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Authors' contributions
EW and KF designed and carried out the experiments, analyzed the data and wrote the manuscript; AK was responsible for statistical analysis, preparation of figures and charts, RNA isolation and data analysis; BZN and KP helped with chromatography and RT-qPCR reactions, respectively, and contributed to the analysis of obtained results; JK was responsible for interpretation of the part of the data and preparing the manuscript

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-octadecatrienoic acid (13-HPOT) and 11(*S*)-hydroperoxy-hexadecatrienoic 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-octadecatrienoic acid [12,13(*S*)-EOT] and 10,11(*S*)-epoxy-octadecatrienoic 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-MACCGGWGG-3′ (forward) and 5′-ACGGCATYTKYTCCATCSCCGMCATCGT-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′-GCTTCAGTTGCGCGTGCTTCGGCTC-3′) and 3′-RACE (5′-GTCGTGAAATCCAACGGCGGGAAAGTCA-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′-CGGAGATGTTTGAGGGTTTG-3′ (forward) and 5′-CATTCAAGCCGACTT-TACCC-3′ (reverse) with hydrolysis probe UPL 134, *InACT4* (65 bp) with the gene specific primers 5′-GGAAATACAGTGTCTGGATTGGA-3′ (forward) and 5′-CCA-CATCTGTTGGAATGTGC-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*).

a

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  1 ATGGCGTCATCTTCATTAGCTGTTCACTTCCAAATCCCTTCTCAGAAATCTTCTTTAACG
    M  A  S  S  S  L  A  V  H  F  Q  I  P  S  Q  K  S  L  L  T     20
 61 TTGAAGCCTTCTAGCCGGCGTTTCAAGATTTGCCCTGTTTCTGCCACCGTGTCGGATACT
    L  K  P  S  S  R  R  F  K  I  C  P  V  S  A  T  V  S  D  T     40
121 CCGCCGTCGGTTTCCTTGTCCCCGGTCCCGGAGAAACTCCCAAAAAGGAAAATACCCGGC
    P  P  S  V  S  L  S  P  V  P  E  K  L  P  K  R  K  I  P  G     60
181 GACTATGGGCTGCCGTTGATCGGGCCGTGGAAAGATCGGCTGGACTATTTCTACAATCAA
    D  Y  G  L  P  L  I  G  P  W  K  D  R  L  D  Y  F  Y  N  Q     80
241 GGAGAGAGGAGTTTTTCCGGTCTCGGGGTTCAGAAATACGGGTCGACGGTTTTCGGACGG
    G  R  E  E  F  F  R  S  R  V  Q  K  Y  G  S  T  V  F  R  T    100
301 AACATGCCACCCGGCCGTCCATTTCCTTCAGCCCTAACGTCCGTGGTTTTGCTCGACGGC
    N  M  P  P  G  P  F  I  S  F  S  P  N  V  V  V  L  L  D  G    120
361 AAGAGTTTTCCGACGCTGTTTGATCCGGGAAAGGTCGAGAAACGGGATCTTCTCACCGGA
    K  S  F  P  T  L  F  D  P  G  K  V  E  K  R  D  L  F  T  G    140
421 ACTTTCATGCCCTCCACGGAACTCACCGGCGGGTACCGGATCCTCTCGTATCTGGACCCG
    T  F  M  P  S  T  E  L  T  G  G  Y  R  I  L  S  Y  L  D  P    160
481 TCGGAGCCGAAGCACGCCGAACTGAGCAGCTGATGTTCTTTCTCCTCTCCTCCCGGCGC
    S  E  P  K  H  A  Q  L  K  Q  L  M  F  F  L  L  S  S  R  R    180
541 GGCCACGTCATACCGGAGTTCCACCGGAGCTTCACGGAGATGTTTGAGGGTTTGGAGAAG
    G  H  V  I  P  E  F  H  R  S  F  T  E  M  F  E  G  L  E  K    200
601 GAGGTAGCTAGCAAGGGTAAAGTCGGCTTGAATGCCGGCTAACGATCAGGCGGCTTTCAAT
    E  V  A  S  K  G  K  V  G  L  N  A  A  N  D  Q  A  A  F  N    220
661 TTCTTGGCCCGATCGTGGTTCGGAGTTGATCCGGCGGGGACGAAGATCGGAAACGACGGC
    F  L  A  R  S  W  F  G  V  D  P  A  G  T  K  I  G  N  D  G    240
721 CCTAATCTCGTCGGGAAATGGGTAGTGTTTAATCTCCATCCTTTATTAGTTCTCGGGCTC
    P  N  L  V  G  K  W  V  V  F  N  L  H  P  L  L  V  L  G  L    260
781 CCTAAGGGTTTAGAGGAAGCTCTCCTCCATACTTTCCGGTTGCCGGCGGCGCTGGTGAAG
    P  K  G  L  E  E  A  L  L  H  T  F  R  L  P  A  A  L  V  K    280
841 AAAGATTACCAGAGGCTCTATGAGTTCTTCTACGCCAACTCAACGGAGATTCTCGACGAG
    K  D  Y  Q  R  L  Y  E  F  F  Y  A  N  S  T  E  I  L  D  E    300
901 GCCGAGAATCTCGGGCTGTCACGTGAGGAGGCCTGTCATAACCTCCTCTTCGCCACGTGC
    A  E  N  L  G  L  S  R  E  E  A  C  H  N  L  L  F  A  T  C    320
961 TTCAACTCGTTCGGAGGTATGAAGAATTTCTTCCCCAACATGATCAAGTGGATCGGCCGC
    F  N  S  F  G  G  M  K  I  F  F  P  N  M  I  K  W  I  G  R    340
021 GGCGGCGCGAAGCTGCACGCGCAGCTGGCGCGGGAGATCCGGTCGGTCGTGAAATCCAAC
    G  G  A  K  L  H  A  Q  L  A  R  E  I  R  S  V  V  K  S  N    360
081 GGCGGGAAAGTCACGATGGCCGGGATGGAACAGATGCCGTTGATGAAGTCGGTGGTGTAC
    G  G  K  V  T  A  G  M  E  Q  M  P  L  M  K  S  V  V  Y    380
141 GAGGCCCTCCGGACTCGAACCGCCGGTCCCGGCCCAATACGGCCGGGCCAAACGCGACTTC
    E  A  L  R  I  E  P  P  V  P  A  Q  Y  G  R  A  K  R  D  F    400
201 GTCGTCGAGTCACGACGACGCCGTATTTGAAGTCAAGGAAGGGGAGATGCTATTCGGGTTC
    V  V  E  S  H  D  A  V  F  E  V  K  E  G  E  M  L  F  G  F    420
261 CAACCGTTCGCCACAAAAGACCCGAAGATCTTCGACCGGGCCGAGGAGTTCGTGCCGGAC
    Q  P  F  A  T  K  D  P  K  I  F  D  R  A  E  E  F  V  P  D    440
321 CGGTTCACCGGCGAGAACGCAAACGAACTCTTGAGCCACGTGTTATGGTCTAACGGCCCG
    R  F  T  G  E  N  A  N  E  L  L  S  H  V  L  W  S  N  G  P    460
381 GAAACGGAGAGCCCCACCGTGAACAACAAACAGTGCGCCGGCAAGGATTTCGTCGTCTTG
    E  T  E  S  P  T  V  N  N  K  Q  C  A  G  K  D  F  V  V  L    480
441 GTTTCACGGCTGATGGTCGTCGAACTTTTCCTCCGGTACGATTCTTTCGACATCGAGGTG
    V  S  R  L  M  V  V  E  L  F  L  R  Y  D  S  F  D  I  E  V    500
501 GGAACGTCGCCGTTAGGTGCTTCCGTCACCGTAACGTCACTGAAGAGAGCCAGTTTCTGA
    G  T  S  P  L  G  A  S  V  T  V  T  S  L  K  R  A  S  F    519
561 cgtggtatatttgcctacagtttattatccttgttgtgaatatgttgggattaatttttt
621 tttttaatgaaatcataattttttatttttaaaaaaaaaaaaa
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b

	aa sequence identity (%)
Lotus japonicus AOS	70
Glycine max AOS	71
Populus trichocarpa AOS	74
Solanum tuberosum AOS	76
Solanum lycopersicum AOS	72
Lonicera japonica AOS	71
Ipomoea nil AOS	
Nicotiana attenuata AOS	74
Nicotiana tabacum AOS	74
Hyoscyamus niger AOS	74
Hevea brasiliensis AOS	70
Arabidopsis thliana AOS	65

Fig. 1 **a** Coding sequence of *InAOS* cDNA and the deduced amino acid sequence. Subsequent nucleotide positions are marked on the left side of the figure, and amino acid positions on the right. The translation initiation point (the start codon) and termination point (the stop codon) are in boldface type. Small letters are used for regions not subject to translation. Four conserved domains characteristic of cytochrome P450 proteins, which are common for all the AOS family are underlined. Light grey denotes the typical transit peptide for chloroplast targeting within deduced N-terminal sequences [4,6,11]. The heme-binding domain preserved among cytochrome P450s and strongly conserved cysteine residue (in boldface type) is marked grey [4,6]. The characteristic KIFF motif within the oxygen-binding domain is in italics and boldface type [13]. **b** The phylogenetic relationship of *InAOS* compared with the AOS from *A. thaliana* and other plant species. A phylogram tree was generated using ClustalW. Percentages placed in the column were generated in BLAST and deducted InAOS amino acid sequence identity when compared with *A. thaliana* and other species of AOS. GeneBank accession numbers from top to bottom: BAJ78216.1, NP_001236432.1, XP_002302453.1, CAD29735.1, NP_001234833.1, ABC17856.1, HM357792.2, CAC82911.1, BAM76723.1, ABS50433.1, AAY27751.1, NP_199079.1.

Determination of endogenous JA

The method described by Wilmowicz et al. [9] for endogenous JA analyses was used. d_5-JA (100 ng) were added to the crude extract as internal standards. SIM GC/MS was performed by monitoring m/z 193, 198, 224, 229.
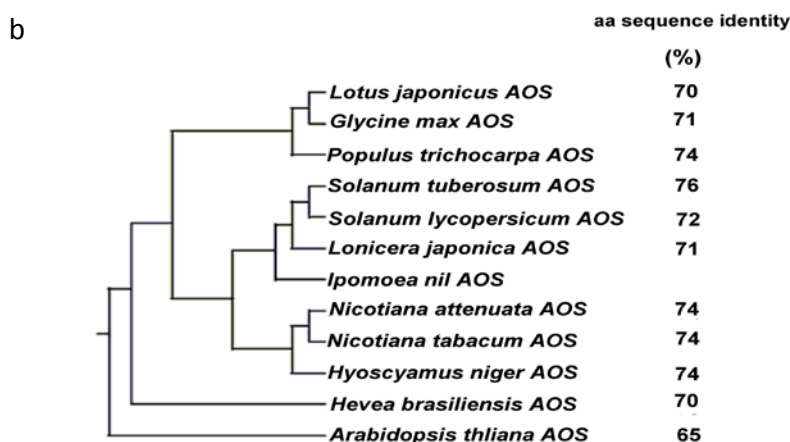
Results

Isolation of cDNA for the allene oxide synthase

The coding sequence of *InAOS* was obtained using 3'RACE-PCR (GeneBank accession number HM357792.2). The full-length *InAOS* cDNA containing start and stop was obtained using 5'RACE-PCR and is composed of 1662 bp and encodes for 519 amino acids (Fig. 1a). A comparison of AOS sequences from *A. thaliana* in the Blast 2.2.25 with InAOS shows that InAOS has 65% amino acid sequence identity to AtAOS (GenBank acc. No. NP_199079.1). Based on amino acid sequence, phylogenetic analysis revealed that InAOS was very closely related to AOS (76%) from *Solanum tuberosum* (GenBank acc. No. CAD29735.1), *Populus trichocarpa* (74%; GenBank acc. No. XP_002302453.1), *Nicotiana attenuata* (74%; GenBank acc. No. CAC82911.1), *Nicotiana tabacum* (74%; GenBank acc. No. BAM76723.1), and *Hyoscyamus niger* (74%; GenBank acc. No. ABS50433.1; Fig. 1b).
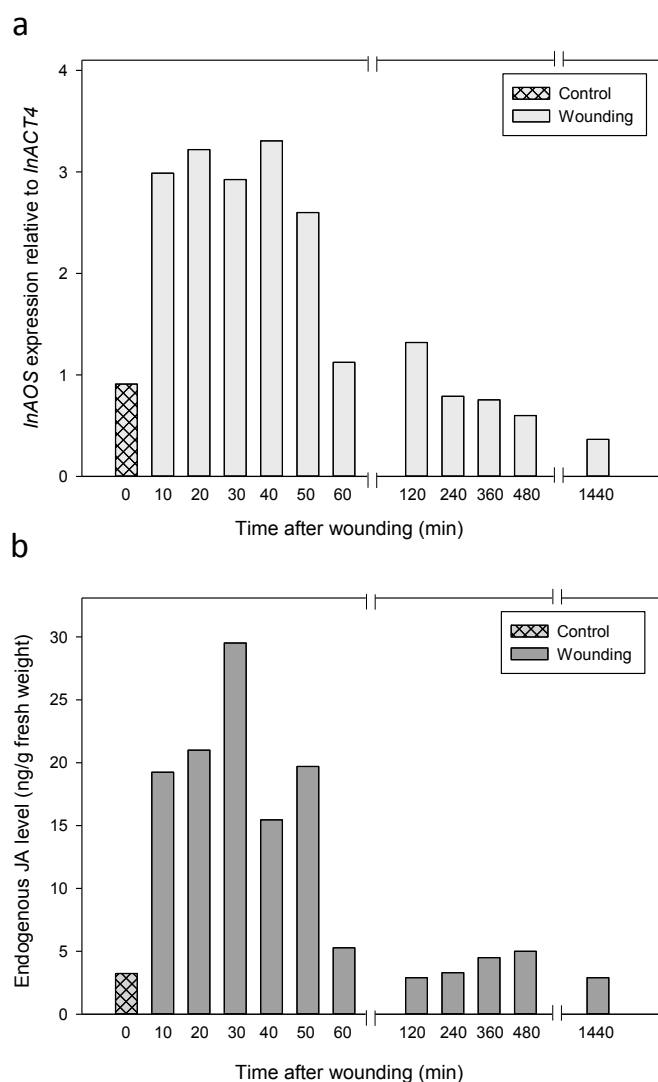
Predicted amino acid InAOS sequence analysis and alignment with others showed that there were four conserved domains characteristic of cytochrome P450 proteins, which are common for all the AOS family. Because InAOS contains motifs that are evolutionarily conserved and characteristic of AOS it can be suspected that this gene encodes for a functional enzymatic protein.

Expression analysis of *InAOS* and endogenous jasmonic acid level in wounded cotyledons of *I. nil*

As shown in Fig. 2a wounding plants resulted in a strong increase in *InAOS* expression level in the cotyledons of

a

b

Fig. 2   **a** The expression level of *InAOS* transcript related to *InACT4* in wounded and control cotyledons of *Ipomoea nil*. The expression activity was measured in three independent replicates. *SE* is marked on the bars. **b** Changes in JA content (ng/g fresh weight) in mechanically wounded cotyledons of *Ipomoea nil*. The control was non-wounded plants. Values are means of three separate samples with two replications for each sample.

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 for a protein similar to that of allene oxide synthases identified 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 an substantial role in JA systemic accumulation in response to wounding.

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