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Theobroxide inhibits stem elongation in *Pharbitis nil* by regulating
jasmonic acid and gibberellin biosynthesis

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Summary

In this study, exogenous factors affecting the elongation growth in the short day plant, *Pharbitis nil*, was investigated. Theobroxide inhibited stem elongation in *Pharbitis nil* both under short day (SD) and long day (LD) conditions. Salicylhydroxamic acid (SHAM), an inhibitor of jasmonic acid (JA) biosynthesis, and GA₃ recovered the inhibitory effect of theobroxide on stem elongation. Quantitative analysis of JA showed that the level of endogenous JA increased significantly in theobroxide treated plants, while exogenously applied GA₃ and SHAM suppressed JA biosynthesis stimulated by theobroxide. The activity of lipoxygenase (LOX, the key enzyme of JA biosynthesis) also was stimulated by theobroxide and this stimulation was nullified by SHAM and GA₃. Quantitative analysis of GA₁ showed that theobroxide suppressed GA₁ biosynthesis. In non theobroxide treated *Pharbitis nil*, SD conditions stimulated JA biosynthesis and LOX activity, while GA₁ biosynthesis was suppressed. All these results suggest that JA probably is involved negatively in the control of stem elongation, and the balance between JA and gibberellin might determine the stem growth in *Pharbitis nil*.

Keywords: Theobroxide; Jasmonic acid; Gibberellin; Stem elongation; Lipoxygenase; *Pharbitis nil*

Abbreviations JA, jasmonic acid; LOX, lipoxygenase; SHAM, salicylhydroxamic

acid; GA, gibberellin; LD, long day; SD, short day; GC-SIM-MS, gas chromatography selected ion monitoring mass spectrometry; JA-Me, methyl jasmonate; Jasmonates, JA and its related compounds

Introduction

Theobroxide, 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 glories (*Pharbitis nil*), induced formation of potato tuber and flower buds, respectively, under non-inductive conditions [2]. Another report showed that theobroxide-induced potato tuber formation was correlated with increased endogenous levels of JA, and elevated LOX activity [3]. Moreover, the inductive effect of theobroxide on potato tuber formation was eliminated by applying SHAM, which was a JA biosynthesis inhibitor [4].

Many recent studies have demonstrated that JA is a significant component of the signaling pathway that regulates the expression of plant defense genes in response to various environmental stresses [5]. Other fundamental and remarkable roles of the jasmonates include their regulation of plant morphogenesis [5]. Needless to say, plant morphogenesis is controlled mainly by the frequency and direction of cell division, and the direction of cell expansion. The production of organs or tissues with defined shapes

must be precisely controlled among the constituent cells. JA and JA-Me are known to participate in the control of the elongation growth of plants [6]. In dwarf rice seedlings, exogenous JA reduces the effect of GA₃ on the elongation of the second leaf sheath [7]. In lettuce, applied JA also diminishes the effect of GA₃ on the elongation of hypocotyls [7]. Exogenously applied JA substantially inhibits IAA-induced elongation of etiolated oat coleoptile segments [8]. In soybean, endogenous JA is involved in the control of the stem growth habit [9]. Exogenous JA-Me inhibits roots and shoots growth in *Pharbitis nil* [10].

Lipoxygenases (LOXs; EC 1.13.11.12) are non-heme iron-containing dioxygenases that are widely distributed in plants and animals. LOX catalyzes the addition of molecular oxygen to polyunsaturated fatty acids containing a (Z,Z)-1,4-pentadiene system to produce an unsaturated fatty acid hydroperoxide. In plants, linolenic and linoleic acids are the most common substrates for LOX [11]. Distinct LOX isozymes preferentially introduce molecular oxygen into either the C-9 or the C-13 of linolenic and linoleic acids. Subsequently, two distinct fatty acid monohydroperoxides are formed and further directed into separate biosynthetic pathways that result in the accumulation of compounds with distinct physiological functions. 13-Hydroperoxy fatty acids are precursors of biologically active compounds such as JA and JA-Me which

serve hormone like regulatory and defense-related roles in plants [11]. In potato, theobroxide increased LOX activity and stimulated biosynthesis of JA and tuberonic acid glucoside, consequently induced potato tuber formation [3, 12].

In present study, we report here the inhibitory effects of theobroxide on stem elongation in *Pharbitis nil*, and the effects of the compound in the presence of SHAM or GA₃ on the stem elongation, JA levels, GA₁ levels and LOX activity in the plant. A mechanism of action of theobroxide is also proposed.

Materials and methods

Plant materials

Seeds of *Pharbitis nil* cv. Violet (Marutane Seed Co, Kyoto, Japan) were treated with concentrated H₂SO₄ for 1 hour, washed with running tap water for another 3 hours, and then soaked in distilled water overnight at 25 °C. 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⁻²s⁻¹), at temperature of 25 °C 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.

Treatments of plants

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]. SHAM was purchased from Sigma Chemical Co., and GA₃ was provided by Prof. Zhou Xie (Nanjing Agricultural University, China). Theobroxide, SHAM, and GA₃ were each dissolved in a small amount of DMSO, and then diluted in water containing 100 ppm Tween 20 to give the concentration of 1 mM. Water containing small amount of DMSO and 100 ppm Tween 20 was used as control solution. Five milliliter theobroxide or SHAM solution was sprayed onto the leaf surface of each seedling at 2-day intervals after plants were transferred. For the theobroxide-SHAM treatment, theobroxide solution was sprayed 30min after the SHAM treatment. For the theobroxide- GA₃ treatment, GA₃ (200ul) was applied to the cotyledons using a soft paint brush 30min before theobroxide spraying. Plant tissues were harvested two weeks after spraying for all treatments, placed immediately in liquid nitrogen and then stored at -80°C until being analyzed. Stem length was measured from cotyledon to the top of the pant. Ten plants were used in each experiment which was repeated at least 2 times.

Determination of endogenous JA

The content of JA was analyzed by the method of Matsuura et al. [13] with small modification. Plant tissues (1g) were ground under liquid nitrogen and suspended in 80% aq. MeOH (10 ml) for 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 H₂O, adjusted to pH 2-3 with 6 N HCl, and extracted with EtOAc (10ml×3). The combined organic layers were washed with saturated NaHCO₃ (10ml×3), adjusted to pH 2-3 with 6N HCl, and extracted with EtOAc (10ml×3). The combined organic layer was concentrated, and the resulting residue was dissolved in 1 ml of H₂O and subjected to the cartridge column of Bond Elut C₁₈ (Varian, CA, U.S.A.). The column was successively washed with H₂O (1ml×2) and MeOH/H₂O (4:1, v/v, 1ml×4). The 80% MeOH/H₂O 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 JA fraction. The fraction was analyzed by 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 °C for 1 min, 80-290 °C at 20 °C/min and 290 °C for 5 min. Injector and detector temperatures were 200 and 280 °C, respectively. The amounts of endogenous JA were calculated from the peak areas of JA in comparison with the corresponding internal standard.

Determination of endogenous GA₁

The extraction and purification procedure of GA₁ was the same as that used in the JA determinations described above. As internal standard, 17,17-d₂- GA₁ was added to the 80% aq. MeOH extracts. GA₁ containing fractions from HPLC were methylated with ethereal diazomethane followed by trimethylsilylation with *N,O*-Bis(trimethylsilyl)acetamide. The derivatives was analyzed by GC-SIM-MS (QP5000 system, SHIMAZU, Japan) using a ZB-1 column (30m×0.25mm, 0.5µm phase thickness). After injection, the oven temperature was kept at 60 °C for 1 min, and then increased to 200 °C with 20 °C min⁻¹ followed by further increment to 300 °C with 4 °C min⁻¹, and finally kept at 300 °C for 10 min. Injector and detector temperatures were both 230 °C. The amounts of endogenous GA₁ were calculated from the peak areas of GA₁ in comparison with the corresponding internal standard.

Lipoxygenase assay

LOX activity was determined as previously described [3]. About 0.1 g of frozen

leaves was homogenized with a polytron in 1 ml of 0.1 M phosphate buffer (pH 7.2). The homogenate was centrifuged for 20 min at 15,000g. The LOX activity in the supernatant was analyzed immediately at 25 °C using 10mM sodium linoleate as the substrate in 0.1 M acetate buffer (pH 4.4) by a Hitachi U3210 spectrophotometer. The increase in absorbance at 234 nm was measured to monitor the formation of conjugated-diene compound according to the method of Axelrod et al. [14]. One unit of LOX activity is defined as 1 μ mol of product formed mg⁻¹ protein min⁻¹. Protein contents were determined by Bradford method using BSA as standard [15]. All extraction procedures were carried out at 4 °C.

Results

*Effects of theobroxide, SHAM and GA₃ on stem elongation of *Pharbitis nil**

Our previous report [3] showed that theobroxide increased LOX activity and stimulated JA biosynthesis in potato (*Solanum tuberosum*. L). It could be reasonably presumed that JA biosynthesis was also enhanced by theobroxide in *Pharbitis nil*, and that JA was probably involved in the control of theobroxide-inhibited stem elongation in *Pharbitis nil*. In order to examine this assumption, we studied the effect of a JA biosynthesis inhibitor, SHAM [4], on the stem elongation of the plants treated with theobroxide both under SD and LD conditions. The solutions of theobroxide (1mM) and

SHAM (1mM) were sprayed on the seedlings of *Pharbitis nil*. Two weeks after treatment, the stem length was examined. The results showed that theobroxide inhibited stem elongation both under SD (10L/14D) and LD (18L/6D) conditions (Fig. 1). Under SD condition, the stem length of theobroxide treated plants (5.50cm) was approximately 40% lower than that of control plants (9.11cm) two weeks after treatment, and similar effect was observed under LD conditions. As expected, the inhibitory effect of theobroxide on stem elongation was partially reversed by application of SHAM both under SD and LD conditions (Fig. 1). Furthermore, to examine the interactions of theobroxide with GA on shoot growth, we applied 1mM of GA₃ to plants once before theobroxide spraying. The results indicated that GA₃ nullified the inhibitory effect of theobroxide on shoot growth both under SD and LD conditions (Fig. 1). In addition, the plant was significantly higher under LD conditions than under SD conditions with treatment even without treatment (Fig. 1).

Endogenous levels of JA after different treatments

SHAM was shown to reverse the inhibitory effects of theobroxide on shoot growth (Fig. 1). We therefore examined the endogenous level of JA in different tissues of *Pharbitis nil* two weeks after treatment. In leaves, the content of JA in theobroxide treated plants was 4 times higher than that in control plants under SD and LD conditions

(Table 1, 2). The effect of theobroxide for increasing JA level was so potent, and in accordance with the previous report [3]. When theobroxide was applied together with SHAM or GA₃, the level of JA was reduced. In cotyledons and shoots, the endogenous levels of JA were also increased by theobroxide both under SD and LD conditions, however JA levels in cotyledons and shoots were lower than that in the leaves (Table 1, 2). The amount of endogenous JA in all tissues examined in this study under SD conditions was higher than those under LD conditions, and the JA content was approximately 1.5 times higher under SD condition than LD condition in control plants. These results suggested that JA levels were related not only to the theobroxide-influenced stem growth but also to the photoperiod regulated stem growth in *Pharbitis nil*.

Effects of theobroxide, SHAM and GA₃ on LOX activity

LOX is the key enzyme in the JA biosynthesis pathway, and we therefore measured LOX activities in leaves treated with theobroxide independently or together with SHAM or GA₃ (Fig. 2). After 2 weeks treatment, the activity of LOX was increased by theobroxide both under SD and LD conditions. In plants treated with theobroxide together with SHAM or GA₃, the LOX activity was decreased to the same level as the control. Furthermore, the LOX activity in control plants under SD conditions was

higher than that under LD conditions. Taken together, these results indicated that theobroxide stimulated LOX activity, and that SHAM and GA3 reduced these stimulatory effects and photoperiod influenced LOX activity.

*Theobroxide inhibited gibberellin biosynthesis in *Pharbitis nil**

As described previously [16], by using prohexadione, an inhibitor of late step of GA biosynthesis, GA₁ was determined to be the active GA in the control of shoot elongation in *Pharbitis nil*. Since the stem elongation was inhibited by theobroxide, GA₁ biosynthesis might be influenced by theobroxide. In order to determine the effect of theobroxide on GA biosynthesis, we analyzed the endogenous content of GA₁ by GC-SIM-MS in *Pharbitis nil* grown under different photoperiods. The results (Table 3) showed that theobroxide reduced the endogenous level of GA₁ both under SD and LD conditions in *Pharbitis nil*. The GA₁ level was also controlled by photoperiods, *i.e.* LD conditions stimulate GA₁ biosynthesis in accordance with the previous report [16]. These results indicated that GA₁ biosynthesis was suppressed by theobroxide, contributing to the inhibition of stem elongation by theobroxide as well as the participation of JA.

Discussion

Based on our previous results [1, 2, 3], we assumed that JA might play an important

role in the control of stem growth in *Pharbitis nil*. This was also suggested by some reports indicating that JA participates in the control of the elongation growth in different species, including rice [7], oat [8], and *Pharbitis nil* [10]. Our assumption was supported by the results obtained here. In theobroxide treated plants, the stem elongation was inhibited (Fig. 1) while the endogenous levels of JA (Table 1, 2) and LOX activity (Fig. 2) were stimulated both under SD and LD conditions. SHAM reversed the inhibitory effect of theobroxide on the stem elongation (Fig.1), and this reversion was in accordance with the reduction of endogenous JA levels (Table1, 2) and LOX activity (Fig. 2). Furthermore, in non theobroxide treated plants, the stem elongation was stimulated by LD conditions while the endogenous JA content and LOX activity were reduced (Fig 1, 2 and table 1, 2). These results suggested that endogenous JA was negatively involved in the control of stem elongation in *Pharbitis nil*.

Gibberellins plays an important role in the plant stem growth, and GA₁ is the active GA in the control of shoot elongation in *Pharbitis nil* [15]. In order to estimate the role of gibberellins in the theobroxide-inhibited stem elongation of *Pharbitis nil*, we analyzed the endogenous content of GA₁ and applied exogenous GA₃. As a result, GA₃ completely recovered the inhibitory effect of theobroxide on stem elongation (Fig. 1) both under SD and LD conditions. Interestingly, GA₃ also suppressed the endogenous

level of JA (Table 1, 2) and LOX activity (Fig. 2). The inhibitory effect of GA₃ on LOX activity was almost same as SHAM (Fig. 2), suggested GA₃ might be an inhibitor of JA biosynthesis. Quantitative analysis of the endogenous content of GA₁ (Table 3) showed that GA₁ biosynthesis was suppressed by theobroxide both under SD and LD conditions. The role of gibberellins in promoting stem elongation is due to an enhancement of cell division or cell elongation [17]. While the inhibition of JA on the stem growth may be the results of the reorganization of shoot meristems, reduction in cell division, inhibition of cell elongation, and premature cell maturation [18]. Thus, the balance of gibberellins and JA may determine the direction of cell differentiation which decides the tendency of stem growth in plants.

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Figure legends

Figure 1. Interactive effect of theobroxide with SHAM or GA₃ on stem elongation in *Pharbitis nil*. SD, plants were growing under SD conditions; LD, plants were growing under LD conditions. The stem length was measured 2 weeks after treatment started. Ten plants were used for each treatment, values were means \pm SE of 3 independent experiment.

Figure 2. Effects of theobroxide with SHAM or GA₃ on LOX activity in the leaves of *Pharbitis nil*. SD, plants were growing under SD conditions; LD, plants were growing under LD conditions. Plants were subjected to different treatment, and leave samples were collected 2 weeks after treatment started. Data were the means \pm SE of three independent replicates.

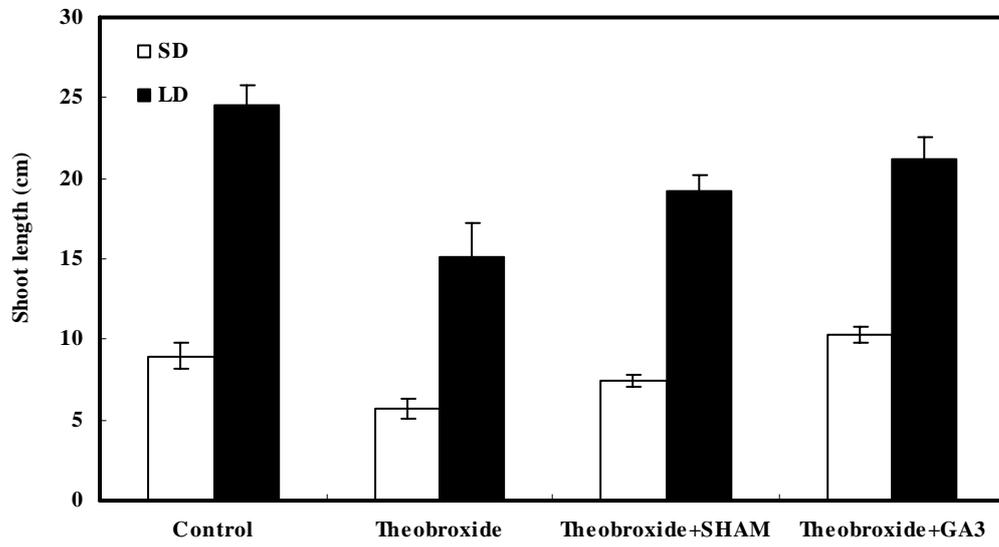


Figure 1

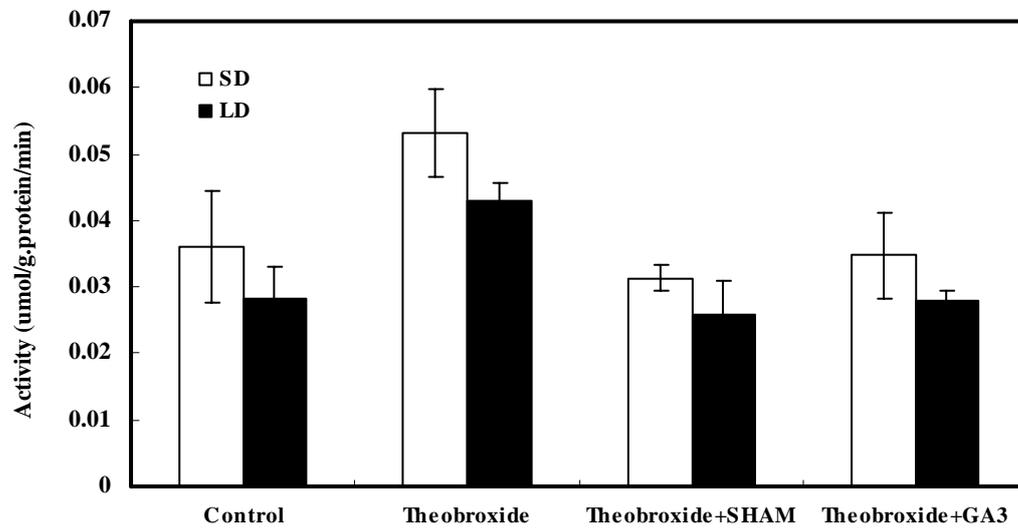


Figure 2

Table 1

Interactive effects of theobroxide with SHAM or GA₃ on endogenous level of JA ($\mu\text{g/g}$

DW) under SD (10L/14D) conditions

Treatment	Leaves	Cotyledons	Shoots
Control	0.34 \pm 0.08	0.32 \pm 0.02	0.87 \pm 0.02
Theobroxide	1.64 \pm 0.12	0.36 \pm 0.03	0.93 \pm 0.04
Theobroxide+SHAM	1.35 \pm 0.13	0.28 \pm 0.03	0.54 \pm 0.03
Theobroxide+GA ₃	1.48 \pm 0.13	0.30 \pm 0.01	0.43 \pm 0.04

Plants were subjected to different treatment, and plant tissues were harvested 2 weeks

after treatment started. Ten plants were used for each treatment, values were means \pm

SE of 3 independent experiment.

Table2

Interactive effects of theobroxide with SHAM or GA₃ on endogenous level of JA (µg/g DW) under LD (18L/6D) conditions

Treatment	Leaves	Cotyledons	Shoots
Control	0.30±0.04	0.10±0.01	0.53±0.04
Theobroxide	1.19±0.01	0.25±0.02	0.60±0.01
Theobroxide+SHAM	0.97±0.10	0.24±0.02	0.40±0.02
Theobroxide+GA ₃	0.82±0.11	0.23±0.01	0.39±0.02

Plants were subjected to different treatment, and plant tissues were harvested 2 weeks after treatment started. Ten plants were used for each treatment, values were means ± SE of 3 independent experiment.

Table 3

Endogenous content of GA₁ (ng/g DW) in the leaves of *Pharbitis nil*

Photoperiods	Experiment 1		Experiment 2	
	Control	Theobroxide	Control	Theobroxide
SD	4.63	3.53	4.85	3.56
LD	7.72	5.72	7.98	5.05

SD, plants were growing under SD conditions; LD, plants were growing under LD conditions. Plant leave samples were collected 2 weeks after treatment started. Values were from two analyses of independent experiments and the results were reproducible.