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Inhibition of stem elongation in spinach by theobroxide

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## **Summary**

In the current studies, we investigated the influences of theobroxide on stem elongation in spinach (*Spinacia oleracea* L.). Our results showed that stem elongation and flower formation were inhibited by spraying spinach plants with theobroxide under inductive, long day conditions (16 h light/8 h dark), while application of exogenous applied GA<sub>3</sub> prevented the effect of theobroxide.

Quantitative analysis showed that theobroxide suppressed GA<sub>1</sub> biosynthesis, whereas the endogenous content of jasmonic acid was unchanged. However, under short day conditions (10 h light/14 h dark), there were no differences in stem length between treated and untreated plants. These results suggest that the inhibition of stem elongation by theobroxide is probably due to the suppression of gibberellin biosynthesis.

**Key words:** Gibberellin, Jasmonic acid, Spinach (*Spinacia oleracea* L.), Stem elongation, Theobroxide

**Abbreviations:** GA, gibberellin; GC-SIM-MS, gas chromatography-selected ion monitoring-mass spectrometry; JA, jasmonic acid; LD, long day; SD, short day; TA, tuberonic acid; TAG, tuberonic acid glucoside.

## **Introduction**

Long day (LD) rosette plants, such as spinach (*Spinacia oleracea* L.), grow vegetatively and generally do not undergo stem elongation when grown under short day (SD) conditions. Stem elongation and flowering can be induced upon transfer to LD conditions (Zeevaart, 1971).

In spinach, there is considerable evidence that gibberellin (GA) biosynthesis increases in rosette plants under LD conditions (Talon and Zeevaart, 1990; Talon et al., 1991). Application of GA to rosette plants under SD conditions promotes stem elongation, whereas treatment with inhibitors of GA biosynthesis suppresses stem growth under LD conditions. Stem elongation is dependent on GA-regulated processes, and GA<sub>1</sub> has been shown to be the active GA for stem elongation in spinach (Zeevaart, 1971; Zeevaart et al., 1993). Stem elongation of *Silene armeria*, another LD plant, is induced under LD conditions and also by the application of GA. The elongation of the stem in this case is mainly due to transverse cell division (Talon et al., 1991).

Using cultures of single-node segments of potato (*Solanum tuberosum* L.) stems, theobroxide (Fig. 1) was isolated from the culture filtrate of *Lasiodiplodia theobromae* as a compound that induces potato tubers *in vitro* (Nakamori et al., 1994). Additionally, spraying theobroxide on the leaflets of

potatoes and the leaves of morning glories (*Pharbitis nil*) under non-inductive conditions (LD) causes potato tuber formation and flower bud induction, respectively (Yoshihara et al., 2000). This theobroxide-induced potato tuber formation is due to the accumulation of jasmonic acid (JA) and tuberonic acid (TA; Fig. 1; Gao et al., 2003). The JA and TA subsequently are metabolized to tuberonic acid glucoside (TAG; Fig. 1), which is the potato tuber-inducing substance (Yoshihara et al., 1989; Yoshihara et al., 1996). Furthermore, theobroxide inhibits stem elongation in *Pharbitis nil*, a SD plant, and JA is a negatively regulator of stem elongation (our unpublished data). Results indicate that the biological activity of theobroxide is equivalent to that of jasmonate. Herein, we examine the effect of theobroxide on stem elongation in spinach, a LD plant, under inductive and non-inductive conditions.

## **Materials and Methods**

### **Plant material and growing conditions**

Prior to experiments, spinach (*Spinacia oleracea* L., var. Solomon) plants were grown for 5 weeks in a 1:1.5 (v/v) mixture of peat moss: perlite in growth chambers (NK System, Biotron NC 350, Japan) under SD (10 h light/14 h dark) conditions. Water was applied to the plants every 2 days, and liquid Hyponex

(1:500 in water; Hyponex Japan Co., Ltd, Japan) was applied once a week. The growth chambers were maintained at 25°C with 60% relative humidity and were illuminated by 20 fluorescent lamps (NEC FL40SEX-N-HG, Japan) to provide light at an intensity of  $90 \mu\text{mol m}^{-2} \text{s}^{-1}$ .

### **Treatment of plants**

Theobroxide used in the experiment was isolated from the filtrate of a *Lasiodiplodia theobromae* culture according to the method of Nakamori et al. (1994). Theobroxide and GA<sub>3</sub> were dissolved at 1 mM in distilled water containing 100 ppm Tween-20. The control plants received the same amount of distilled water containing only 100 ppm of Tween-20. The test solutions (20 mL per seedling) were applied with plastic spray bottles to the leaf surface every two days. For LD treatment, 20 boxes (10 boxes for control and 10 boxes for theobroxide treatment) were transferred to LD conditions (16 h light/8 h dark) after pre-planting, and for SD treatment, 20 boxes (10 boxes for control and 10 for theobroxide treatment) were kept in SD conditions (10 h light/14 h dark) after pre-planting. Spraying was started after pre-planting. For the theobroxide/GA<sub>3</sub> treatment, seedlings were sprayed with GA<sub>3</sub> (three times  $\times 1.5$  mL) 30 min before spraying with theobroxide under LD conditions. Stem

height was determined from the base of the stem to the tip of the stem or to the inflorescence (Zeevaart et al., 1971). Plant tissues were harvested at the indicated time, placed immediately in liquid nitrogen, and stored at -80°C until analysis. The experiment was repeated at least three times, with 10 plants for each treatment.

### **Determination of endogenous GA<sub>1</sub> content**

Plant tissues (10 g) were ground under liquid nitrogen and suspended overnight in 100 mL of 80% aqueous methanol. This mixture was filtered, and 50 ng 17,17-d<sub>2</sub>-GA<sub>1</sub> was added as an internal standard. The filtrate was concentrated under reduced pressure, dissolved in H<sub>2</sub>O, adjusted to pH 2-3 with 6 N HCl, and extracted with ethyl acetate (10 mL × 3). The combined organic layers were washed with saturated NaHCO<sub>3</sub> (10 mL × 3), adjusted to pH 2-3 with 6 N HCl, and extracted with ethyl acetate (10 mL × 3). The combined organic layer was concentrated, and the resulting residue was dissolved in 1 mL of H<sub>2</sub>O and then subjected to chromatography using a Bond Elut C<sub>18</sub> cartridge column (Varian, CA, U.S.A.). The column was washed successively with H<sub>2</sub>O (1 mL × 2) and 4:1 (v/v) methanol/H<sub>2</sub>O (1 mL × 4). The 80% methanol/H<sub>2</sub>O eluant was concentrated *in vacuo*, and the residue was



loaded onto a Bond Elut DEA column (Varian). The column was washed successively with methanol (1 mL × 2) and 1 N acetic acid/methanol (1 mL × 4). The acetic acid/methanol eluant was evaporated, and the residue was dissolved in methanol and purified by using a L7100 HPLC (Hitachi, Japan) equipped with a YMC-Pack ODS column (300 mm × 10 mm; YMC Co, Ltd., Japan) to isolate the GA<sub>1</sub> fraction. The GA<sub>1</sub>-containing fraction was methylated with ethereal diazomethane followed by trimethylsilylation with *N,O*-bis(trimethylsilyl)acetamide. The derivatives were analyzed by gas chromatography-selected ion monitoring-mass spectrometry (GC-SIM-MS) using a QP5000 system (Shimadzu, Japan) with a ZB-1 column (30 m × 0.25 mm; 0.5-μm phase thickness). After injection, the oven temperature was kept at 60°C for 1 min, increased to 200°C at 20°C min<sup>-1</sup>, to 300°C at 4°C min<sup>-1</sup>, and finally maintained at 300°C for 10 min. The injector and detector temperatures were both 230°C. The amounts of endogenous GA<sub>1</sub> were calculated by comparing the peak areas for GA<sub>1</sub> with that of the GA<sub>1</sub> internal standard.

### **Determination of endogenous JA content**

The content of JA was analyzed by the method of Matsuura et al. (2002) with some modifications to the HPLC and GC-SIM-MS procedures. The

pre-purified JA-containing sample was dissolved in methanol, methylated with ethereal diazomethane, and purified with a L7100 HPLC (Hitachi) equipped with a YMC-Pack ODS column (300 mm × 10 mm; YMC Co, Ltd.) to isolate the JA fraction. The fraction was analyzed by GC-SIM-MS on a QP5000 system (Shimazu) with a ZB-1 column (30 m × 0.25 mm; 0.5- $\mu$ m phase thickness). The GC temperature program was 80°C for 1 min, followed by 80-290°C at 20°C/min and 290°C for 5 min. Injector and detector temperatures were 200°C and 280°C, respectively. The amounts of endogenous JA were calculated by comparing the peak areas for JA with that of the JA internal standard.

## **Results and Discussion**

### **Inhibitory effects of theobroxide on stem elongation and flowering in spinach**

Based on our previous reports (Nakamori et al., 1994; Yoshihara et al., 2000; Gao et al., 2003), we suspected that theobroxide mimics a plant hormone and thereby regulate developmental processes that are influenced by photoperiod. To confirm this hypothesis, we investigated the effect of theobroxide on stem elongation in spinach, an LD plant. After two weeks of theobroxide treatment

under LD conditions, the stem length was recorded as a function of time. As shown in Fig. 2, stem elongation was inhibited by theobroxide under inductive (LD) conditions. Furthermore, we found that stems developed in both treated and untreated plants but that theobroxide inhibited stem elongation relative to the control. Application of exogenous GA prevented the inhibitory effect of theobroxide on stem elongation (Fig. 2). Meanwhile, theobroxide also inhibited flower formation, an effect similarly reversed by the application of GA (Table 1). In contrast, under SD conditions, there was no apparent difference between the control and the theobroxide-treated plants with respect to stem length or flower formation (data not shown). This difference can be explained by a failure of the stem to develop under SD conditions as a result of insufficient accumulation of GA<sub>1</sub> (Zeevaart et al., 1993).

### **Effects of theobroxide on endogenous content of GA<sub>1</sub> and JA**

Because GA<sub>1</sub> is the active form of GA for stem elongation in spinach (Zeevaart et al., 1993), we suspected that theobroxide inhibits stem elongation by suppressing the biosynthesis of GA<sub>1</sub>. To test this hypothesis, we analyzed endogenous the GA<sub>1</sub> content by GC-SIM-MS. We found that the endogenous GA<sub>1</sub> level in spinach was reduced by theobroxide treatment under LD

conditions (Fig. 3), suggesting that the inhibition of stem elongation by theobroxide was due to a reduction in GA<sub>1</sub> biosynthesis. In potato, theobroxide induces tuber formation by stimulating JA and TA biosynthesis, and the JA and TA are then converted into the potato-inducing compound, TAG (Yoshihara et al., 1996; Gao et al., 2003). In morning glory, theobroxide inhibits stem elongation and significantly stimulates JA biosynthesis. In this case, JA probably negatively regulates theobroxide inhibition of stem elongation (our unpublished data). It is reasonable to assume that JA biosynthesis can also be stimulated by theobroxide in spinach because the effect of theobroxide on stem elongation was similar to that in morning glory. Therefore, we examined the endogenous content of JA in spinach by GC-SIM-MS. However, unlike in potato and morning glory, theobroxide did not change the endogenous content of JA in spinach (Fig. 4). This could be due to an ability of theobroxide to stimulate JA biosynthesis in SD but not LD plants. Thus, the mechanisms of defense against exogenous stimuli may differ between SD and LD plants and between plant species.

In conclusion, theobroxide inhibits stem elongation in spinach, an LD plant, under LD conditions (16 h light/8 h dark). This results in a reduction in flower

formation. Exogenous application of GA prevents this inhibitory effect of theobroxide on stem elongation and flower formation. Quantitative analysis showed that theobroxide suppresses GA<sub>1</sub> biosynthesis but does not affect the level of endogenous JA. These results suggest that the inhibition of stem elongation by theobroxide in spinach is probably due to a suppression of GA biosynthesis. Theobroxide may be therefore be useful as an inhibitor of plant bolting (stem elongation), which is an agricultural problem in spinach and beet.

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**Table 1** Flowering percentage of spinach plants after 6 weeks of treatment

under LD conditions

Treatment	Percentage of flowering plants			Average
	Experiment 1	Experiment 2	Experiment 3	percentage
Control	100% (9/10)	100% (9/10)	100% (8/10)	83.3%
Theobroxide	50% (5/10)	40% (4/10)	40% (4/10)	43.3%
Theobroxide +GA <sub>3</sub>	90% (9/10)	100% (10/10)	90% (9/10)	93.3%



Legends of figures

**Figure 1.** The structures of theobroxide, TAG, TA, and JA.

**Figure 2.** Interactive effect of theobroxide and GA<sub>3</sub> on stem elongation in spinach under LD (16 h light/8 h dark) conditions. The plants were grown for five weeks under SD conditions, transferred to LD conditions, and then treated with theobroxide and GA<sub>3</sub>. The stem length was measured after two weeks of theobroxide treatment. Values represent means  $\pm$  SE (n= 10).

**Figure 3.** Effects of theobroxide on endogenous levels of GA<sub>1</sub> (ng/g DW) in leaves under LD (16 h light/8 h dark) conditions. The plants were grown for five weeks under SD conditions, transferred to LD conditions, and then treated with theobroxide. Leaves were harvested at the indicated times. Ten plants were used for each treatment, and values represent means  $\pm$  SE of three independent experiments.

**Figure 4.** Effects of theobroxide on the endogenous level of JA ( $\mu$ g/g DW) in leaves under LD (16 h light/8 h dark) conditions. The plants were grown for five weeks under SD conditions, transferred to LD conditions, and then treated with theobroxide. Leaves were harvested at the indicated times. Ten plants were used for each treatment, and values represent the means  $\pm$  SE of three

independent experiments.

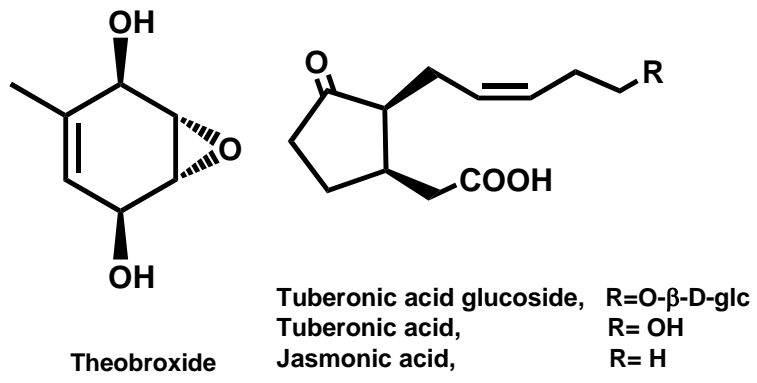


Figure 1

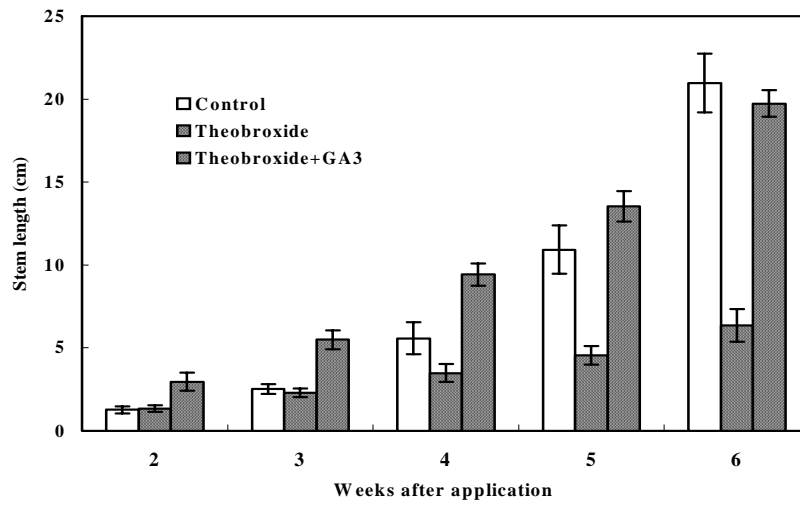


Figure 2

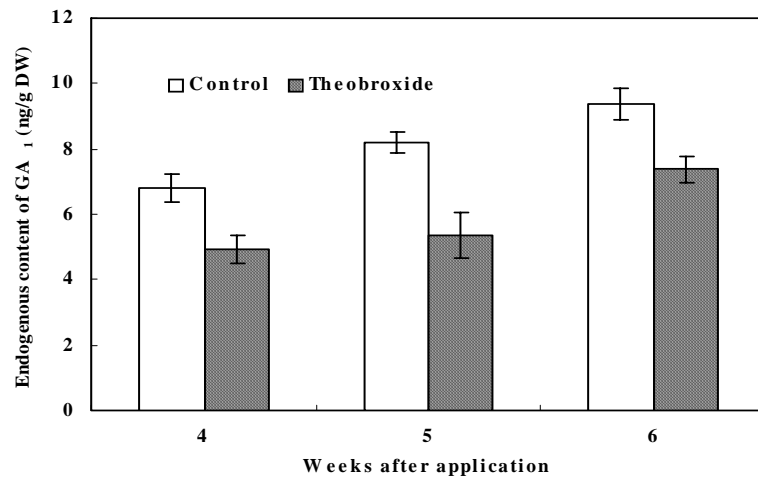


Figure 3

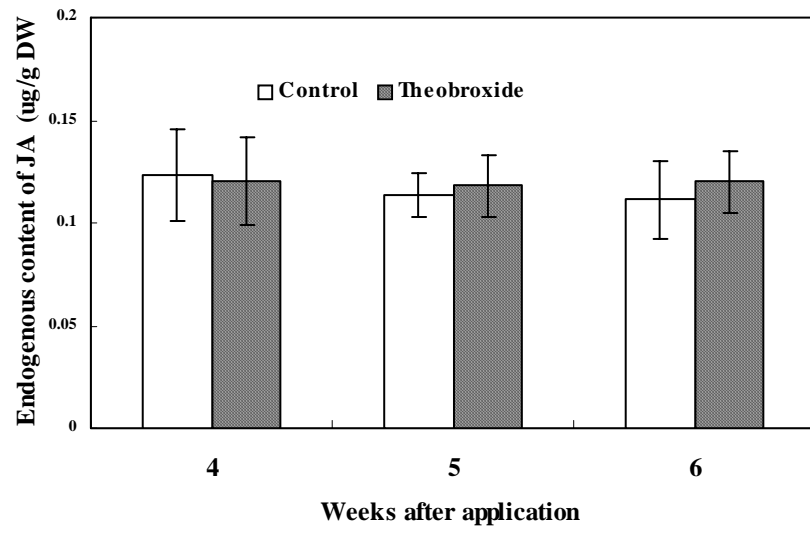


Figure 4