO1* and the two new *TAOs* with other known plant AOs reveals a high degree of similarity (Fig. 1). TAO2 and TAO3, similar to TAO1 and AOs in other plant species, do not have the Gly–Tyr–Arg (GYR) NAD binding site that is conserved in all xanthine dehydrogenases (XD) [15]. The three putative functional TAOs have more than 80% identity in their amino acid sequences and 76–85% identity in their DNA sequences, and they share 50–60% similarity to other plant AOs in both DNA and amino acid sequences. The two putative TAO pseudogenes also

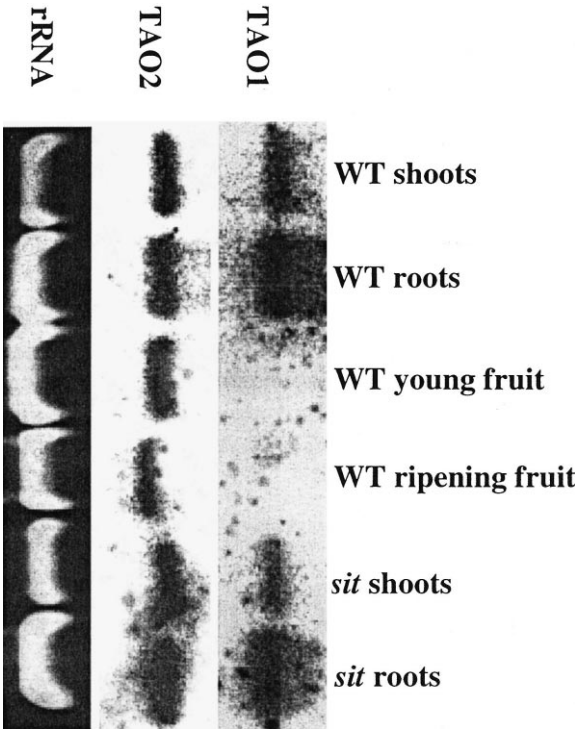

Fig. 3. Expression patterns of *TAO1* and *TAO2* in different tissues of tomato. Total RNA (15 μg/lane) was extracted from shoots and roots of 3-week-old wild-type (cv. Moneymaker) and *sit* mutant seedlings, and young and ripening fruit from wild-type adult plants. RNA samples were subjected to electrophoresis in 1.2% (w/v) agarose–2.2 M formaldehyde gels, and were stained with ethidium bromide. After transfer, the Hybond N^+ membrane (Amersham Life Science, Amersham, UK) was hybridized with ^{32}P-labeled DNA probes (Random primer labeling system, Amersham/Pharmacia Biotech, Piscataway, NJ) and washed under stringent conditions.

share quite high similarity to other TAOs (64–91%) and to AOs from other plant species (52–56%) at the cDNA level; in particular, *TAO5* and *TAO3* share 90% identity. A phylogenetic analysis composed of all known plant AOs and *Arabidopsis* XDs clearly demonstrates that the two novel functional tomato AOs (TAO2 and TAO3) are more closely related to TAO1 than to other plant AOs, and all plant AOs are more closely related to each other than are XDs and AOs from even the same species (Fig. 2).

To examine expression patterns and levels of each AO in different organs of wild-type plants and mutant plants, total RNA was isolated from roots and shoots of 3-week-old seedlings of Moneymaker and *sit*, and young fruit as well as ripening fruit of Moneymaker adult plants. To avoid cross hybridization, fragments of the 3′ untranslated regions of *TAO1*, *TAO2* and *TAO3*, which are relatively low in homology, were amplified by PCR using their cDNAs as templates and gene specific primers (for *TAO1*: 5′-CAGAGAAGTTCGTAGAAAC-3′ and 5′-ATAAATCACCTCATTATATG-3′; for *TAO2*: 5′-TGGAGAAATACTTGGAAAC-3′ and 5′-GGGATCTTGTATGTCCAAG-3′; for *TAO3*: 5′-TAGAGAAATATTTGGAAAG-3′ and 5′-TCAGTATCTATGTTGCTCGG-3′). The PCR products were purified with a QIAquick PCR purification kit (QIAGEN, Valencia, CA) and labeled by [^{32}P]dCTP and then used as probes. Northern analysis showed that *TAO1* was mainly expressed in shoots and roots and *TAO2* was expressed in all the tissues we examined including shoots, roots, young fruit and ripening fruit (Fig. 3). *TAO1* expression was not detectable in fruit. There was no difference in the expression levels of *TAO1* and *TAO2* between wild-type and *sit* mutant plants. *TAO3* expression was not detected in any tissue monitored by Northern analysis, but its expression was detectable with RT–PCR in both shoots and roots (data not shown). These results indicate that each AO may play different roles in the regulation of tomato growth and development.

In summary, we have cloned two novel full-length putative functional AO genes (*TAO2* and *TAO3*) and two putative AO pseudogenes (*TAO4* and *TAO5*), and have also correctly identified the 5′-end of *TAO1*. A search of the TIGR tomato gene index (http://www.tigr.org) did not reveal any new AO-related gene. Our results are consistent with the finding of at least three AO activity bands in roots and two bands in shoots by native polyacrylamide gel electrophoresis with indole-3-adehyde or acetaldehyde as a substrate [6]. The molecular cloning of these AO genes in tomato provides a molecular basis for AO genetic mapping and functional examination that may aid in the identification of the *SIT* gene. The functional characterization of each AO via expression in a yeast system is in progress.

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