SHORT COMMUNICATION

Wounding stimulates ALLENE OXIDE SYNTHASE gene and increases the level of jasmonic acid in Ipomoea nil cotyledons

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Abstract

Allene oxide synthase (AOS) encodes the first enzyme in the lipoxygenase pathway, which is responsible for jasmonic acid (JA) formation. In this study we report the molecular cloning and characterization of InAOS from Ipomoea nil. The full-length gene is composed of 1662 bp and encodes for 519 amino acids. The predicted InAOS contains PLN02648 motif, which is evolutionarily conserved and characteristic for functional enzymatic proteins. We have shown that wounding led to a strong stimulation of the examined gene activity in cotyledons and an increase in JA level, which suggest that this compound may be a modulator of stress responses in I. nil.

Keywords

allene oxide synthase; gene expression; jasmonates; phytohormones; wounding; Ipomoea nil

Introduction

The biosynthesis of jasmonates (JAs) is initiated by lipoxygenase (LOX) or α-dioxygenase (DOX), which catalyze the formation of hydroperoxides 13(S)-hydroperoxy-octadecatetraenoic acid (13-HPOT) and 11(S)-hydroperoxy-hexadecatetraenoic acid (11-HPHT), respectively [1]. The committed step of JA biosynthesis is catalyzed by allene oxide synthases (AOS), an atypical family of cytochrome P450, designated CYP74. They use preoxygenated fatty acid hydroperoxide substrates both as oxygen donor and as a source for reducing equivalents. Dehydration of 13-HPOT by AOS results in the formation of unstable short-lived allene oxide intermediates 12,13(S)-epoxy-octadecatetraenoic acid [12,13(S)-EOT] and 10,11(S)-epoxy-octadecatetraenoic acid [10,11(S)-EHT], which are converted by allene oxide cyclase (AOC) to the cyclopentanone ring-containing 12-oxophytodienoic acid (OPDA) or dinor-OPDA (dnOPDA), respectively. Both OPDA and dinor-OPDA undergo three cycles of β-oxidation in the peroxisomes, to generate JA [1,2].

It was known that LOX and OPR genes encode enzymes crucial for the regulation of JAs biosynthesis. However, the complementation analysis of Arabidopsis thaliana delayed-dehiscence2-2 male-sterility mutant reveals that the damage is caused by interruption of the gene sequence encoding AOS, another of the main JA biosynthesis pathway enzymes [3]. AOS genes were also identified in many other plant species [4-6]. The AOS mRNA level depends on both internal and external factors such as phytohormones, pest attack and wounding.

In this paper we identified InAOS cDNA encoding a member of the CYP74 P450 gene family, involved in JA biosynthesis. The expression pattern in cotyledons after wounding of this gene was measured. We also examined the correlation between changes in AOS mRNA level and endogenous JA in cotyledons after wounding.
Material and methods

Plant materials and growing conditions

Preparation of plant material (Ipomoea nil, Chois ‘Violet’; Marutane Seed Co., Kyoto, Japan) was made according to Wilmowicz et al. [7]. The seedlings were grown in a growth chamber under the conditions described by Wilmowicz et al. [7]. Cotyledons of plants were perforated at regular intervals 5 × 5 mm.

Plant material for cloning InAOS cDNA and determination of JA level

For experiment examining the wounding effect on the level of endogenous JA and expression of InAOS the cotyledons were collected 20, 30, 40, 50, 120, 240, 360, 480, and 1440 minutes after the beginning of the stress stimulus action, immediately frozen in liquid nitrogen and stored at −80°C. Each experiment was repeated at least three times. All data are presented as mean ± standard error (SE).

Molecular cloning of InAOS cDNA

The identification of full-length cDNA coding for InAOS was performed according to Frankowski et al. [8]. PCR, using degenerated primers 5’-TGCCGTCRACKGAACT-MACCCGGWG-3’ (forward) and 5’-ACGGGATYTKYTCCCATCSCGMATCCTG-3’ (reverse) constructed on the basis of conserved sequences encoding AOSs in Arabidopsis thaliana, Lycopersicon esculentum, and Solanum tuberosum was performed in the T3 Thermocycler (Biometra, Göttingen, Germany). A 693 bp amplified cDNA fragment was isolated from an agarose gel, cloned and sequenced. Gene specific primers for 5’-RACE (5’-GCTTCAATGCGGATGCTTCCTGCT-3) and 3’-RACE (5’-GTCGTGAAAATCCACGGGGAAAGTCA-3) were picked from a cDNA sequence of the identified clone using FastPCR software (a public version of the FastPCR is available at: http://www.biocenter.helsinki.fi/bi/programs/fastpcr.htm). Full-length InAOS sequence obtained here have been deposited at GeneBank under accession number HM357792. Data analyses were performed in ClustalW (http://www.ebi.ac.uk/clustalw), BLAST 2.2.25 (http://www.ncbi.nlm.nih.gov/BLAST), and ExPASY (http://www.expasy.org). The phylogenetic analysis was performed using the Phylogeny.fr web service (http://www.phylogeny.fr/simple_phylogeny.cgi).

Quantitative real-time RT-PCR analysis of InAOS gene expression

The gene expression analyses were performed by real-time PCR (RT-qPCR) with a LightCycler 2.0 Carousel-Based System (ROCHE Diagnostics GmbH, Germany) and the LightCycler TaqMan Master Kit (ROCHE Diagnostics GmbH, Germany). All expression procedures were performed according to Frankowski et al. [8]. Gene-specific primers and UPL probe were designed using the Universal ProbeLibrary Assay Design Center (http://www.roche-applied-science.com/sis/rtPCR/upl). Actin (InACT4) was used as a reference endogenous control for normalization purposes. qPCR analyses were performed with the gene specific primers: InAOS (60 bp) 5’-CGGAGATGTTGTGGGGTGGTGG-3’ (forward) and 5’-CATCCAAGCCGACTT-TACCC-3’ (reverse) with hydrolysis probe UPL 134, InACT4 (65 bp) with the gene specific primers 5’-GGAATACAGTGTTGCTCGATTGGA-3’ (forward) and 5’-CCA- CATCTGTTGAATGTGC-3’ (reverse) with hydrolysis probe UPL 139 (ROCHE Diagnostics GmbH, Germany). Relative quantification was performed using standard curves from serial dilutions of cDNA. The efficiency tested was >99%. The computer application used for the analysis was LCS4.0 (ROCHE Diagnostics GmbH, Germany) and for the calculations and graphs – MS Office Excel (Microsoft). PCR reactions were performed in triplicate for each RNA sample. All data are presented as mean ± standard error (SE).
The coding sequence of \textit{InAOS} was obtained using 3RACE-PCR (GenBank accession number HM357792.2). The full-length \textit{InAOS} cDNA containing start and stop was obtained using 5RACE-PCR and is composed of 1662 bp and encodes for 519 amino acids (Fig. 1a). A comparison of AOS sequences from \textit{A. thaliana} in the Blast 2.2.25 with \textit{InAOS} shows that \textit{InAOS} has 65\% amino acid sequence identity to \textit{AtAOS} (GenBank acc. No. NP_199079.1). Based on amino acid sequence, phylogenetic analysis revealed that \textit{InAOS} was very closely related to AOS (76\%) from \textit{Solanium tuberosum} (GenBank acc. No. CAD29735.1), \textit{Populus trichocarpa} (74\%; GenBank acc. No. XP_002302453.1), \textit{Nicotiana attenuata} (74\%; GenBank acc. No. CAD29735.1), \textit{Nicotiana tabacum} (74\%; GenBank acc. No. CAD29735.1), and \textit{Hyoscyamus niger} (74\%; GenBank acc. No. AB550433.1; Fig. 1b). Predicted amino acid \textit{InAOS} sequence analysis and alignment with others showed that there were four conserved domains characteristic of cytochrome P450 proteins, which are common for all the AOS family. Because \textit{InAOS} contains motifs that are evolutionarily conserved and characteristic of AOS it can be suspected that this gene encodes for a functional enzymatic protein.

Expression analysis of \textit{InAOS} and endogenous jasmonic acid level in wounded cotyledons of \textit{l. nil}

As shown in Fig. 2a wounding plants resulted in a strong increase in \textit{InAOS} expression level in the cotyledons of
5-d-old seedlings of *I. nil* during the first hour after stimulus action and the reaction is accompanied by an increase in the endogenous JA level (Fig. 2b). The highest *InAOS* transcript accumulation in reference to the control plants (0 h) was observed 20 minutes after the wounding. The expression of this gene remained on a similar, high level 30, 40, and 50 minutes after stress and after that it decreased (120 min) and was similar to that in non-wounded plants.

We also showed that JA content directly after wounding cotyledons of *I. nil* was 6-fold higher than in the control plants (Fig. 2b). The level of endogenous JA increases over 50 minutes and reaches a maximum value of about 30 ng/g fresh weight in 30 min, which was 10 times higher than for non-wounded cotyledons. Beginning from 120 minutes after the stimulus action, the level of JA gradually decreases and although it slightly oscillates, the results were not significantly different from the control value (0 h).

### Discussion

Maintenance of a JAs level appropriate for the control of various processes is possible by multi-level regulation of their biosynthesis, in which a key role is played by, inter alia, *LOX*, *AOS*, *AOC*, and *OPR3*. *AOS*, the first enzyme of the octadecanoid biosynthesis pathway, is a major site of pathway control, which proceeds through feedback amplification of its biosynthesis from downstream metabolites. The expression of this gene is tissue-specific and correlates with endogenous JA level.

In this study we obtained the AOS cDNA from cotyledons of *I. nil* (*InAOS*) and analyzed its sequence (Fig. 1a). The *InAOS* gene encodes a functional enzymatic protein, which may have similar functions as in other plant species [4,5,10] (Fig. 1b). The *InAOS* contains a conserved domain with heme-binding cysteine (Cys-470), which is characteristic for cytochrome P450s [4]. This suggests that *InAOS* encodes for a functional enzymatic protein, which may have similar functions as in other plant species.

Expression of *AOS* was shown to be tightly linked with elevated JA content during the wound response in *A. thaliana* [11]. In wounded cotyledons of *I. nil*, a transient increase in *AOS* mRNA level was observed, with a maximum between 20 and 50 min after stimulus (Fig. 2a). The corresponding level of JA during the first 50 min is shown in Fig. 2. Wound-induced expression of the *AOS* gene was also observed in leaves of several plants, e.g., tomato, barley [5,11]. The JAs content in plants influences the level and specificity of the defence responses. These hormones are some of the main messengers in the cell (local response) or at the systemic level. The rate of changes in the level of endogenous jasmonates is different for individual plant species, ranging from several seconds to several minutes after the stress stimulus [12].

In this paper we characterized *InAOS* gene encoding the first allene oxide synthase identified in *Ipomoea nil* involved in JAs biosynthesis, which may play a substantial role in JA systemic accumulation in response to wounding.
References


