Molecular Cloning of Allene Oxide Cyclase

THE ENZYME ESTABLISHING THE STEREOCHEMISTRY OF OCTADECANOIDS AND JASMONATES*

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Allene oxide cyclase (EC 5.3.99.6) catalyzes the stereospecific cyclization of an unstable allene oxide to (9S,13S)-12-oxo-(10,15Z)-phytodienoic acid, the ultimate precursor of jasmonic acid. This dimeric enzyme has previously been purified, and two almost identical N-terminal peptides were found, suggesting allene oxide cyclase to be a homodimeric protein. Furthermore, the native protein was N-terminally processed. Using degenerate primers, a polymerase chain reaction fragment could be generated from tomato, which was further used to isolate a full-length cDNA clone of 1 kilobase pair coding for a protein of 245 amino acids with a molecular mass of 26 kDa. Whereas expression of the whole coding region failed to detect allene oxide cyclase activity, a 5′-truncated protein showed high activity, suggesting that additional amino acids impair the enzymatic function. Steric analysis of the 12-oxophytodienoic acid formed by the recombinant enzyme revealed exclusive (>99%) formation of the 9S,13S enantiomer. Exclusive formation of this enantiomer was also found in wounded tomato leaves. Southern analysis and genetic mapping revealed the existence of a single gene for allene oxide cyclase located on chromosome 2 of tomato. Inspection of the N terminus revealed the presence of a chloroplastic transit peptide, and the location of allene oxide cyclase protein in that compartment could be shown by immunohistochemical methods. Concomitant with the expression of the N-terminated allene oxide cyclase mRNA was transiently induced after wounding of tomato leaves.

Jasmonic acid (JA) and its methyl ester, collectively named jasmonates, consist of a cyclopentanoid ring where an acetic acid and a pentenyl side chain are attached (Fig. 1). These side chains are either oriented in the cis (3R/7S) or the trans form (3R/7R), and a number of structurally related compounds have been described and found to occur ubiquitously in plants (1). The first physiological role of JA found in 1971 was inhibition of plant growth (2). Since then, jasmonates were identified as signals of altered gene expression in various plant responses to biotic and abiotic stresses as well as of distinct stages of plant development (3, 4). In tomato leaves, JA was recognized as an essential intermediate in the wound-induced signaling cascade following herbivore attack (5), and for numerous cell suspension cultures JA was described as a signal of elicitor-induced phytoalexin synthesis (6). Beside expression of defense genes such as proteinase inhibitors (5), defensins (7), or thionines (8), the synthetases of phytoalexins (9), alkaloids (10), and volatiles such as terpenoids (11) are the most intriguing JA responses, caused in most cases by up-regulation of specific enzymes (9, 12). JA responses were identified by means of altered expression of specific genes, by JA-insensitive and JA-deficient mutants, by JA-deficient transgenes, or by corresponding endogenous rises of jasmonates including inhibitor studies. More recently, the JA precursor (9S,13S)-12-oxo-(10,15Z)-phytodienoic acid (OPDA) was suggested to be the preferential signal of JA-mediated responses such as tendril coiling (13) or terpenoid biosynthesis (11). Among developmental processes, pollen maturation (14) and seedling growth (15) are JA-dependent.

The biosynthesis of JA proceeds via an oxylipin pathway (Fig. 1), starting with the lipoxygenase-catalyzed insertion of molecular oxygen into position 13 of linolenic acid followed by the dehydration of the resulting fatty acid hydroperoxide ((13S)-hydroperoxy-(9Z,11E,15Z)-octadecatrienoic acid) by allene oxide synthase (AOS; EC 4.2.1.92) to an allene oxide (1). This allene oxide is then cyclized by allene oxide cyclase (AOC; EC 5.3.99.6) to OPDA. After reduction of the ring double bond by a reductase and three rounds of β-oxidation, (+)-7-iso-JA, i.e. (3R,7S)-JA, is formed. Vick and Zimmerman (16) already proposed a similar biosynthetic scheme in 1983, but the formation of OPDA from (13S)-hydroperoxy-(9Z,11E,15Z)-octadecatrienoic acid was believed to be the result of a single enzyme called hydroperoxide cyclase. In 1988, Hamberg (17) showed that this step was performed by two enzyme activities. One of them, a membrane-bound activity purified later as AOS (18), catalyzed the formation of an unstable allene oxide, which rapidly decays by chemical hydrolysis with a half-life of 25 s to α- and γ-ketol and OPDA (Fig. 1). OPDA only amounts to 10–15% of the total degradation products and is racemic, containing the trans double bond at position 15 of the OPDA backbone (18). The other enzyme activity, purified as AOC, catalyzes the stereospecific cyclization of the allene oxide to OPDA (18).
sisting of the cis isomers 9S,13S and 9R,13R. However, in the presence of a second, soluble enzyme activity (AOC) that was purified recently (19), the amount of α- and γ-ketols decreased, and the 9S,13S enantiomer of OPDA was formed exclusively. This specificity of AOC determines the stereochemistry of the side chains in the naturally occurring jasmonate structure.

In addition, the lipoxygenase-derived products can be converted by a divinyl ether synthase, a reductase, a peroxygenase, and a hydroperoxide lyase (20). Due to these facts and the unspecific ketol formation following the AOS step, the AOC can be regarded as the first enzyme specific for JA synthesis. Interestingly, correct isomeric structure of OPDA formed by AOC is kept only by one of two reductases isolated so far (21).

Several forms of lipoxygenases, AOSs, and OPDA reductases have been cloned from different plant species and have been characterized biochemically, leading to hints of their physiological significance (21–25). So far, for AOC only biochemical data of the purified enzyme including substrate specificity are available (19, 26). In order to analyze the physiological importance of that step of JA biosynthesis, we cloned AOC. Here, we describe a full-length cDNA isolated from tomato leaves coding for a protein of 26 kDa that was localized in chloroplasts and whose 5′-truncated version exhibited AOC activity.

**EXPERIMENTAL PROCEDURES**

**Plant Material**—Corn (*Zea mays* L. cv. Boss), barley (*Hordeum vulgare* L. cv. Salome) and tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) were grown in soil under greenhouse conditions with 16 h light (with a minimum intensity of 130 μmol·m⁻²·s⁻¹) at 25 °C. Primary barley leaves were harvested 7 days after germination, cut into 5-cm segments starting 1 cm below the leaf tip, and floated in Petri dishes on a 1 M sorbitol solution. Developing corn seeds were harvested 2 weeks after anthesis. Tomato plants were grown for 8 weeks, and the second leaf was excised and wounded using a fabric pattern wheel and subsequently floated on distilled water under continuous white light (120 μmol·m⁻²·s⁻¹) for the indicated times.

**Analysis of Endogenous JA and OPDA**—Quantitative determination of levels of nonesterified OPDA and linolenic acid and steric analysis of OPDA were performed in unwounded leaves of tomato (15–20 g) or leaves (3–5 g) that had been wounded *in situ* for 0.5 h (OPDA) and 3 h (linolenic acid), respectively, by use of a hemostat. The tissue was shock-frozen in liquid nitrogen, and the powder was extracted with ethanol. [2H₅]OPDA and [2H₅]linolenic acid were added to a small part of the extracts, and the levels of OPDA and linolenic acid were determined by mass spectrometry. The remaining parts of the extracts were subjected to solid phase extraction and reversed-phase high pressure liquid chromatography, and the isolated OPDA was subjected to steric analysis as described by Ziegler et al. (26). JA levels were determined as described by Kramell et al. (27).

**FIG. 1.** Scheme of jasmonate biosynthesis. The reaction sequence after the lipoxygenase-catalyzed formation of the linolenic acid hydroperoxide is shown. This scheme includes the enzymatic conversion of the allene oxide (12,13S)-epoxyoctadecatrienoic acid to enantiomeric OPDA and later to jasmonic acid as well as the chemical decomposition to α- and γ-ketol and racemic OPDA, which has, up to now, only been shown to occur in vitro. 13(S)-HPOT, (13S)-hydroperoxy-(9Z,11E,15Z)-octadecatrienoic acid.

**FIG. 2.** Nucleotide sequence of tomato allene oxide cyclase cDNA and deduced amino acid sequence. Sequence stretches obtained by amino acid sequencing of the purified corn protein are underlined. Solid arrows, sequences used for reverse transcriptase-PCR; broken arrows, sequences used to amplify a fragment encoding a truncated version of allene oxide cyclase used for overexpression. Double underlines, putative cleavage sites for chloroplastic signal peptide as predicted by the ChloroP program, version 1.1 (available on the World Wide Web).
Determination of Peptide Sequences and PCR Cloning—AOC was purified from corn seeds as described (19). The purified protein was subjected to SDS-PAGE and electroblotted on a polyvinylidene difluoride membrane. The protein band was excised and subjected to N-terminal sequencing as well as to internal sequencing after Lys C digestion by automated Edman degradation.

For PCR cloning total RNA was extracted from developing corn seeds, barley leaves, and tomato leaves according to standard protocols (28). The RNA of all three tissues was used in a reverse transcriptase-PCR kit (Titan TM, Roche Molecular Biochemicals) with the following primers: upstream, 5′-CAA GAA TTC TAG GTA(C/G/T) TG(A/C) GA(A/ G)-3′; downstream, 5′-dT(dG)TG(A/C/G/T)-3′. The following temperature program was used: 30 min at 52 °C for the reverse transcription reaction, followed by 30 cycles of 94 °C for 1 min, 48 °C for 1.5 min, 72 °C for 2 min, and a final extension at 72 °C for 10 min. The PCR products were blunt end-ligated into pBSK Bluescript (Stratagene) and sequenced. The clone pTomAOC4 was used as a probe to screen 5000 plaques. The latter were almost identical and only differed by one additional Lys residue at the N-terminal end. Previously, a dimeric nature of AOC was suggested by the molecular size of about 48 kDa as estimated by gel filtration compared with the migration of the AOC protein as a 22-kDa band on SDS-PAGE (19). The finding of two almost identical N-terminal peptides supports the suggestion that AOC is a homodimeric protein. Additionally, both N-terminal sequences lack the start methionine residue. This could be caused by post-translational processing, which might be necessary for dimer formation or for the transport across intracellular membranes.

The obtained peptide sequences served as templates for the generation of oligonucleotides primers to perform a reverse transcriptase-PCR-based cloning approach for AOC. As upstream primers, degenerate oligonucleotides directed against different regions of the N-terminal sequence were used, whereas for the downstream primer, an oligo(dT) anchor was chosen. Since developing corn seeds showed a high AOC activity, we used the RNA from that tissue as a template. Irrespective of the primer combinations and PCR conditions, no specific PCR fragment could be amplified, suggesting that either the peptide information is not specific enough to amplify the desired product or that the mRNA is of very low abundance or even absent in this tissue. In order to exclude the second possibility as far as possible, we focused on tissues, where an accumulation of endogenous JA levels can be induced, presumably preceded by an increase in AOC expression. Therefore,
sorbitol-stressed barley leaf segments (32) and wounded tomato leaves (33) were used as a source for RNA. No specific PCR products were obtained with the barley RNA, but reverse transcriptase-PCR with RNA from wounded tomato leaves resulted in a weak band of about 850 bp. Sequencing of this PCR fragment revealed that it also encodes the internal sequence of six amino acids obtained from the purified corn protein. This PCR product was used as a probe to screen a tomato cDNA library, resulting in the isolation of a 1-kilobase pair clone. This size approximately corresponds to the size of the signal detected on the Northern blots, suggesting that a full-length cDNA clone was obtained. The first start codon in frame with the peptide sequences from the purified corn AOC is located at position 47 and is preceded by a stop codon at position 16. The protein coding region encompasses 732 bp encoding a protein of 244 amino acids with a calculated molecular mass of 26 kDa (Fig. 2). The difference of about 4 kDa between the deduced molecular mass of the tomato protein and that from the subunit of the purified corn enzyme as determined by SDS-PAGE could, in part, be due to the post-translational removal of amino acids at the N terminus.

**Overexpression of AOC**—In order to identify the protein encoded by the cDNA clone as an AOC, we performed overexpression to measure AOC activity. At first, the whole coding region was cloned into the expression vector pJC20 and, for purification, into the His tag vector pJC40. After induction by isopropyl-β-thiogalactopyranoside, only a low expression of the recombinant protein was observed on SDS-PAGE, but after Ni²⁺-nitritotriacetic acid-agarose chromatography of the His-tagged protein, one band of the expected size of 26 kDa could be detected. However, neither the crude bacterial extracts nor the purified protein showed AOC activity. Considering that the purified protein from corn was N-terminally processed, the lack of enzymatic activity in the full-length tomato protein could be due to the failure of the right post-translational modification in the bacteria. To explore this possibility, we amplified a fragment from the tomato sequence, which produces a truncated protein starting at amino acid residue 64. This corresponds in length to the processed N terminus of the purified corn protein. In that case, the start codon was introduced by a NdeI restriction site. After induction of the bacteria, SDS-PAGE revealed a prominent band of 22 kDa that was absent in control bacteria transformed only with the empty vector (Fig. 3). The same band was also observed in noninduced bacteria, which might be due to an insufficient repression of the bacterial expression system in the absence of inducer. The bacterial extracts were then examined for AOC activity. As shown in Table I, the control bacteria showed no activity at all, whereas in induced, transformed bacteria a high specific activity of about 15,000 nmol of OPDA/mg of protein was detected. As expected from the protein pattern on SDS-PAGE, the noninduced bacteria also showed AOC activity, but in this case a 10-fold lower specific activity was obtained. The AOC activity of the recombinant protein was sensitive to proteinase K digestion and was inhibited by 12,13-epoxyoctadecenoic acid, a specific AOC inhibitor (19, 26), which further supports the specificity of the recombinant protein. One special feature of the AOC reaction is the competition between the chemical decomposition of the unstable allene oxide substrate, giving rise to racemic OPDA and the enzymatic conversion to enantiomeric OPDA. Therefore, the ultimate identification for a protein as AOC can only be achieved by the proof that the enzyme specifically forms (9S,13S)-OPDA. The steric analysis of the reaction products showed that the recombinant protein almost exclusively forms the (9S,13S)-OPDA enantiomer. Proteinase K digestion and the addition of 12,13-epoxyoctadecenoic acid decreased the amount of that OPDA enantiomer to levels seen after chemical decomposition. Altogether, the results on the AOC activity showed that the isolated clone from tomato codes for AOC. Interestingly, the specific activity of the N-terminal, His-tagged protein expressed in the pJC40 vector was about 10-fold lower than that of the untagged protein. Together with the observation that

<table>
<thead>
<tr>
<th>Specific AOC activity</th>
<th>Percentage (S/S) of total OPDA</th>
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<tbody>
<tr>
<td>nmol of OPDA × mg⁻¹ protein⁻¹</td>
<td>%</td>
</tr>
<tr>
<td>pJC20/pJC40 Only chemical</td>
<td>0</td>
</tr>
<tr>
<td>pJC20 + insert 1202</td>
<td>7</td>
</tr>
<tr>
<td>pJC20 + insert + IPTG 15,741</td>
<td>100</td>
</tr>
<tr>
<td>with proteinase K 944</td>
<td>6</td>
</tr>
<tr>
<td>with inhibitor (100 μM) 1259</td>
<td>8</td>
</tr>
<tr>
<td>pJC40 + insert + IPTG 1302</td>
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* Due to the assay design, AOC activity could only be calculated by end point measurements of OPDA.

**Fig. 4. Immunocytochemical localization of allene oxide cyclase protein in tomato leaves.** Cross-section of leaves floated on 50 μM jasmonic acid methyl ester for 24 h were probed with preimmune serum (A) or with anti-AOC-antibody raised against purified recombinant AOC of tomato (B), followed by a labeled secondary antibody. In contrast to the yellow-brown label upon treatment with preimmune serum, a strong green fluorescence label within chloroplasts in B is indicative of the AOC protein. Starch granules within chloroplasts seen in B as nonfluorescent areas were visualized by differential interference contrast image (C). Bar, 10 μm.
only the truncated protein was active, it seems possible that additional amino acids at the N terminus might somehow impair dimer formation.

**Intracellular Localization of AOC**—Most enzymes of the oxy-

lipin pathway have been reported to reside in the chloroplast. N-terminal amino acid sequence analysis of cloned lipoxygen-

ase from barley (34), Arabidopsis (35), and tomato (36) as well as partially performed import studies (35, 36) indicated the occurrence and function of putative chloroplast signal peptides. Also, the AOS from flax (23) and occurrence and function of putative chloroplast signal peptides. Also, the AOS from flax (23) and tomato (36) as well as partially performed import studies (35, 36) indicated the occurrence and function of putative chloroplast signal peptides. Also, the AOS from flax (23) and Arabidopsis (37) carry a putative chloroplast signal peptide, and the barley AOS co-

purified with chloroplasts (25). Biochemical data revealed the presence of the enzyme activities of lipoxygenase, hydroperox-

ide lyase, and AOS in the outer envelope membrane (38). In case of AOS and lipoxygenase, their chloroplastic location was also shown immunocytochemically (12, 25). Inspection of the N-terminal region of the tomato AOC also revealed structural features for a chloroplastic signal peptide. It is highly enriched in Ser residues (26%) in the first 50 amino acids; the start Met is followed by an Ala residue, and the first 10 amino acids are devoid of any charged residue (39). Computer analysis of the first 100 amino acids using the ChloroP version 1.1 program (available on the World Wide Web) predicts a chloroplastic localization with a putative cleavage site between position 93 and 94. However, this cleavage site is highly unlikely, since the purified mature protein from corn starts at amino acid residue 64 in the tomato sequence. Other predicted possible cleavage sites would be at residues 41, 52, and 60. To establish the localization of AOC experimentally, we performed an immuno-

cytological approach using an antibody directed against the recombinant AOC. Cross-sections of tomato leaf tissues probed with that antibody showed a significant fluorescence label in the chloroplasts (Fig. 4). The autofluorescence of chloroplasts is shown by cross-section of tissues that were treated with pre-

immune serum. This confirms the data from the sequence analysis indicating that AOC is a chloroplastic protein. In contrast to AOS, which was co-purified with outer envelope membranes (38), AOC is a soluble protein (17, 19). Since its substrate is highly unstable, it seems reasonable to expect AOC to be in close proximity to AOS in order to convert the substrate efficiently to (9S,13S)-OPDA. In order to study this point further, the levels and stereoconfiguration of endogenous OPDA were determined in tomato leaves. In a typical experiment using unwounded leaves, the levels of OPDA and nonesterified linolenic acid were 2 and 206 ng/g, fresh weight, respectively. Levels of OPDA and linolenic acid increased to 187 ng/g (90-fold) and 1813 ng/g (9-fold), respectively, within 30 and 180 min, respectively, upon mechanical wounding, which was in the range observed previously (40). Steric analysis of the wound-induced OPDA showed it to be due exclusively (>99%) to the (9S,13S)-stereoisomer. Additionally, no ketalts or racemic OPDA have been detected in plants up to now (41), suggesting that chemical degradation of the allene oxide is improbable in vivo. One may suggest, therefore, that AOS and AOC are either localized close to each other or even loosely associated. This putative interaction of AOS and AOC is now under study using the corresponding clones in a yeast two-hybrid system.

**Genomic Analysis and Mapping of the AOC Gene**—Southern blot analysis with genomic DNA from tomato revealed a hy-

bridization pattern that is in agreement with a single gene for AOC in the tomato genome (Fig. 5A). Clear polymorphism was detected with several restriction enzymes in the parents of the standard tomato mapping population (30). All segregating fragments could be mapped to a single locus on chromosome 2 of tomato (Fig. 5B).

**Physiology of AOC Expression**—The accumulation of JA has been shown to be an important part of a signal transduction cascade in response to wounding (33, 40, 42, 43). In tomato, a chloroplastic lipoxygenase is up-regulated upon wounding (36), and Arabidopsis plants co-suppressed with a specific chloro-

plastic lipoxygenase failed to respond with increased JA levels upon wounding (35). Moreover, the strict spatial and temporal regulation of the second enzyme of the pathway, AOS, during the wounding response in Arabidopsis underscores the importance of the activation of JA biosynthetic enzymes in the accumulation of JA (24, 44). Because AOC is the enzyme that catalyzes...
the first committed step in the sequence leading to JA, establishing the stereochemistry of the naturally occurring enantiomer, we were interested in knowing whether the expression of AOC coincides in time with the increase in JA levels after wounding. As seen in Fig. 6, AOC mRNA levels start to increase 30 min after wounding of tomato leaves. The maximum induction was observed after 2 h, and at 8 h the control level was reached again. This correlated well with the JA levels measured after wounding, which also showed a transient accumulation with a peak at 1 h (40, 43, 45). Moreover, this corresponds to the exclusive formation of the (9S,13S)-stereoisomer 30 min after wounding mentioned above. This result suggests an important physiological function also for AOC in the regulation of JA levels during the wound response in tomato.

Another, highly attractive function for AOC might be in the development of floral organs. A data base search with the cloned tomato AOC revealed its identity with the tomato clone TP15 (46). This yet unidentified clone was isolated by differential hybridization of tomato pistil cDNA libraries and was found to be highly expressed in pistils, mature petals, red fruits, and, to a lower level, in stamens. In young, developing flower buds, no expression was detected. Arabidopsis mutants shown to be defective in JA signaling, such as the coi1 mutant, or JA-deficient, such as the fad3–2 fad7–2 fad8 mutant, are both male sterile (14, 47), indicating the importance of JA in flower development. It was also shown that the AOS gene is highly transcribed in floral organs of Arabidopsis thaliana, suggesting that JA might be produced in flowers (44). This corresponds to elevated levels of JA repeatedly found in flowers (48). It will be interesting to analyze the expression of AOC in floral organs as well as during development and its correlation to the corresponding levels of jasmonates and octadecanoids.

**Conclusion**—Through the pioneering work of Vick and Zimmermann (16) and Hemberg (17), the enzymes for JA biosynthesis were elucidated. In the last decade, the characterization and cloning of these enzymes have been greatly advanced, and clones of lipoxigenase (22), AOS (23, 25, 37), and OPDA reductases (49, 50) are already available. With the described isolation of a cDNA clone coding for AOC, all enzymes leading to the first physiologically active cyclopentenone, OPDA, are now cloned. Additionally, this enzyme may be of major importance, since it determines the stereochemistry of the cyclopentanones and has a pivotal role in directing one of the oxylipin metabolic pathways to the biosynthesis of the jasmonates. Using the AOC clone and those for the other enzymes, extensive physiological studies and biotechnological applications are now possible to reveal the participation of each of the biosynthetic enzymes in the stress-induced or developmentally regulated levels of JA.

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**References**

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