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Citation	Biochemical and Biophysical Research Communications, 336(4), 1150-1156 https://doi.org/10.1016/j.bbrc.2005.08.246
Issue Date	2005-11-04
Doc URL	http://hdl.handle.net/2115/8514
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Type	article (author version)
File Information	Fanjiang Kong-BBRC.pdf

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Allene oxide cyclase is essential for theobroxide-induced jasmonic acid biosynthesis in *Pharbitis nil*

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Abstract

Theobroxide, a natural product, strongly stimulates the biosynthesis of jasmonic acid (JA) in *Pharbitis nil.* In this study, we investigated the accumulation of protein by the immunoblot analysis of lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC), key enzymes in JA biosynthesis, and how the endogenous levels of JA in *Pharbitis nil* are affected by theobroxide. The effect of JA on the accumulations of these proteins was monitored simultaneously. The results show that theobroxide treatment led to a high level accumulation of JA, which is due to high accumulations of LOX, AOS and AOC proteins induced by theobroxide treatment both under short day (SD) and long day (LD) conditions. However, under SD conditions AOS and AOC proteins are not enhanced by JA treatment. Kinetic analysis of protein levels show that a biphasic activation of AOC protein by theobroxide is displayed and the first activation of AOC protein together with elevated JA levels are observed within 30 minutes after treatment. Meanwhile, AOS and LOX proteins are activated by theobroxide later than AOC protein, suggesting that AOC plays an essential role in the initial JA formation induced by theobroxide. Since theobroxide-increased JA levels also show a biphasic manner similar to AOC activation and AOS, LOX proteins are activated later than AOC, and thus we propose a positive JA feedback regulation.

Interestingly, AOS protein, which is also the enzyme for the biosynthesis of 9,10-ketol-octadecadienoic acid (KODA, a flowering inducing factor), accumulates markedly due to the simultaneous involvement of theobroxide and SD conditions, suggesting that AOS probably plays a role in flower bud formation in *Pharbitis nil*.

Keywords: Allene oxide cyclase; Allene oxide synthase; Jasmonic acid; Lipoxygenase; *Pharbitis nil*; Theobroxide

Abbreviations: AOC, allene oxide cyclase; AOS, allene oxide synthase; JA, jasmonic acid; KODA, 9,10-ketol-octadecadienoic acid; LOX, lipoxygenase; LD, long day; SD, short day; GC-SIM-MS, gas chromatography selected ion monitoring mass spectrometry.

Introduction

Theobroxide (Fig 1), a potato tuber inducing compound, was isolated from the culture filtrate of *Lasiodiplodia theobromae* [1]. Interestingly, spraying theobroxide on the leaflets of potatoes (*Solanum tuberosum* L.) and the leaves of morning glory (*Pharbitis nil*), induced potato tuber and flower buds formation, respectively, under non-inductive conditions [2]. Furthermore, theobroxide was shown to inhibit stem elongation in spinach (*Spinacia oleracea* L.) [3] and morning glory [4, 5]. In morning glory, theobroxide was proven to inhibit stem elongation through suppressing

gibberellin biosynthesis and stimulating jasmonic acid biosynthesis [4]. Another report also showed that theobroxide strongly induced JA biosynthesis in morning glory [6]. In potato, theobroxide-induced tuber formation is correlated with increased endogenous levels of JA, and elevated LOX activity [7].

JA is synthesized by one of seven different branches of the lipoxygenase (LOX) pathway, which is initiated by the LOX-catalyzed oxygenation of α-linolenic acid (α-LeA) [8]. Insertion of oxygen can take place at position 13 by a 13-LOX or at position 9 by a 9-LOX. The 13-LOX product with α-LeA as a substrate is a hydroperoxide (13S-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid, 13-HPOT) (Fig. 1). 13-HPOT can be metabolized by an allene oxide synthase (AOS) into an unstable allene oxide, which is cyclized by an allene oxide cyclase (AOC) to *cis*-(+)-OPDA (9*S*, 13*S*) carrying the enantiomeric structure of the naturally occurring JA. The subsequent reduction of the cyclopentenone ring and three steps of β-oxidation lead to JA (Fig. 1). In morning glory, JA involves the theobroxide-inhibited stem elongation [4], while in potato, theobroxide induces tuber formation by stimulating JA and tuberonic acid (TA, Fig. 1) biosynthesis. Subsequently, JA and TA are then converted into the potato tuber-inducing compound, tuberonic acid glucoside (TAG, Fig 1) [9, 10, 7].

As a flower inducing factor, 9-hydroxy-10-oxo-12(*Z*),15(*Z*)-octadecadienoic acid

(9,10-ketol-octadecadienoic acid, KODA, Fig. 1) was isolated from *Lemna paucicostata*. There is a close relationship between flower induction and the transient elevation of the endogenous KODA level during the dark period, suggesting that KODA may be involved in flower induction in *P. nil* [11]. KODA is presumed to be formed from 9-hydroperoxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid (9-HPOT), generated from linolenic acid by 9- LOX and AOS (Fig 1) [12, 13]. Thus, AOS probably plays a role in flower bud formation in *P. nil*.

Theobroxide strongly induces JA formation and flower bud formation in morning glory, but these mechanisms remain to be elucidated. Therefore, in this study, we investigated the protein accumulations of the enzymes in JA biosynthesis by immunoblot analysis and the changes in the endogenous JA levels. The action mechanisms of theobroxide for inducing JA biosynthesis and flower bud formation will be discussed.

Results

Organ specificity of LOX, AOS and AOC expression in P. nil

Immunoblot analysis of total proteins, extracted from various organs revealed the abundant appearance of LOX in leaves, cotyledons and flower buds of mature *P. nil* plants. Much less protein occurs in stems and petioles and almost undetectable levels

could be found in flowers and roots. AOS protein is predominantly found in the leaves and low levels are detected in cotyledons. Unlike in Arabidopsis [14], AOS protein is undetectable in flower organs in *P. nil*. AOC protein is also observed at high levels in the leaves, less protein occurs in the cotyledons and flower buds and, no protein is found in other organs (Fig. 2). All these results suggested that the leaf is the main site for the biosynthesis of JA in *P. nil*. Therefore, we selected leaves as the materials for further analysis.

Accumulation of LOX, AOS and AOC proteins and JA levels in P. nil induced by theobroxide

Our previous results [4, 6] demonstrated that theobroxide strongly stimulates JA biosynthesis in *P. nil*. However, the mechanism of enhancement of JA biosynthesis by theobroxide remains unclear. Therefore, in this study we investigated the accumulations of three enzymes in JA the biosynthesis pathway affected by theobroxide via an immunoblot approach. In comparison, the effect of JA on protein accumulation was also evaluated. One week old seedlings of *P. nil* were treated with theobroxide or JA every 2 days until the leaves were harvested. Total proteins were extracted and the accumulation levels of LOX, AOS and AOC were determined; meanwhile, the endogenous levels of JA were also quantified. The immunoblot analysis showed that LOX, AOS and AOC

proteins accumulated to high levels after 1 and 2 weeks of theobroxide treatment in *P. nil* under LD conditions (Fig. 4). However, under SD conditions, AOS and AOC proteins accumulated markedly due to theobroxide treatment, while LOX protein remained constant (Fig 4a). The corresponding levels of JA affected by theobroxide are shown in Figure 3. The levels of JA increased dramatically after 1 and 2 weeks of theobroxide treatment both under SD and LD conditions. This is in accordance with the increased accumulations of LOX, AOS and AOC proteins, but LOX accumulation was seemingly not necessary for the theobroxide induced JA production under SD conditions since the LOX protein remained unchanged. Similarly, under LD conditions, JA induced high accumulations of LOX, AOS and AOC proteins (Fig 4). Under SD conditions, unlike the effect of theobroxide, JA only induced the accumulation of LOX, while the accumulation of AOS was reduced and the accumulation of AOC was unaffected after 1 and 2 weeks treatment. These results suggest that the effects of theobroxide and JA on the protein accumulations of JA synthesis enzymes are different and a somewhat complicated process was proposed.

AOC is essential for theobroxide-stimulated jasmonic acid biosynthesis in P. nil

Theobroxide treatment led to the accumulation of high levels of LOX, AOS, AOC proteins and endogenous JA after 1 and 2 weeks treatment in *P. nil*. How these

enzymes are regulated by theobroxide remains unclear. Therefore, we investigated the kinetic accumulation of relative proteins treated with theobroxide by the immunoblot approach. The results show how under SD conditions (Fig 5a), in the biphasic activation of AOC protein, the first and second activation are displayed at 30 minutes and 6 hours after theobroxide application, respectively. While LOX protein accumulation was enhanced by theobroxide later (1 hour after theobroxide treatment) than AOC (30 minutes after treatment), LOX accumulation decreases gradually (Fig 5a), while AOS accumulation remains almost constant. Conversely, under LD conditions (Fig 5b), the AOC protein accumulation induced by theobroxide showed a biphasic activation manner, but the second activation was earlier (2 hours after treatment) than under SD conditions (6 hours after treatment), and remained constant until 24 hours. Unlike that under SD conditions, AOS protein accumulation was enhanced by theobroxide 1 hour after application. In the case of LOX protein accumulation, stimulation occurred within 2 hours after theobroxide treatment, which was late in comparison with the SD conditions (1 hour after treatment). It became clear that AOC is the first protein activated by theobroxide, but not AOS and LOX proteins. The quantitative analysis results showed that JA levels were increased by theobroxide 30 minutes after application (Fig 5c) and at this time only the AOC protein is activated (Fig 5b),

suggesting that the rise of JA level was due to activated AOC accumulation. Similar to the activation pattern of AOC protein, a biphasic rise of the JA level induced by theobroxide was also displayed. After about 6 hours of theobroxide application, the JA levels were increased again and this second rise of the JA levels was attributed to the enhanced LOX, AOS and AOC protein accumulations, suggesting the occurrence of positive JA feedback regulation. The results we obtained here show that the first peak of theobroxide-induced JA accumulation was dependent on the enhancement of AOC protein accumulation directly simulated by theobroxide, while the second peak of JA accumulation was probably due to the feedback activation of LOX, AOS and AOC protein accumulations by the initially elevated JA accumulation.

In order to test the effect of JA on LOX, AOS and AOC accumulation in the short term, we applied JA onto the seedlings under LD conditions and performed immunoblot analysis. Unlike the effect of theobroxide, slight effects of JA on AOS and AOC protein accumulation could be observed. However, LOX protein accumulation was strongly enhanced by JA treatment and this enhancement was earlier (1 hour after treatment) than that of theobroxide (2 hours after treatment, Fig 5b). These results show that the effects on LOX, AOS and AOC protein accumulation of JA and theobroxide are different, suggesting the presence of different effects of JA and theobroxide on JA

biosynthesis.

Correlations between AOS protein expression and flower bud formation in P. nil

KODA is involved in flower induction in *P. nil* [11] and is synthesized through catalyzing 9-hydroperoxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid by AOS protein (Fig 1) [12, 13], suggesting that AOS protein might be involved in the flower bud formation induced by theobroxide and SD conditions. Our results show that after 2 weeks, flower bud formation was induced by theobroxide in *P. nil* under non-inductive conditions, LD conditions (Fig 7a). At this time, extremely high levels of AOS protein are accumulated by theobroxide application (Fig 4b). Conversely, under flower inductive conditions (SD conditions), more flower buds were formed in theobroxide treated plants compared with the control plants (Fig 7a), which is also closely related to the high accumulation of AOS protein by theobroxide treatment (Fig 4b). Interestingly, AOS proteins accumulated markedly under SD conditions, while AOS proteins were almost undetectable under LD conditions (Fig 7b). This is in accordance with the fact that SD conditions induce flower bud formation in *P. nil* [15], which was also observed in this experiment (Fig 7a), but no flower buds were found, even after 20 days under LD conditions. Moreover, by applying a JA analogue, methyl jasmonate, flower bud formation was inhibited in *P. nil* under SD conditions [16, 17]. This inhibition was also

in agreement with our results that JA reduces flower bud numbers under SD conditions (Fig 7a). This was in accordance with the immunoblot results (Fig. 4b), the AOS protein levels were markedly reduced by JA treatment under SD conditions. All these results suggest that AOS proteins are closely related to flower bud formation in *P. nil*.

Discussion

Theobroxide induces the tuber formation of potato, induces flower bud formation and inhibits stem elongation in *P. nil* [2, 4]. Theobroxide-induced potato tuber formation and theobroxide-inhibited stem elongation in P. nil are dependent on the stimulation of JA biosynthesis by theobroxide [4, 7]. It is less understood, however, how this JA stimulation is regulated by theobroxide. JA and its methyl ester, collectively named jasmonates, are ubiquitously occurring plant growth regulators. The stress-induced rise of jasmonates is a common phenomenon in all plants analyzed so far. It has been reported several times that following a stimulus such as elicitation [18] or wounding [19], JA levels increase significantly and often in a complex, biphasic manner (for a review see [20]). This is also the case for theobroxide activated JA accumulation in *P. nil* (Fig. 5c). We have shown here that AOC protein expression is first activated by theobroxide following a transient JA burst after 30 minutes and, subsequently a positive JA feedback regulation probably occurs. Later LOX, AOS and AOC protein

expressions are enhanced, following the second rise of JA levels (Fig. 5). These results show a preferential role of AOC in the regulation of JA biosynthesis, suggesting the AOC is the major control point for theobroxide-induced JA formation. This is in accordance with the previous results that only the accumulation of AOC protein, but not that of LOX and AOS proteins is affected in Arabidopsis mutant *opr3*, in which JA downstream biosynthesis enzyme, OPDA reductase, was blocked [21]. The AOC-catalyzed step is regarded as the crucial step in jasmonate biosynthesis, because only this enantiomeric form is the substrate for the naturally occurring JA, which is formed after the reduction of OPDA by a specific OPDA reductase and three cycles of β-oxidation [22].

Regulation of JA biosynthesis is a quite complex process. In Arabidopsis, LOX, AOS and AOC proteins, which are compartmentalized within the chloroplast [14, 23], might function by substrate channeling during basal JA formation, and stimuli may allow substrate accessibility by the relevant enzymes, thereby increasing JA formation. This model was discussed by Froehlich et al. [24] and is in accordance to previous data on 35S::LOX2*antisense* plants [23], which exhibited a decrease in wound-induced JA formation only, but were still able to form residual levels of JA. Another possibility in the regulation of JA biosynthesis would be an activity control by post-translational

modifications of the pre-existing enzymes, such as AOC. Such a control would allow the plant to respond rapidly upon wounding or pathogen/insect attack or theobroxide application by the formation of JA, which functions subsequently as a signal in defense gene expression or for feedback regulation of JA biosynthesis enzymes. The functional role of the subsequent transcriptional up-regulation of JA-biosynthetic enzymes remains to be elucidated.

P. nil (violet) is a typical short-day plant, in which flowering is induced when the day length becomes shorter than a critical value. The process is very sensitive; flowering is induced by exposing a seedling to a single 16 hour dark period after cultivation under continuous light [15]. Under non inductive conditions, theobroxide strongly induces flower bud formation in *P. nil* [2, 7]. However, how theobroxide induces flower bud formation in *P. nil* remains unclear. KODA reacts with catecholamines to generate products that strongly induce flowering and a close relationship between flower induction and the transient elevation of the endogenous KODA level during the dark period was observed, suggesting that KODA may be involved in flower induction in *P. nil* [11, 25, 26]. AOS catalyzes 9(S)-hydroperoxy-octadecatrienoic acid (9-HPOT) to 9,10-epoxylinolenic acid, a short-lived allene oxide that forms KODA automatically[12, 13] suggesting AOS may

involve in flower bud formation in *P. nil*. Here, we show that under inductive conditions (SD conditions or LD conditions with theobroxide application), AOS protein is strongly induced (Fig 7b, Fig 4b), which correlates with the flower bud induction (Fig. 7a). Under SD conditions, JA was shown to inhibit flower bud formation (Fig. 7a) [16, 17], which is in accordance with our observations that AOS accumulation is reduced by JA (Fig 4b). All these results suggest that there is a close relationship between AOS protein expression and flower bud formation, indicating that AOS is probably involved in flower bud formation in *P. nil*.

Materials and Methods

Plant materials and growing conditions

Seeds of Pharbitis nil cv. Violet (Marutane Seed Co, Kyoto, Japan) were treated with concentrated H_2SO_4 for 1 hour, washed with running tap water for another 3 hours, and then soaked in distilled water overnight at 25 ºC. The swollen seeds were sown in pots filled with peat moss and perlite (1:1 v/v) under LD (18 hour light/6 hour dark) conditions in growth chambers (NK System, Biotron NC 350, Japan) before treatment. The chambers were equipped with 20 slip fluorescent lamps (NEC FL40SEX-N-HG, Japan) to provide white light (150 μ mol $m^{-2}s^{-1}$), at a temperature of 25 ºC and relative humidity of 60%. Seven-day-old seedlings were treated with different solutions. Water

and liquid Hyponex (diluted to 500 times with water, Hyponex Japan Co. Ltd., Japan) were applied to the plants at every two 2 days and once a week, respectively.

Plant treatments

The photoperiods for SD and LD conditions were set in 10L/14D and 18L/6D, respectively. Theobroxide was isolated from the culture filtrate of *Lasiodiplodia theobromae* [1]. Theobroxide and JA were each dissolved in a small amount of DMSO, and then diluted in water containing 100 ppm Tween 20 to give a concentration of 1 mM. Water containing a small amount of DMSO and 100 ppm Tween 20 was used as a control solution. Five milliliters of theobroxide or JA solution was sprayed onto the leaf surface of each seedling at 2-day intervals after the plants were transferred. Plant tissues were harvested two weeks after spraying, placed immediately in liquid nitrogen and then stored at -80ºC until being analyzed. Ten plants were used in each experiment which was repeated at least 3 times.

Scoring of the flowering response

The timing date of flower buds was scored and the number of flower buds and flowers per plant in *P. nil* were counted every two days. Ten plants were used for each treatment, and the average number of flower buds per seedling is presented. Each treatment was repeated at least three times.

Protein extraction

About 0.1 g of frozen leaves was homogenized with a polytron in 1 ml of 0.1 M phosphate buffer (pH 6.6). The homogenate was centrifuged for 20 min at 15,000g and the supernatant was used as the enzyme source. Protein contents were determined by the Bradford method, using BSA as standard [27].

Electrophoresis and Immunoblot analysis

Proteins were analyzed in 10% SDS-polyacrylamide gel according to Laemmli [28]. In the transfer of electrophoretically separated proteins from the gels to nitrocellulose sheets, we followed the method of Towbin et al. [29]. Blots were developed using chemiluminescence reagent (PerkinElmer Life Sciences, Inc.) according to the manufacturer's instructions. Rabbit polyclonal antibodies raised against potato LOX [30], the recombinant AOS of *A. thaliana* [14] and the recombinant AOC2 of *A. thaliana* [21], were used. Antibodies were used in the following dilutions: anti-LOX, 1: 5000; anti-AOS, 1: 5000; anti-AOC, 1: 5000.

Extraction and quantification of endogenous JA

The content of JA was analyzed according to the method of Matsuura et al. [31] with small modifications. Plant tissues (1g) were ground under liquid nitrogen and suspended in 80% aq. MeOH (10 ml) overnight. The mixture obtained was filtered and

500 ng deuterium-labeled JA (internal standard) was added. The filtrate was concentrated under reduced pressure, re-dissolved in H_2O, adjusted to pH 2-3 with 6 N HCl, and extracted with EtOAc (10ml×3). The combined organic layers were washed with saturated $NaHCO_3$ (10ml×3), adjusted to pH 2-3 with 6N HCl, and extracted with EtOAc (10ml×3). The combined organic layers were concentrated and the resulting residue was dissolved in 1 ml of H_2O and subjected to the cartridge column of Bond Elut C_{18} (Varian, CA, U.S.A.). The column was successively washed with H_2O (1ml×2) and MeOH/H_2O (4:1, v/v, 1ml×4). The 80% MeOH/H_2O eluant was concentrated *in vacuo*, and the residue was subjected to Bond Elut DEA (Varian, CA, U.S.A.). The column was successively washed with MeOH (1ml×2) and 1N AcOH/MeOH (1ml×4). The AcOH/MeOH eluant was evaporated. The residue was dissolved in MeOH, methylated by ethereal diazomethane and purified by HPLC (Hitachi L7100, HITACHI, Japan) equipped with a column of YMC-Pack ODS (300 mm×10 mm; YMC Co, Ltd., Japan) to give a JA fraction. The fraction was analyzed by a GC- SIM-MS (QP5000 system, SHIMAZU, Japan) using a ZB-1 column (30m×0.25mm, 0.5μm phase thickness). The GC temperature program was 80 ºC for 1 min, 80-290 ºC at 20 ºC/min and 290 ºC for 5 min. Injector and detector temperatures were 200 and 280 ºC, respectively. The amounts of endogenous JA were calculated from the peak areas of JA

in comparison with the corresponding internal standard.

Acknowledgements

We thank Dr. C. Wasternack for the supply of anti-AOC-antibodies, Dr. F. Schaller and Prof. E.W.Weiler for the supply of anti-AOS-antibodies, Prof. J. J. Sanchez-Serrano for anti-LOX-antibodies, Mr. Li Li, Dr. Baohui Liu and Dr. Shinya Mitsuhashi for their help with immunoblot analysis. Dr. C. Wasternack and Dr. F. Schaller are gratefully acknowledged for their critical readings of the manuscript and helpful suggestions.

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Figure legends

Figure 1. Chemical structure of theobroxide and putative linolenic acid cascade.

Figure 2. Immunoblot showing the relative abundance of LOX, AOS and AOC proteins in different organs of 3-week-old *P. nil* plants growing under SD conditions. A total of 20 μg protein was applied to each lane. Immunoblots were probed with anti-LOX antibodies, with anti-AOS antibodies and anti-AOC antibodies all at dilution of 1:5000, respectively.

Figure 3. The effect of theobroxide on endogenous JA levels of leaves growing under SD conditions (A) and LD conditions (B). One-week-old seedlings were subjected to theobroxide treatment every 2 days, leaf samples were collected 1 and 2 weeks after treatment started. Data are the means ± s.d. of n=3 independent experiments.

Figrue 4. Immunoblot analysis of LOX (A), AOS (B) and AOC (C) proteins in *P. nil* following treatment with theobroxide and JA. One-week-old seedlings were subjected to theobroxide and JA treatment every 2 days, leaf samples were collected 1 and 2 weeks after treatment started. Cont, plants were treated with H_2O; Theo, plants were

treated with theobroxide; JA, plants were treated with JA. A total of 20 μg protein was applied to each lane. The dilution of antibodies was described at figure 2.

Figure 5. Kinetics of LOX, AOS and AOC proteins levels of *P. nil* growing under SD (A) and LD (B) conditions or endogenous levels of JA following treatment with Theobroxide. Two-week-old seedlings were subjected to theobroxide treatment, leaf samples were collected at the times indicated. For immunoblot analysis, a total of 20 μg protein was applied to each lane. The dilution of antibodies is described in figure 2. For JA quantification (C), identical samples as in (B) were used for JA extraction. Data are the means ± s.d. of n=3 independent experiments.

Figure 6. Kinetics of LOX, AOS and AOC proteins levels of *P. nil* growing under LD conditions following treatment with JA. Two-week-old seedlings were subjected to JA treatment, leaf samples were collected at the indicated times. A total of 20 μg protein was applied to each lane. The dilution of antibodies was described at figure 2.

Figure 7. Numbers of flower buds in *P. nil* (A) and immunoblot analysis of AOS protein under different photoperiods (B). For (A), one-week-old seedlings were subjected to

theobroxide and JA treatment every 2 days. The number of flower buds was counted at the indicated time. SD-Cont, plants treated with water under SD conditions; SD-Theo, plants treated with theobroxide under SD conditions; SD-JA, plants treated with JA under SD conditions. For (B), one-week-old seedlings were growing under SD or LD conditions, after 1 and 2 weeks, plant leaves were collected and proteins were isolated. A total of 20 μg protein was applied to each lane. Immunoblots were probed with anti-AOS antibodies at a dilution of 1:5000.

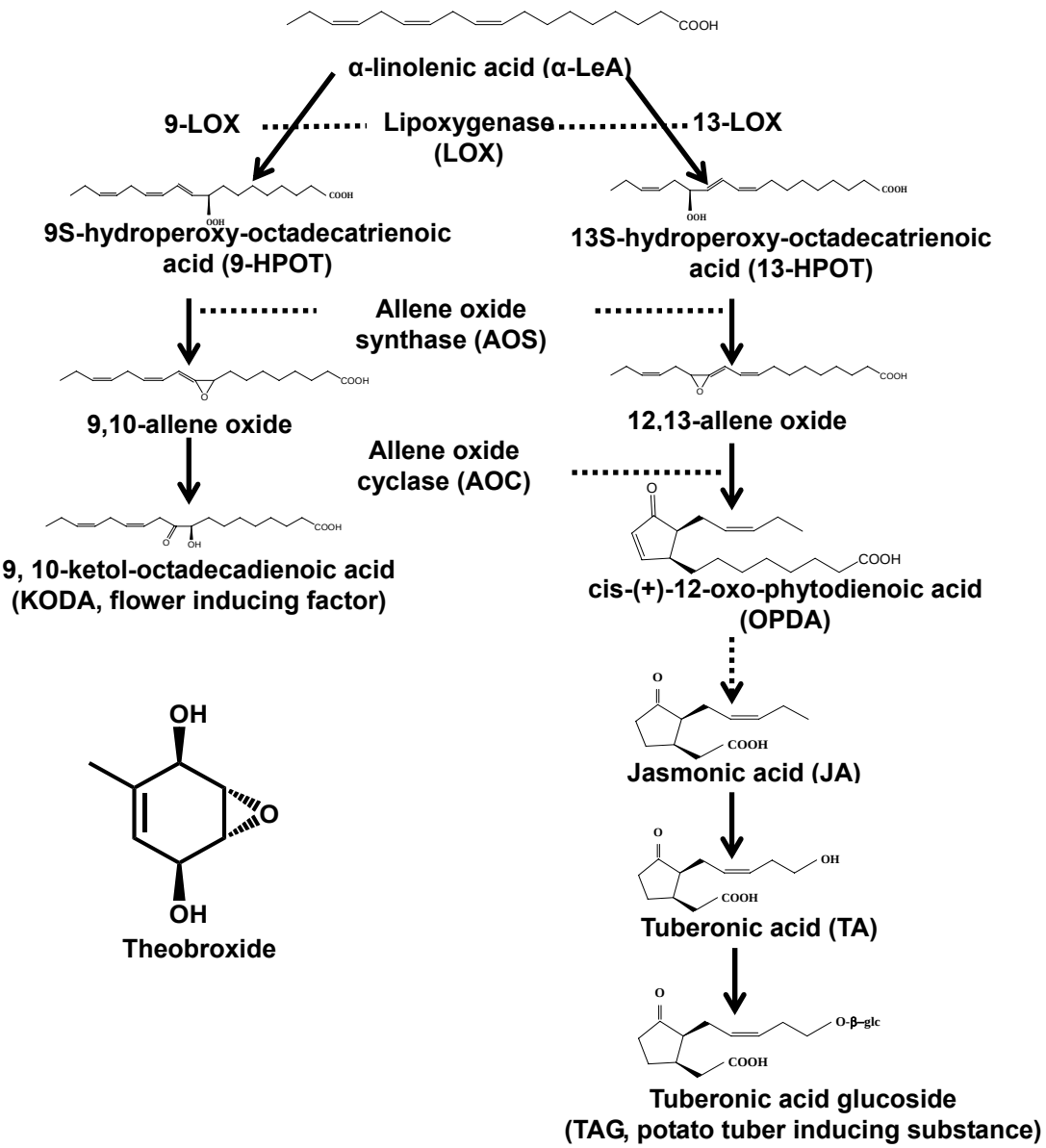
α-linolenic acid (α-LeA)
9-LOX
Lipoxygenase (LOX)
13-LOX
9S-hydroperoxy-octadecatrienoic acid (9-HPOT)
13S-hydroperoxy-octadecatrienoic acid (13-HPOT)
Allene oxide synthase (AOS)
9,10-allene oxide
12,13-allene oxide
Allene oxide cyclase (AOC)
9, 10-ketol-octadecadienoic acid (KODA, flower inducing factor)
cis-(+)-12-oxo-phytodienoic acid (OPDA)
Jasmonic acid (JA)
Tuberonic acid (TA)
Tuberonic acid glucoside (TAG, potato tuber inducing substance)
Theobroxide

Figure 1

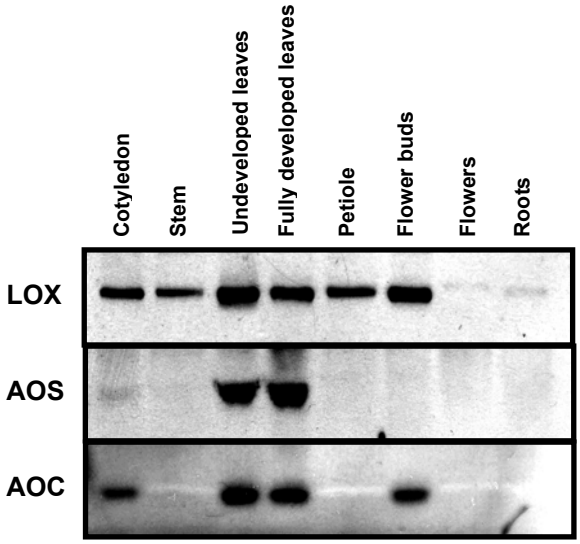
Cotyledon
Stem
Undeveloped leaves
Fully developed leaves
Petiole
Flower buds
Flowers
Roots
LOX
AOS
AOC

Figure 2

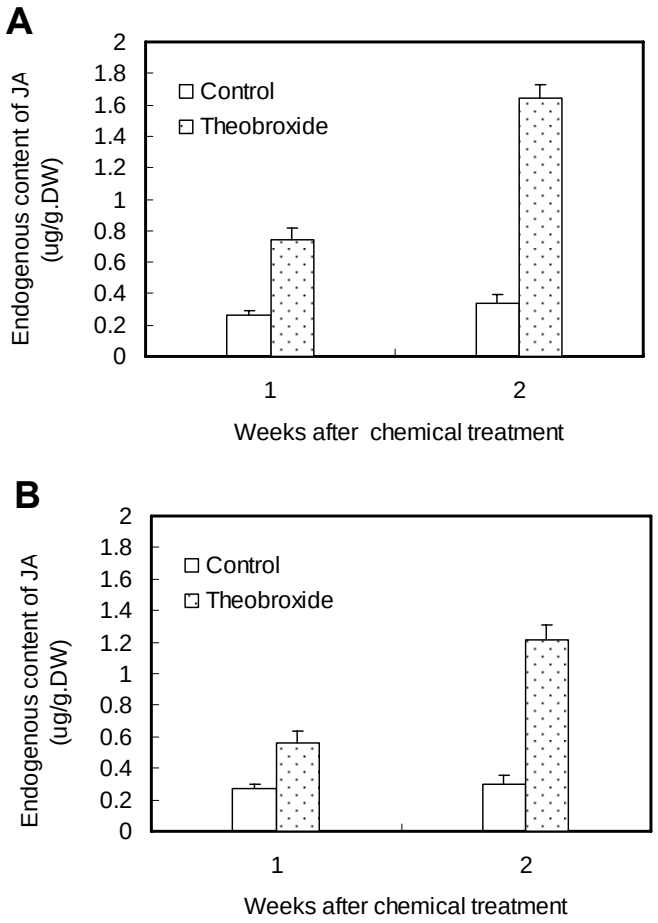
A
Endogenous content of JA (ug/g.DW)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
Theobroxide
1
2
Weeks after chemical treatment
B
Endogenous content of JA (ug/g.DW)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
Theobroxide
1
2
Weeks after chemical treatment

Figure 3

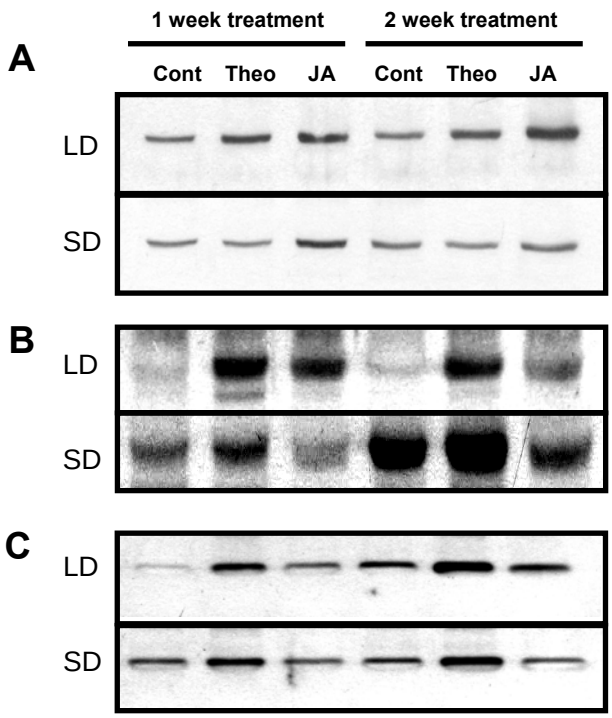
1 week treatment
2 week treatment
A
Cont
Theo
JA
Cont
Theo
JA
LD
SD
B
LD
SD
C
LD
SD

Figure 4

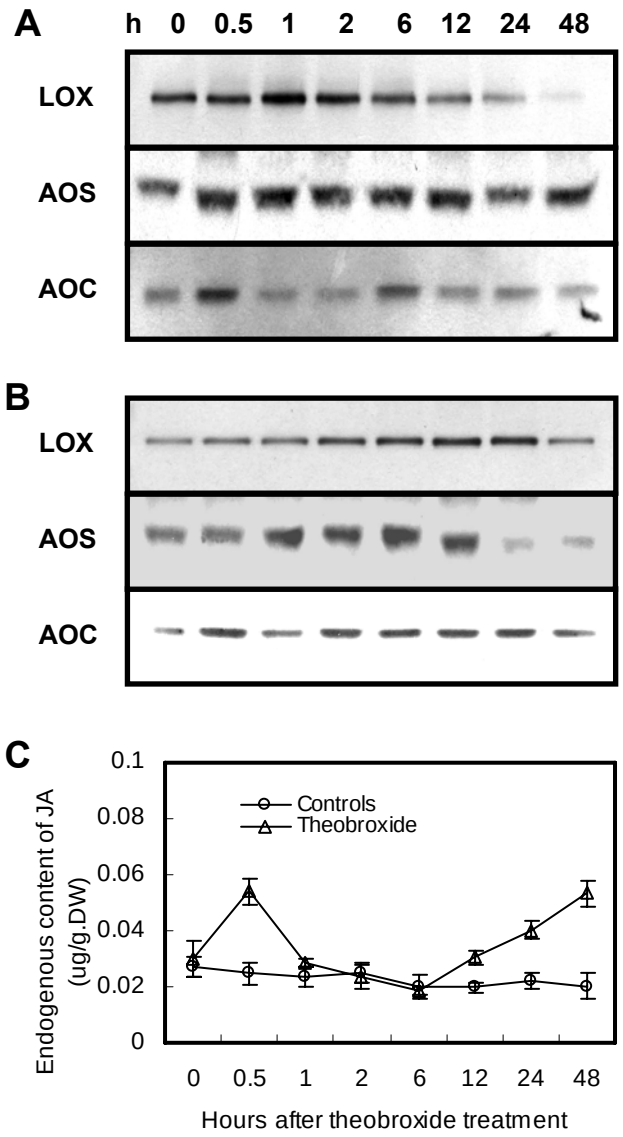
A
h 0 0.5 1 2 6 12 24 48
LOX
AOS
AOC
B
LOX
AOS
AOC
C
Endogenous content of JA (ug/g.DW)
0.1
0.08
0.06
0.04
0.02
0
Controls
Theobroxide
0 0.5 1 2 6 12 24 48
Hours after theobroxide treatment

Figure 5

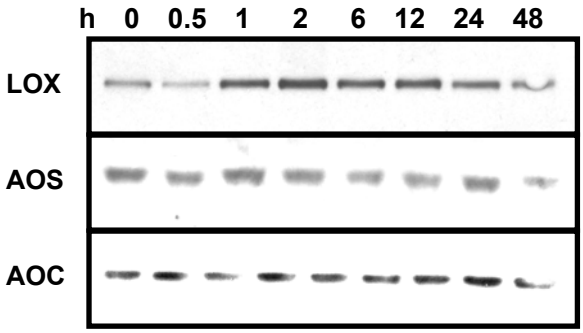

Figure 6

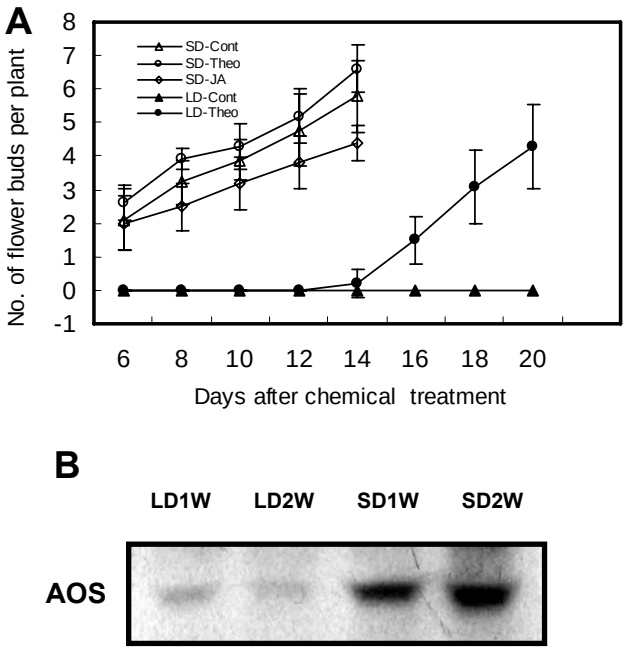
A
No. of flower buds per plant
8
7
6
5
4
3
2
1
0
-1
SD-Cont
SD-Theo
SD-JA
LD-Cont
LD-Theo
6
8
10
12
14
16
18
20
Days after chemical treatment
B
LD1W
LD2W
SD1W
SD2W
AOS

Figure 7