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Original Paper

Transient accumulation of α-ketol linolenic acid (KODA) in immature flower buds of some ornamental plants

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Abstract 9-Hydroxy-10-oxo-12(*Z*),15(*Z*)-octadecadienoic acid (KODA) induces flowers in *Lemna paucicostata* after reacting with catecholamine (Yokoyama et al. 2000; Yamaguchi et al. 2001), and changes in endogenous KODA levels during the flower-inductive phase in short-day-induced cotyledons are closely related to flower induction (Suzuki et al. 2003). Here, we examined the change of KODA level after the flower induction period. KODA showed a transient increase in immature flower buds in all the plants we examined, i.e., *Pharbitis nil, Dianthus caryophyllus* L., *Dendranthema grandiflorum* Kitam. and *Eustoma russellianum* Griseb. No such increase of KODA was seen in foliar buds of *P. nil.* These phenomena indicate that KODA may be involved in flower formation, as well as flower induction.

Key words: *Dendranthema grandiflorum* Kitam., *Dianthus caryophyllus*, *Eustoma russellianum* Griseb., Flowering, Flower bud, KODA, Linolenic acid, Oxylipin, *Pharbitis nil.*

α-Ketol linolenic acid [KODA, 9,10-ketol-octadecadienoic acid, or 9-hydroxy-10-oxo-12(*Z*),15(*Z*)-octadecadienoic acid] was identified as a stress-induced factor in *Lemna paucicostata* (Yokoyama et al. 2000; see also Takimoto et al. 1994). It reacts with catecholamines to generate many products that strongly induce flowering in *L. paucicostata* (Yamaguchi et al. 2001). Using *Pharbitis nil* 'Violet', which is a typical short-day plant in which flowering can be induced by exposing seedlings cultivated under continuous light to a single 16-h dark period, we showed that changes of KODA level in the cotyledons are closely associated with flower induction (Suzuki et al. 2003).

KODA is presumably formed from 9-hydroperoxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid (9-HPOT), which is generated from linolenic acid by 9-specific lipoxygenase (9-LOX) (Vick and Zimmerman 1987). Indeed, we have shown that KODA can be synthesized from linolenic acid *in vitro* with 9-LOX from rice germ and allene oxide synthase (AOS) from flax seed (Yokoyama et al. 2000; Yamaguchi et al. 2001). KODA is the first substance to have been identified as an active metabolite of 9-HPOT (Yokoyama et al. 2000), although it was already known to be one of the various oxidative products formed from linolenic acid by LOX (Graveland 1973).

Here, we show that the level of endogenous KODA transiently increases in immature flower buds following the initial elevation in the flower-inducing phase (dark period) of *Pharbitis nil* (Suzuki et al. 2003).

Materials and methods

Plant cultivation

Campanula garganica was grown in the open air in Ibaraki prefecture until the middle of December, then either cultivated at temperature higher than 15°C in a greenhouse at our research center in Yokohama city to promote flowering, or cultivated in the open air at the same location to delay flowering. These plants were used for analysis of endogenous KODA in Feburary, when the plants in the greenhouse started to form flowers while those grown outside did not. *Dianthus caryophyllus* was grown in a greenhouse after the transfer of small nursery plants into pots in late July. Top pinching was carried out twice, in the middle of December and at the beginning of

Abbreviations: AOS, allene oxide synthase; 9-HPOT, 9-hydroperoxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid; 13-HPOT, 13-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid; I.S., internal standard; JA, jasmonic acid; KODA, 9-hydroxy-10-oxo-12(*Z*),15(*Z*)-octadecadienoic; LD, long-day condition; 9-LOX, 9-specific lipoxygenase; 13-LOX, 13-specific lipoxygenase; OPDA, 12-oxophytodienoic acid; SD, short-day condition; TFA, trifluoroacetic acid.

February, to induce extension of axillary buds. *D. cariophyllus* was used for analysis of KODA levels in the middle of March, when the plants were still in the vegetative stage, and at the beginning of April, when many small buds had differentiated. *Dendranthema grandiflorum* Kitam. (chrysanthemum), which is a cultivar that forms multiple flowers per plant, is a short-day plant, i.e., flowering is induced by short daylight times. Flower-induction of *D. grandiflorum* was suppressed by exposing the plants to artificial light for 4 hr in the middle of the night until the end of September, when the experiments started. Then the night-breaking illumination was stopped in order to induce flower bud formation, while control plants continued to receive the night-breaking illumination. Flower buds were detected under leaves by 2 weeks after exposure of the plants to the short-day environment, and protruded from the leaves after more than 3 weeks. *Eustoma russellianum* was transplanted to plant boxes from the nursery cell trays in the end of January. Flowers forming in May were used for the experiments. *Pharbitis nil* 'Violet' was germinated and cultivated in liquid medium as reported before (Suzuki et al. 2003). Germinated seedlings were grown on wet sea sand under continuous light (the irradiance and temperature were the same as those used for the cultivation following dark exposure; see below) for 4 days. Then they were transplanted to Nakayama's culture solution (Nakayama and Hashimoto 1973), and subjected to a single dark exposure at 25°C during 16 hr. After the dark treatment, the seedlings were cultivated under continuous light provided by cool-white fluorescent tubes; the irradiance level varied from 18.1 to 23.7 W m^{-2} (average, from 400 to 700 nm), as evaluated with an optical power meter (Anritsu, Tokyo, Japan), at 26°C. Flower buds were recognized 2 weeks after re-illumination. KODA was analyzed at the 1st, 2nd and 3rd weeks after the dark treatment.

Analyses of endogenous KODA level

Endogenous KODA was analyzed as previously described (Suzuki et al. 2003). Target parts of each plant were harvested, powdered in liquid nitrogen and stored at −80°C until use. Each sample (about 1 g) was sonicated in $CHCl_3$-methanol (2 : 1) with 1 μg of I.S. in ice-water. After evaporation of the solvent, the residue was dissolved in diethyl ether, and then this solvent was replaced with methanol (200 μl). Five μl of the methanol solution was mixed with 45 μl of 0.05% 9-anthryldiazomethane (Funakoshi, Tokyo, Japan) in acetone for one hour at room temperature, and 5 μl of the solution was injected into an HPLC instrument (NANOSPACE SI-1, Shiseido, Tokyo, Japan) using a CAPCELL PAK C_{18} column (250 mm×4.6 mm I.D., Shiseido) coupled in series to a fluorescence detector (Ex 365 nm, Em 412 nm, Jasco, Tokyo, Japan). The mobile phase consisted of the following gradients; elution starting with 60% aqueous CH_3CN (0.1% TFA) isocratic for 8 min; 60 to 80% CH_3CN from 8 to 23 min; 80% CH_3CN isocratic from 23 to 38 min; and 80 to 100% CH_3CN from 38 to 43 min, at 1 ml min^{-1}. Typical retention times were 31.8 min for KODA-methylanthrylate and 38.6 min for the I.S. (15-hydroxypentadecanoic acid) methylanthrylate. The detection limit of KODA-methylanthrylate was 3 pg (0.01 pmole) as KODA and the quantification limit was 37 pg (0.1 pmol) as KODA.

Results

The KODA content in aerial parts of *C. garganica* and *D. caryophyllus* was first examined in both the vegetative and flowering stages. *C. garganica* was analyzed in the middle of December when it was in the vegetative stage, and in the middle of February when plants in the greenhouse were in the flowering stage and those grown outside was still in the vegetative stage. KODA levels were 40.6 (in December, vegetative stage), 761 (in February, flowering stage) and 86.8 pmol/g-tissue (in February, vegetative stage). *D. caryophyllus* was analyzed in the middle of March when the plant was in the vegetative stage or at the beginning of April when it had already formed many small buds. The KODA levels were 59.6 in the vegetative stage and 359 pmol/g-tissue in the plants differentiating many flower buds. Thus, KODA was clearly increased at the inflorescent stage of *C. garganica* and *D. caryophyllus.*

We next analyzed the KODA content in shoot tops (5 cm long) of *D. grandiflorum*, a short-day plant, at 0, 2, 4, and 6 weeks after exposure to short-day conditions or after no exposure (Figure 1). The KODA level of the plant exposed to short-day conditions was increased by about 20-fold compared with that of the control (still under night-breaking illumination) at the 2nd week, when flower buds had been evoked. The level thereafter gradually declined with growth of the flower buds. On the other hand, the KODA level in control plants remained unchanged throughout the experiment, except for an increase at the 6th week. These data indicate that KODA increased at a very early stage in floral organs. Therefore, we examined the KODA level in flower buds of *D. caryophyllus* at various stages (Figure 2A). KODA was present in flower buds and seemed to be highly accumulated in immature buds. We reexamined the KODA content at various stages of flower budding in more detail using *E. russellianum* (Figure 2B). We prepared flower buds of various sizes, down to the smallest that we could remove. Again, higher KODA levels were seen in immature (shorter) flower buds.

We next examined the KODA content in foliar or flower buds of *P. nil* (Figure 3). KODA has been reported to show a distinct, transient increase in cotyledons at the

end of the dark period (Suzuki et al. 2003). Thereafter, no increase was seen in cotyledons for three weeks (data not shown). In foliar buds, the KODA level remained at 10 to 35 pmol/g-tissue during the experiment. On the other hand, the KODA level rose to 314 pmol/g-tissue in very small flower buds with a length of less than 3 mm, which appeared at the 2nd week. By the 3rd week, KODA had returned to the basal level.

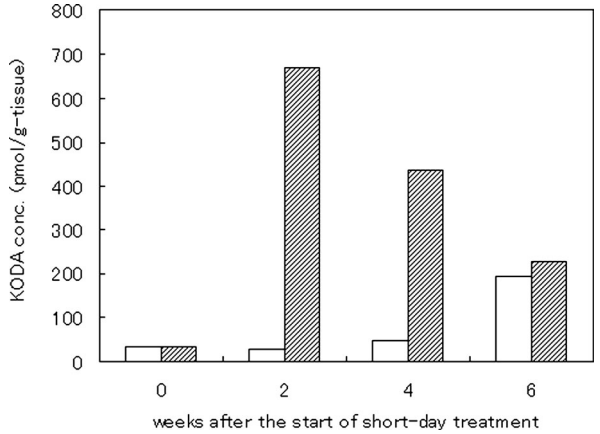

Figure 1. Changes in the amount of endogenous KODA in the shoot top region of *Dendranthema grandiflorum.* Flower buds were recognized at the 2nd week, although they were still covered with leaves. Top flowers grew to a couple of millimeters out of the leaves at the 4th week, and to over 5 mm at the 6th week, with slightly smaller axillary flower buds. The acro-region (5 cm from the shoot tip) of plants which had been exposed to a short-day condition in September (hatched column) or kept under night-breaking illumination (blank column) (see Materials and Methods) was cut off every two weeks and immediately powdered in liquid nitrogen. About 1 g of the powder was used for analyzing KODA. Each value is from an individual plant. A separate experiment yielded similar data.

Discussion

We previously showed that the KODA level in *P. nil* cotyledons sharply increased during the last 2 hrs of a 16-hr dark period (flower-inductive phase) and decreased to the basal level by 1 hr after light re-irradiation (Suzuki et al. 2003). This study revealed that the KODA level was increased in immature flower buds, but not in foliar buds of *P. nil* (Figure 3). We found a similar increase of KODA level in immature flower buds in other species, i.e., *D. caryophyllus* L., *D. grandiflorum* Kitam. and *E. russellianum* Griseb. At least in these plant species, the elevation in KODA in apical buds is related to flower formation.

We also found that in foliar shoots of *D. grandiflorum*

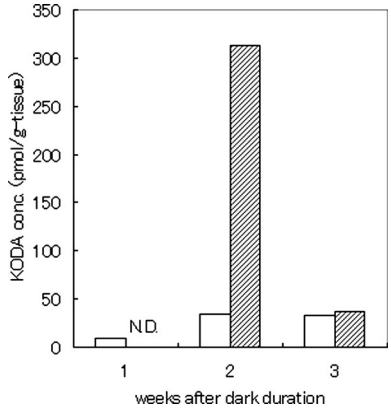

Figure 3. KODA content in foliar or flower buds of *Pharbitis nil.* The KODA content in foliar buds (blank column) and flower buds (hatched column) was measured at every week after dark exposure. At the first week, KODA content in flower buds was not analyzed (N.D.) since flower buds were not visible. Flower buds appeared between the 1st and 2nd week after re-lighting. Length of flower buds was below 5 mm at 2nd week. A separate experiment yielded similar data.

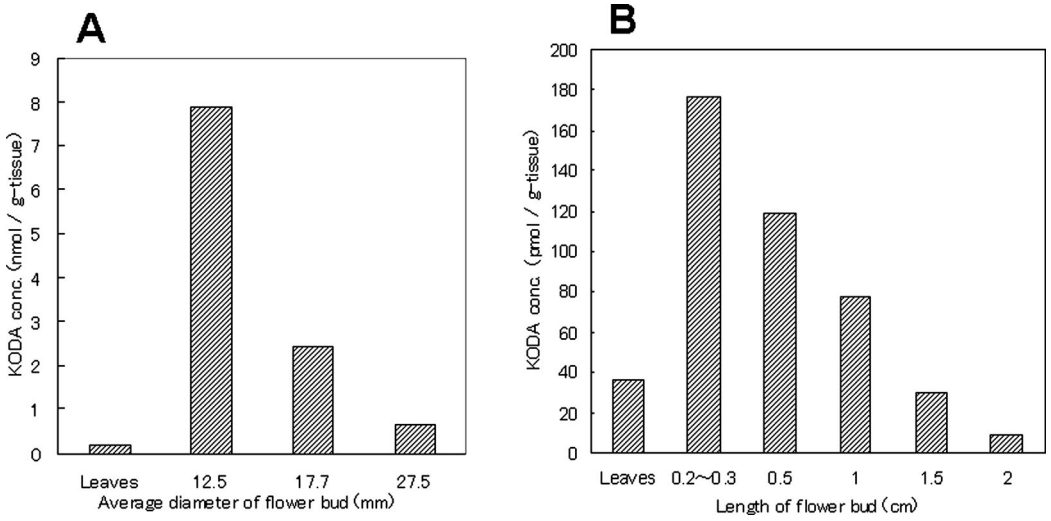

Figure 2. Contents of endogenous KODA in flower buds of *Dianthus caryophyllus* (A) and *Eustoma russellianum* (B) at various developmental stages. Flower buds were cut and sorted according to development stage, which was defined in terms of the diameter of flower buds in *D. caryophyllus* or the longitudinal length (not including the length of awn) of flower buds in *E. russellianum.* The flower buds were gathered in amounts of at least 1 g fresh weight, except for the groups of 0.2–0.3 and 0.5 cm length of flower buds in *Eustoma russellianum* (around 150 mg each). A separate experiment yielded similar data.

under the 4 hr night-breaking illumination, the KODA level tended to increase during the experiment (Figure 1). This might have resulted from autonomous flowering, or the night-breaking illumination may have been insufficient to completely inhibit flower induction, although no flower formation was observed during the experiment. The sensitivity to day length of the various cultivars of *D. grandiflorum* differs, but even under a long-day condition, most of them ultimately transform into a kind of reproductive stage and generate abnormal flowers (Kawada et al. 1987).

It was not clear when the KODA accumulated in immature flower buds was synthesized. As far as we observed, apparently the synthesis of KODA did not take place concomitantly with the development of flower buds. The decrease of KODA could be explained by the increase of tissue volume, because the fresh weight of flower buds increased by about ten-fold during the period from the 2nd to the 3rd week, with a corresponding decrease in the KODA content in flower buds of *P. nil*.

JA, like KODA, is one of the products (oxylipins) derived from linolenic acid via the lipoxygenase pathway. Flowers of dicotyledonous plants contain high levels of jasmonates, including amino acid conjugates or amides (Miersch et al. 1997; Schmidt et al. 1990). Hause et al. (2000) showed that JA, 12-oxophytodienoic acid (OPDA) and JA isoleucine conjugate were accumulated in flowers of developing plants at much higher concentration (20 nmol/g-tissue) than in leaves. In accordance with this, immunocytochemical analysis revealed the specific occurrence of allene oxide cyclase, which converts allene oxide to OPDA, in ovules, the transmission tissue of the style and vascular bundles of receptacles, flower stalks, stems, petioles and roots (Hause et al. 2000). This observation suggests that JA and related compounds may play some role in plant development, in addition to the response to external stimuli, such as wounding (Conconi et al. 1996a; Pena-Cortes et al. 1995), pathogen attack (Penninckx et al. 1996) or UV-induced stress (Conconi et al. 1996b). On the other hand, the KODA level is transiently increased at the very early stage of flower budding in *D. caryophyllus*, *E. russelianum* and *P. nil*. Although the content of JA has not been examined in flower buds at such an early stage, our observation that KODA induced expression of the OPDA reductase gene in *Arabidopsis thaliana* (unpublished data) suggests that KODA may be involved in the regulation of the synthesis of JA in plants.

Kolomiets et al. (2001) showed by means of an RNA hybridization technique that the accumulation of Lox1, which primarily produces 9-hydroperoxide (Royo et al. 1996), was restricted to developing tubers, stolons and roots, and that mRNA accumulation correlated positively with tuber initiation and growth. In situ hybridization showed that Lox1 transcripts accumulated in the apical and subapical regions of the newly formed tuber, specifically in the vascular tissue of the perimedullary region, the site of the most active cell growth during tuber enlargement. Yoshihara et al. (2000) found that a cyclohexene compound, theobroxide, promoted the tuberization of potato at similar concentrations to the effective concentration of JA. Theobroxide activated lipoxygenase, and this was presumed to result in the synthesis of JA and tuberization. Theobroxide also activated flowering in morning glory (Yoshihara 2003). *P. nil* 'Sunsmile', which was sprayed with theobroxide, formed flower buds in all individuals after 34 days under a long-day condition, while control plants failed to form flowers even after 77 days under the same condition. Since JA inhibited flowering in *P. nil* (Maciejewska and Kopcewicz 2002) and in *P. nil* 'Violet' (Yokoyama et al. 2000), theobroxide might have increased the level of KODA by activating LOX, and then KODA promoted the flowering.

King and Evans (1968) observed that juvenile flower buds, having bract, sepal and anther primordia, backed down to leaves when *P. nil* was transferred to a lighted location at a higher temperature (28°C instead of 21°C). It is therefore possible that even if flower buds are being formed, physiological differentiation may not be completed. Stress may also be involved in flower induction, since many stress conditions, such as low temperature, poor nutrition, senescence and the like, are known to induce flowering (Thomas 1993). KODA was released from *L. pausicostata* during recovery from stress, such as draught, rather than during exposure to the stress (Yokoyama et al. 2000). KODA was also found to improve drought tolerance (unpublished data). KODA might play a role in flower formation during recovery from stress in just-evoked juvenile flower buds.

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