

Effects of Theobroxide, a Natural Product, on the Level of Endogenous Jasmonoids

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The natural potato microtuber inducing substance, theobroxide, strongly induces the formation of tuber of potato (*Solanum tuberosum* L.) and flower bud of morning glory (*Pharbitis nil*) plants under non-inducing conditions (long days) (Yoshihara *et al.*, 2000). In the present study, theobroxide was evaluated for its effect on the level of endogenous jasmonoids in different tissues of such two plants. An *in vitro* bioassay using cultures of single-node segments of potato stems was performed with the supplement of theobroxide in the medium. The endogenous jasmonic acid (JA) and its analogue tuberonic acid (TA, 12-hydroxyjasmonic acid) in segments and microtubers were quantitatively analyzed. The increase in the endogenous JA level caused by theobroxide was observed in both segments and microtubers. Endogenous TA was only detected in segments, and the content increased with the concentration of theobroxide. As for morning glory, the whole plant was sprayed with theobroxide for 1 ~ 5 weeks under different photoperiods and endogenous JA in the leaves was quantitatively analyzed. Theobroxide spraying increased the level of endogenous JA in the leaves of the plants grown under both long and short days.

Key words: Theobroxide, Jasmonoids, Photoperiod-dependent Plants

Introduction

The formation of certain plant organs, including tubers, bulbs and flower buds, is controlled by photoperiod. Potato (*Solanum tuberosum* L.) tuber is formed under short days. Onion (*Allium cepa* L.) bulb formation occurs under long days. Morning glory (*Ipomea* and *Pharbitis*) produces flower buds under short days and vegetative growth under long days.

Other factors such as endogenous growth regulators also affect the formation of such organs of photoperiod-dependent plants. Jasmonic acid (JA), a well-known biological modulator, is reported to induce tuberization of potato stolons *in vitro* (Yoshihara *et al.*, 1989; Koda *et al.*, 1991; Pelacho and Mingo-Castel, 1991) and stimulates shoot and bulb formation of garlic (*Allium sativum*) *in vitro* (Ravnikar *et al.*, 1994). In soil-grown potato plants, it

has been found that the JA content was highest at tuber-set time (Abdala *et al.*, 2002). Jasmonoids seem to play an important role in the regulation of cell division and expansion, leading to the re-orientation of cortical microtubules (Koda, 1997). In contrast, gibberellins are known to inhibit potato tuberization (Castro *et al.*, 2000). The existence of a specific potato tuber-inducing stimulus, which would be formed in the leaves during short days and be translocated to the top of the stolon had been postulated and confirmed by grafting and other experiments (Chapman, 1958; Kumar and Wareing, 1973). This postulated potato tuber-inducing stimulus has been isolated from the leaves of potato (*S. tuberosum* L.) and named as tuberonic acid glucoside (TAG). The structure of the active compound was determined to be 12-hydroxyjasmonic acid glucoside (Koda *et al.*, 1988; Yoshihara *et al.*, 1989). Both TAG and its aglycone tuberonic acid (TA) are structurally related to JA (Fig. 1).

For ten years, we have been concentrated in the search for a potato tuber-inducing substance from

Abbreviations: JA, jasmonic acid; TA, tuberonic acid (12-hydroxyjasmonic acid); TAG, tuberonic acid glucoside (12-hydroxyjasmonic acid glucoside).

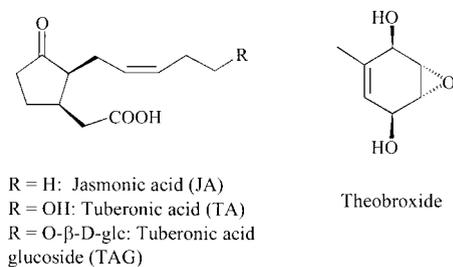


Fig. 1. Structures of jasmonic acid, tuberonic acid, tuberonic acid glucoside and theobroxide.

pathogenic fungus with the guidance of a bioassay using cultures of single-node segments of potato stems (Koda and Okazawa, 1988). Theobroxide is an epoxy cyclohexene natural product (Fig. 1) isolated from the culture filtrate of *Lasiodiplodia theobromae* and it induces potato microtubers formation *in vitro* at concentrations as low as 5×10^{-6} M (Nakamori *et al.*, 1994). This activity is similar to that of JA (10^{-6} M) (Pelacho and Mingo-Castel, 1991). Moreover, theobroxide exhibits significant activity in potato tuber formation and flower induction of morning glory under non-inducing conditions (Yoshihara *et al.*, 2000). Up to date, there is no evidence to support that theobroxide itself is a single trigger for the formation of tubers and flowers, because in contrast to [^{14}C] (\pm) JA (Yoshihara *et al.*, 1996) neither metabolism nor transportation occurred in an experiment with the application of [$3,6\text{-}^3\text{H}$] (\pm) theobroxide to the leaves of potato plants (unpublished data). The mechanism of theobroxide has not been fully understood.

Recently, we reported that theobroxide increased the endogenous levels of JA and TA, and lipoxygenase (LOX) activity in potato leaves. And this inductive effect was inhibited by salicylhydroxamic acid (SHAM), a JA biosynthesis inhibitor, associated with a reduction in tuber formation. These results suggest that theobroxide probably achieves its inductive effect on potato tuberization by stimulating JA and TA synthesis (Gao *et al.*, 2003).

The bioassay containing cultures of single-node segments of potato stems is a well-defined *in vitro* tuberization system. It has been used to evaluate the inductive activity of substance on *in vitro* potato tuber formation based on the observation of the yield of microtubers. In such a system, theobroxide shows similar activity of potato microtu-

ber induction at 10^{-5} M as JA at 10^{-6} M. But the relationship between theobroxide and JA in potato microtuber formation has not been examined. Herein, we report the effect of theobroxide on the endogenous levels of JA and TA in the segments and microtubers obtained from the bioassay cultures.

In a previous paper (Yoshihara *et al.*, 1996), we presented information showing that the formation of tubers and flower buds of potato may be the result of the same mechanism. Other reports also suggest that the signals involved in floral induction are similar to those involved in tuberization (Chailakhyan *et al.*, 1997; Martin *et al.*, 1982). Hence, in a sense, it is significant to investigate whether theobroxide influences the content of endogenous JA in the process of morning glory flower bud formation even though the role of JA in floral induction is still not clear. Quantitative analysis of endogenous JA in morning glory leaves was carried out after the whole plant was sprayed with theobroxide.

Materials and Methods

Chemicals

Theobroxide was isolated and purified from *Lasiodiplodia theobromae* in our laboratory. Deuterium-labeled JA ($[^2\text{H}_1\text{-}10, ^2\text{H}_2\text{-}11, ^2\text{H}_3\text{-}12]\text{JA}$) and deuterium-labeled TA ($[^2\text{H}_1\text{-}10, ^2\text{H}_2\text{-}11, ^2\text{H}_2\text{-}12]\text{TA}$) were kindly given by Dr. H. Matsuura. Mega Bond Elut C_{18} cartridge (6 CC/1 GRM) and Bond Elut LRC DEA cartridge (10 CC/500 MG) were purchased from Varian (Harbor City, USA).

Application of theobroxide to *in vitro* cultures of potato single-node segments

Single-node segments from potato plants were cultured *in vitro*, as reported previously (Koda and Okazawa, 1988). In brief, single-node segments prepared from etiolated potato shoots (*Solanum tuberosum* L. cv. Irish Cobber) were sterilized in a 1% solution of sodium hypochlorite for 1 h. Three segments were planted in a 100 ml flask that contained 10 ml of White's medium. The medium was adjusted to pH 5.6 and solidified with 0.6% Bactoagar. The concentration of sucrose in the medium was 2% by weight. Theobroxide was supplemented to such basal medium before sterilization at different concentrations of 2×10^{-3} , 10^{-3} , 10^{-4} and 10^{-5} M. A medium without theobroxide was used as the control. Forty-eight replicates of each

culture were allowed to stand for 4 weeks under dark condition at 25 °C, and the ratio of tuberization was calculated as the number of tuberized laterals divided by the total number of laterals that had emerged.

Extraction and purification of JA and TA from tissues obtained from in vitro cultures of potato single-node segments

After grown for 4 weeks in the dark, the forty-eight replicates of each culture were divided into two groups. Each group contained twenty-four replicates. The twenty-four replicates were harvested together and separated into two tissue fractions: segments and microtubers. Thus at each culture condition, every tissue sample has two replicates. Each tissue sample was weighed, ground in a mortar with a pestle, then immersed in 80% EtOH/H₂O (100 ml for segments and 50 ml for microtubers) for 10 d. After filtration, the filtrate was added internal standards. Deuterium-labeled JA (1.0 µg) and deuterium-labeled TA (2.0 µg) were used. The filtrate was concentrated under reduced pressure to dryness. The residue was partitioned with 30 ml of EtOAc and 20 ml of water for twice. The combined organic layers, in which free JA and TA were expected to be present, were concentrated *in vacuo*. The residue was dissolved in water (4 ml) and subjected to a Mega Bond Elut C₁₈ cartridge that was successively prewashed with MeOH and water. The cartridge was eluted with 80% MeOH/H₂O (10 ml). The 80% MeOH/H₂O eluent was concentrated *in vacuo*. The residue was dissolved in MeOH (2 ml) and subjected to a Bond Elut LRC DEA cartridge that was successively prewashed with 1 N AcOH/MeOH and MeOH. The cartridge was eluted with 1 N AcOH/MeOH (10 ml). The eluent from the Bond Elut LRC DEA cartridge that contained JA and TA was evaporated and subjected to LC-MS quantitative analyses.

Theobroxide treatment on morning glory plants

Morning glory (*Pharbitis nil* cv. Violet) seeds were obtained from Marutane Seed Co. (Kyoto, Japan). Seedlings were grown for 34 d after sowing in a mixture of peat moss and perlite (2:1) in 400 ml pots under long days (18 h light/6 h dark, 25 °C) in the growth chamber. Thirty-two seedlings were divided into four groups and subjected to different treatments. Group S-T was grown un-

der short days (10 h light/14 h dark, 25 °C) and sprayed with theobroxide solution (10⁻³ M, in 100 ppm Tween 20) at an interval of 2 d, whereas group S-N was grown under short days and sprayed with the same volume of Tween 20 solution (100 ppm). Groups L-T and L-N were grown under long days (18 h light/6 h dark, 25 °C) and treated with the same method as that of S-T and S-N, respectively. The treatments lasted for five weeks, and after two weeks spray, the leaves began to be picked up at an interval of one week. The whole leaves of the chosen seedling were picked up and used as a sample for quantitative analysis. Every sample had a replicate.

Extraction and purification of JA from leaves of morning glory

The leaves were frozen at - 84 °C, crashed, soaked in CH₃CN for 24 h and filtered. After the addition of 10 µg deuterium-labeled JA, the filtrate, in which methyl esters of JA-related compounds were expected to be present, was hydrolyzed to free acids with 1 N KOH for 12 h at room temperature, then concentrated *in vacuo*. The residue was partitioned with 100 ml diethyl ether and 40 ml H₂O, the water layer was acidified with 1 N HCl (pH 2) and extracted again with diethyl ether (2 × 50 ml). After concentration, the ether extract was dissolved in 3 ml water and subjected to a Bond Elut C18 column that was prewashed with water and eluted with 4 ml MeOH. The MeOH eluent was concentrated and further purified with 1 N AcOH/MeOH on a Bond Elut LRC DEA column prewashed with MeOH. The 1 N AcOH/MeOH (4 ml) eluate was concentrated and separated by HPLC on 5C18 (Wakosil-II, 10 × 300 mm; flow rate 2.5 ml/min; UV detection at 210 nm). Elution with a gradient mode of 60% methanol/40% water containing 0.2% acetic acid to 100% methanol yielded a fraction containing JA (RT 15 ~ 18 min).

Determination of JA and TA content by LC-MS

LC-MS analysis was carried out using a M-1200AP LC-MS system (HITACHI, Japan). The analytical conditions for HPLC were as follows: column Wakosil 5C8 (4 × 250 mm; Wako Pure Chemical Industries, Ltd.); solvent system: MeOH/0.2% aq. AcOH, 35:65 for TA and 55:45 for JA; flow rate, 0.5 ml/min. Under this HPLC conditions, the retention time of non-labeled JA and TA

were 21.6 and 19.5 min, and those of deuterium-labeled JA and TA were 21.2 and 19.0 min, respectively. The analytical conditions for MS were as follows: nebulizer, 170 °C; desolvator, 400 °C; aperture 1 Htr, 120 °C; aperture 2 Htr; heater ON; needle 2700 V; polarity, negative; multiplier, 2300 V; drift, - 40 V; needle voltage, 2700 V; focus, - 120 V. Each jasmonoid was detected by the m/z value of $[M-H]^-$ ion, because the system was run under negative mode. Peaks due to non-labeled JA and TA were monitored at m/z 209 and 225, and those of deuterium-labeled JA and TA were monitored at m/z 215 and 230. The amounts of endogenous JA and TA were determined from the ratios of the peak areas of the ions that corresponded to the endogenous and deuterium labeled JA and TA.

Results

Induction of potato microtuber formation by theobroxide

Theobroxide supplementation in bioassay medium induced the microtuber formation. The induction correlated with the concentration of theobroxide. At a concentration of 10^{-5} M, theobroxide yielded a 12.5% tuberization ratio, whereas the control medium only afforded a 9.7% tuberization ratio. As the concentration of theobroxide was increased to 10^{-4} M, the tuberization ratio was 31.5%. The tuberization ratio at 10^{-3} M (64.5%) was more than six times that of the control (9.7%). At 2×10^{-3} M, the tuberization ratio was a little bit higher than that at 10^{-3} M. On a fresh microtuber weight basis, the highest induction was obtained at 2×10^{-3} M. The total fresh weight of microtubers in 2×10^{-3} M theobroxide medium was about five

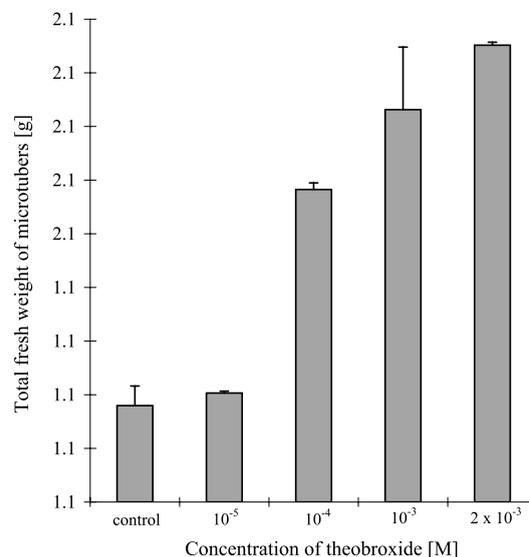


Fig. 2. Total fresh weight of microtubers harvested from twenty-four *in vitro* cultures of potato single-node stem segments grown in the dark at 25 °C for four weeks. The medium was supplemented with theobroxide of different concentrations. Values are the means of two replicates.

times that of the microtubers grown in control medium (Fig. 2).

Effect of theobroxide on the level of JA and TA in tissues obtained from in vitro cultures of potato single-node segments

The effect of theobroxide on the level of endogenous JA was examined in segments and microtubers. JA levels increased in a theobroxide-concentration-dependent manner (Table I) in segments. The level of JA in segments increased continuously at concentrations of 10^{-5} M and 10^{-4} M and

Table I. Effect of theobroxide on the level of endogenous jasmonoids in different tissues obtained from twenty-four *in vitro* cultures of potato single-node stem segments. The cultures were kept in the dark at 25 °C for four weeks before harvest. Data are the means of two replicates.

Concentration of theobroxide in culture medium [M]	Content of endogenous JA [ng/g FW]		Content of endogenous TA [ng/g FW]	
	Microtuber	Segment	Microtuber	Segment
0	ND	4.25 ± 0.60	ND	ND
10^{-5}	ND	6.30 ± 0.20	ND	ND
10^{-4}	19.36 ± 2.33	12.15 ± 0.60	ND	ND
10^{-3}	19.24 ± 1.17	70.30 ± 17.35	ND	41.95 ± 8.16
2×10^{-3}	14.14 ± 1.38	26.75 ± 1.61	ND	23.45 ± 2.55

ND: not detected.

reached a maximum at a concentration of 10^{-3} M. In microtubers, the highest level of JA was observed under a 10^{-4} M theobroxide condition. There was no significant difference of the result between concentrations of 10^{-3} M and 10^{-4} M. In both microtubers and segments, enhancing the theobroxide concentration from 10^{-3} M to 2×10^{-3} M, did not yield a relevant increase in the JA level.

Endogenous TA was only detected in segments. The level of TA increased sharply and reached a maximum when theobroxide was supplemented at a concentration of 10^{-3} M. The level of TA at 2×10^{-3} M theobroxide concentration was much lower than that at 10^{-3} M (Table I).

Induction of theobroxide on flower bud formation of morning glory plants

In the plants grown under non-inducing conditions, namely long days, the induction on flowering by theobroxide was very notable. Two weeks after spray initiation, the L-T group (grown under long days and treated with theobroxide) was observed to form flower bud. The number of flowers per seedling was 1.1. On the other hand, the L-N group (grown under long days and treated with water) didn't form flower buds even after five weeks. Additional application of theobroxide to the plants grown under inducing conditions (short days) produced more flowers compared with non-sprayed plants. Plants grown under short days formed more flowers than those grown under long days (Table II). Four weeks after experiment initiation, both groups S-N (grown under short days

and treated with water) and S-T (grown under short days and treated with theobroxide) began flowering, whereas group L-T still remained in the bud stage.

Effect of theobroxide on the level of JA in leaves of morning glory

Under long days condition, the endogenous content of JA in control plants tended to increase with time, and theobroxide treatment caused an obvious increase in the level of JA. The level of JA in seedlings sprayed with theobroxide for two and three weeks was more than twice that of the controls (Fig. 3A).

It is interesting to note that under short days condition, two weeks after spray initiation, the endogenous JA content in both control and theobroxide-treated plants were uncommonly high. This might be due to "JA burst" caused by the abrupt change of photoperiod from long days to short days. As described in Materials and Methods, before being subjected to the spray experiment under short days, the morning glory plants had been grown under long days for 34 d. From week 3, the endogenous content of JA in both control and theobroxide-treated plants seemed to increase with time. Theobroxide spraying caused a general increase in the JA level, and the increase ranged from one fold to two fold (Fig. 3B).

It can be concluded that theobroxide spray treatment increased the level of endogenous JA in morning glory plants grown under both long and short days. The increase in the JA level caused by theobroxide spray in seedlings under long days was more notable than that in seedlings under short days. Additionally, the content of JA in the plants kept under long days was generally lower compared to that in the plants kept under short days, for both the treatments and the controls.

Table II. The total number of flowers produced in the period indicated by one seedling of morning glory *P. nil* cv. Violet grown under different photoperiods with and without theobroxide.

	S-N ^a	S-T ^b	L-N ^c	L-T ^d
2 weeks	2.1	2.3	0	1.1
3 weeks	4.5	5.1	0	2.3
4 weeks	6.9	7.7	0	6.0
5 weeks	8.4	10.2	0	6.0

^a Grown under short days and sprayed with Tween 20 (100 ppm).

^b Grown under short days and sprayed with theobroxide (10^{-3} M in 100 ppm Tween 20).

^c Grown under long days and sprayed with Tween 20 (100 ppm).

^d Grown under long days and sprayed with theobroxide (10^{-3} M in 100 ppm Tween 20).

Discussion

The remarkable dual activity of theobroxide on potato tuber and morning glory flower bud formation has been well demonstrated by us previously (Yoshihara *et al.*, 2000). Anyway, theobroxide didn't transport and metabolize when it was applied to potato leaves (unpublished data). It was supposed that theobroxide might stimulate the biosynthesis of JA, a common plant growth regulator, which plays an important role in the induction of potato tuberization. This assumption was sup-

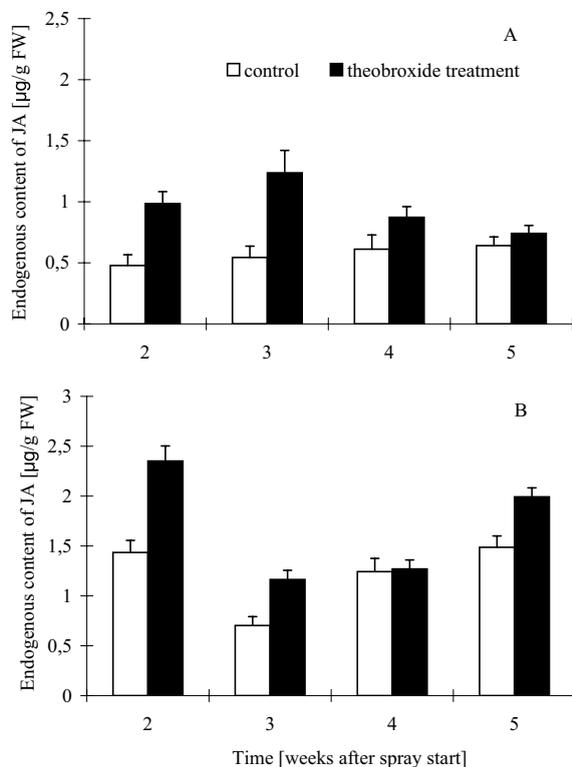


Fig. 3. Effect of theobroxide on the level of endogenous JA in the leaves of morning glory. (A) □: control plants under long days; ■: theobroxide treated plants under long days. (B) □: control plants under short days; ■: theobroxide treated plants under short days. Values are the means of two replicates.

ported by the observation that theobroxide increases the endogenous levels of JA during potato development (Gao *et al.*, 2003). But it has been reported that exogenous JA spraying did not induce tuberization of potato plants (Jackson and Willmitzer, 1994), even though this report lacks persuasion because the increase of endogenous JA levels in those plants was not reported. However, it is better to use a model, which can reflect both inductive activities of theobroxide and JA on potato tuberization to elucidate the relationship between them. It is known that in the *in vitro* tuberization system, cultures of single-node segments of potato stems, theobroxide and JA show similar activity on microtuber formation at 10^{-5} and 10^{-6} M. We sought to use this system to analyze the effect of theobroxide on the endogenous JA levels.

The result of the present study shows that supplement of theobroxide in the bioassay medium

increased the level of endogenous JA in segments and the produced microtubers. Endogenous TA was only detected in segments. This might be due to the low content of TA in plants. Although TA has been detected by GC-MS together with 11-hydroxyjasmonic acid in *S. demissum* (Helder *et al.*, 1993), free TA is rarely detected in plants as shown in the literature. The isolation of tuber-inducing substances from the leaves of both Jerusalem artichoke (*H. tuberosus* L.) and monocot yam (*Dioscorea batatas* Decne. cv. Hontokkuri) plants did not give TA (Matsuura *et al.*, 1993; Koda and Kikuta, 1991). In this report, free endogenous TA was only detected in segments, and the level was also significantly increased by the treatment of theobroxide, from undetected at non-, 10^{-5} and 10^{-4} M theobroxide condition to 42 ng/g at 10^{-3} M theobroxide condition.

As described above, in this *in vitro* potato tuberization bioassay system, the relationship between theobroxide treatment and the increase in the level of endogenous jasmonoids in the tissues yielded from four-week-culture was well elucidated. However, the time course of the level of endogenous jasmonoids during microtuber formation, and the effect of theobroxide on this time course has not been investigated. To do that, tissues including segments and microtubers obtained at different culture times would be required. However, the segments planted in control medium didn't form microtubers until four weeks after cultivation. Therefore, the segments and the microtubers could be harvested only at this time, then the comparison of the levels of endogenous jasmonoids between the treatment and the control was carried out. We are currently setting up a more ideal system for sampling in different stages of microtuber formation, in order that we can monitor the fluctuation of the level of endogenous JA induced by theobroxide treatment. No doubt the system will prove a much more useful way of understanding the relationship of theobroxide and JA in the microtuber formation event.

As shown in Fig. 3, the content of endogenous JA in the leaves of morning glory was also affected by the treatment of theobroxide. It can be summarized that theobroxide spray treatment increased the level of endogenous JA in plants under both long and short days. Furthermore, the content of JA in the plants grown under short days (which stimulates flower bud formation) was higher than that in the plants under long days. It is not certain

that the increase in the level of endogenous JA caused by theobroxide treatment is the reason for the induction of flowering. Because up to date, the role of JA in floral induction is still not clear. It is thought that JA can be hydroxylated to TA then glucosylated to TAG. Our previous paper (Yoshihara *et al.*, 1996) showed that JA fed to the potato leaf was metabolized to TAG and transported to all parts of the plant. A high accumulation of TAG in tubers and flower buds was found. In the present report, theobroxide spray caused the increase in the

level of JA in morning glory plants. It is reasonable to assume that theobroxide spray might cause the increase in the level of TAG. Further work on quantitative analysis of TAG is now in progress.

Although our data showed that theobroxide stimulated endogenous levels of JA in potato microtuber and morning glory leaves, in a future study it will be necessary to investigate the effect of theobroxide on the expression of some genes related to JA biosynthesis.

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