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ORIGINAL PAPER

Effects of fruit load, shading, and 9,10-ketol-octadecadienoic acid (KODA) application on *MdTFL1* and *MdFT1* genes in apple buds

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Abstract The effects of fruit load, shading, and 9, 10-ketol-octadecadienoic acid (KODA) application on the expression of *MdTFL1* and *MdFT1* genes were investigated in apples (*Malus domestica* Borkh.). The expression of *MdTFL1* in apical buds from 21 to 63 days after full bloom (DAFB) in plants subjected to heavy crop treatment (HCT) was higher than that in plants subjected to flower thinning treatment (FTT). In contrast, the expression of *MdFT1* did not show a clear difference between HCT and FTT. The shading treatment increased the expression of *MdTFL1* at 35, 49, and 80 DAFB. However, *MdFT1* did not show a clear difference between shading and non-shading treatments. KODA application decreased the expression of *MdTFL1* at 49 DAFB, but it did not have a clear effect on the expression of *MdFT1* from 21 to 91 DAFB. KODA application did not influence endogenous gibberellic acid (GA) concentrations in apical buds. These results show that KODA may be related to flower bud formation through its influence on *MdTFL1*. The relationship between KODA and GA with regard to the flower bud formation of apples was also discussed.

Keywords 9,10-Ketol-octadecadienoic acid (KODA) · Gibberellic acid (GA) · *Malus domestica* · *MdTFL1* · *MdFT1*

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Introduction

Flower bud formation is one of the most important physiological processes in higher plants, and flowering time is controlled by photoperiod, temperature, endogenous signals, and other factors. Many fruit trees have an extended juvenile phase. For example, the juvenile period can last 2–10 years for citrus (*Citrus sinensis* L.), 5–8 years for apple (*Malus domestica* Borkh.), and even longer for pear trees (*Pyrus communis* L) before these trees start to flower (Ryugo 1986). This characteristic is the primary factor that limits the efficient breeding of fruit trees. Recently, it was shown that 9,10-ketol-octadecadienoic acid (KODA) is related to flower induction of *Lemna paucicostata* and *Pharbitis nil* (Yokoyama et al. 2000; Suzuki et al. 2003). The transient elevation of the endogenous KODA level during a dark period is also related to flower induction (Suzuki et al. 2003; Yokoyama et al. 2005). KODA is formed from 9-hydroxy-10-oxo-12 (*Z*), 15(*Z*)-octadecadienoic acid (9-HPOT) and is generated from linolenic acid by 9-lipoxygenase (9-LOX) (Fig. 1). The presence of endogenous gibberellic acid (GA) from the seeds as well as the application of GA are known to inhibit flowering in apples (Ramírez et al. 2004). In contrast to GA, endogenous KODA concentrations were high in the apical buds of trees whose rate of flower bud formation was also high

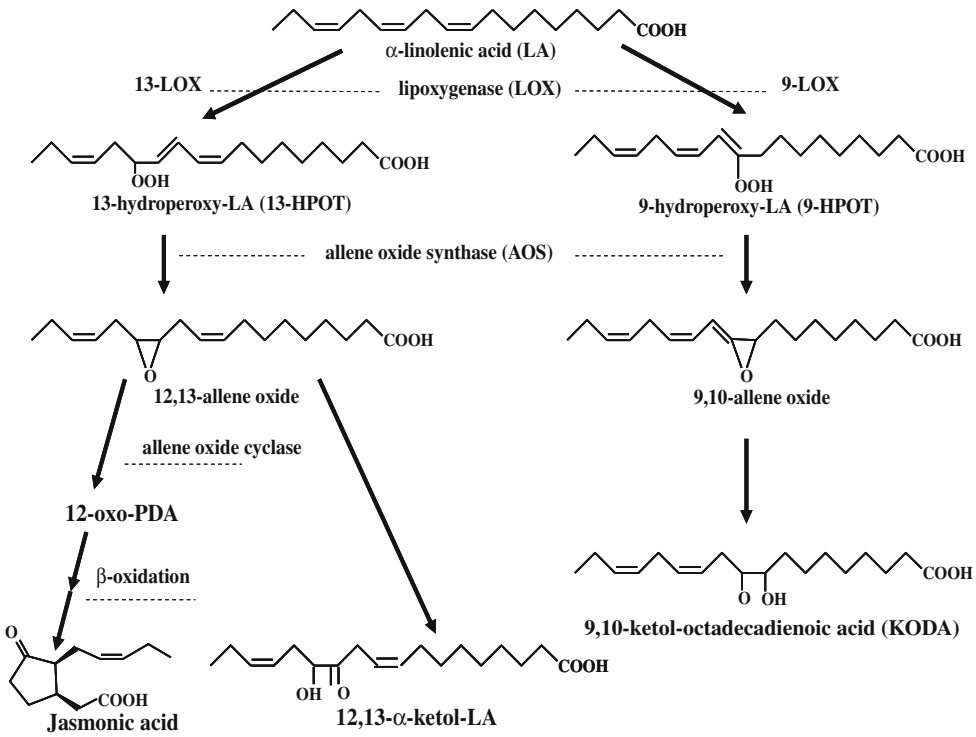

Fig. 1 The pathway for KODA biosynthesis in higher plants

(Kittikorn et al. 2010). Therefore, KODA may be associated with flower bud formation in apples.

Studies have established the molecular mechanisms of flowering in *Arabidopsis* (Bradley et al. 1997) and some fruit trees (Carmona et al. 2007; Esumi et al. 2007). In *Arabidopsis*, genes such as *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF CONSTANS 1* (*SOC1*), *LEAFY* (*LFY*), *APETALA 1* (*AP1*), and *TERMINAL FLOWER 1* (*TFL1*) play central roles in determining the inflorescence architecture (Ratcliffe et al. 1998; Henderson and Dean 2004). *TFL1* gene expression repressed flowering and maintained the identity of inflorescence meristems by preventing the expression of *AP1* and *LFY* (Ratcliffe et al. 1998, 1999). Over-expression of *MdTFL1* generated late flowering phenotypes in apples (Kotoda and Wada 2005). In addition, *MdTFL1* was detected in vegetative tissues such as apical buds, stems, and cotyledons, but not in reproductive tissues such as floral organs (sepals, petals, and stamens), suggesting a key role in maintaining the juvenile and/or vegetative phase in apples (Kotoda et al. 2006). Contrary to the functional role of *TFL1*, *FT1* promoted flowering by altering the expression of other endogenous genes (Kardailsky et al. 1999; Esumi et al. 2007). Environmental factors such as fruit load and shading influence flower bud formation in apples (Rom 1996; Kittikorn et al. 2010). However, there is no report on the relationship between environmental factors and the expression of genes that are associated with flower bud formation. Also, it is unclear whether KODA's effect on these genes is related to flower bud formation.

In the present study, we examined the effects of fruit load, shading, and KODA application on the expression of *MdTFL1* and *MdFT1* genes in apples.

Materials and methods

Chemicals

KODA (9,10-ketol-octadecadienoic acid) was prepared as described previously (Kittikorn et al. 2010). New Osmac® was purchased from Hayashi Chemical Co., Tokyo, Japan. [$^2H_0$] GA_1, [$^2H_0$] GA_4, [$^2H_2$] GA_1, and [$^2H_2$] GA_4 were purchased from the Research School of Chemistry of Australian National University (Canberra, Australia).

Plant materials

Experiments were conducted using 'Fuji' apple trees grafted onto *Malus prunitoria* (Willd.) Borkh. rootstocks growing in an open field at Chiba University, which is located at 36°N, lat., 139°E, long., and at an alti. of 747 m.

Experiment 1: Effect of fruit load on the expression of *MdTFL1* and *MdFT1* genes

Two groups of three 32-year-old trees were selected randomly: one that underwent flower thinning treatment (FTT), in which all flowers were removed at full bloom, and one that underwent heavy crop treatment (HCT), in

which the number of leaves per fruit was adjusted to 20 at 20 days after full bloom (DAFB). At 21, 49, 63, and 103 DAFB, ninety apical buds (30 buds per tree) were randomly collected for analysis of the expression of *MdTFL1* and *MdFT1* genes.

Experiment 2: Effect of shading on the expression of *MdTFL1* and *MdFT1* genes

Three 18-year-old trees, randomly selected, were covered with cheesecloth (50% shading) at 20 DAFB. The untreated control was three non-shaded trees, also randomly selected. The number of leaves per fruit was adjusted to 40 at 20 DAFB. This number of leaves per fruit is usual fruit load not causing biennial bearing. At 35, 49, and 80 DAFB, ninety apical buds (30 buds per each tree) were randomly collected for analysis of the expression of *MdTFL1* and *MdFT1* genes.

Experiment 3: Effect of KODA application on the expression of *MdTFL1* and *MdFT1* genes, and on the GA concentrations

Three 32-year-old trees were randomly selected. One-year-old shoots of 240 (720 apical buds in three trees), also randomly selected at 7 DAFB, in each tree were sprayed with 100 μM KODA with surfactant New Osmac at 7, 21, 35, and 49 DAFB. On each day of treatment, the tips of the shoots were sprayed once until runoff. The untreated controls were one-year-old shoots that were not sprayed with KODA. The number of leaves per fruit was adjusted to 40 at 20 DAFB. At 7, 21, 35, 49, 63, and 91 DAFB, 120 apical buds (40 buds per tree) were randomly collected for analysis of the expression of *MdTFL1* and *MdFT1* genes and GA concentrations.

To determine the rate of flower bud formation in experiments 1, 2, and 3, we randomly collected 90 apical buds (three replications of 30 buds per tree) after defoliation. Each apical bud was longitudinally dissected, and the rate of flower bud formation was determined with a stereomicroscope. In accordance with a previously reported method (Kittikorn et al. 2010), buds in which the apical meristem was dome-shaped were counted as flower buds. The rate of flower bud formation was decided by the number of flower buds in 90 apical buds.

RNA extraction and cDNA synthesis

Total RNA from apical buds was extracted following the protocol described by Kotoda et al. (2000). A 2-μg aliquot of total RNA of apical buds at several developmental stages was treated with 5U of DNase I (Takara Bio, Otsu, Japan) and used for first-strand cDNA synthesis. For reverse transcription-polymerase chain reaction (RT–PCR), cDNA was synthesized in 20-μl reaction volume using ReverTraAce (Toyobo Co., Osaka, Japan) according to the manufacturer's instructions. RNA extraction and cDNA synthesis were performed three times on each sampling date. The apple cDNAs of *MdTFL1* and *MdFT1* used in this study were deposited in the DNA data bank of Japan (DDBJ) with accession numbers AB052994 and AB161112, respectively (Kotoda and Wada 2005).

Analysis of gene expression in apical buds

RT–PCR analysis was performed by amplifying 1 μL of cDNA with 15 pmol of specific primers for *MdTFL1* (5′-CTCTTAAAATGAAAAGAGCCTCGG-3′, forward; 5′-TGTGGCATACATTGTAAATA-3′, reverse) and *MdFT1* (5′-ATGCCTAGGGATAGGGACCCC-3′, forward; 5′-TTATCTTCTCCTTCCACCGGA-3′, reverse) (Kotoda and Wada 2005). These primers amplified fragments of 699 and 530 bp, respectively. The PCR conditions were: 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s for *MdFT1*, and 40 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 60 s for *MdTFL1*. As a positive control, the extracted RNA was confirmed by amplification of ACTIN homologs with 35 cycles of RT–PCR (data not shown) (Gasic et al. 2004). For each sample, 5 μL of the amplification reaction was size-fractionated onto 1% agarose gel and stained with ethidium bromide. Transcript levels of *MdFT1* and *MdTFL1* in the samples were compared directly against an ACTIN homolog control by using the NIH Image Program (http://rsb.info.nih.gov/nih-image/). The entire amplification process was replicated three times with total RNA from other sample tissues, to evaluate the expression of each gene.

Analysis of endogenous GAs

Endogenous gibberellins were extracted and quantified according to the procedure described by Kittikorn et al. (2010). As an internal standard, 200 ng each of [$^2H_2$] GA_1 and [$^2H_2$] GA_4 was added to the 80% MeOH extracts. GA_1 and GA_4 containing fractions from analytical reverse-phase high performance liquid chromatography (HPLC) were methylated with ethereal diazomethane and trimethylsilylated with Deriva-sil (20 μl; Chrompak, Raritan, NJ, USA) at 70°C for 10 min before gas chromatography-mass spectrometry (GC–MS) analysis. The derivatives were analyzed by GC–MS-SIM (selected ion monitoring) (QP 5000; Shimadzu, Japan) using a CP-Sil 5 CB column (Chrompack, Middelburg, Netherlands; 0.25-mm i.d. × 25 m, 0.25-μm film thickness); linear He flow at 50.2 cm·sec^{-1}, column temperature step gradient, 60°C for 2 min, 60 to 270°C at 10°C·min^{-1}, and 270°C for 35 min;

electron potential, 70 eV. The amount of endogenous GAs was determined from the peak area ratios of non-deuterated to deuterated GAs. The endogenous concentrations of GA_1 and GA_4 were calculated from the peak area ratios of *m/z* 286/284 and 508/506, respectively.

Statistical analysis

Data were presented as the means ± SE of three replications, subjected to analysis of variance procedures, and separated by Fisher's least significant difference, $P \leq 0.05$ (SAS Institute, Cary, NC, USA).

Results

Effects of fruit load and shading on the expression of *MdTFL1* and *MdFT1* genes and the rate of flower bud formation

Transcript levels of *MdTFL1* genes in HCT were significantly higher than those in FTT (Fig. 2a). The expression of *MdTFL1* in HCT was strongly detected at 21 and 49 DAFB, but decreased after 63 DAFB in both HCT and FTT. The expression of *MdFT1* genes was detected throughout the measuring dates in both HCT and FTT (Fig. 2b). However, the relationship between the expression levels of *MdFT1* in HCT and FTT fluctuated at each date (Fig. 2b). The flower bud formation rate in FTT was significantly higher than that in HCT (Fig. 2c).

In shading treatment, the expression of *MdTFL1* was higher than that in non-shading treatment throughout the measuring dates (Fig. 3a). The expression of *MdFT1* at 35 and 49 DAFB in shading treatment was higher than that in non-shading treatment, but the opposite result was found at 80 DAFB (Fig. 3b). The shading treatment significantly decreased the flower bud formation rate (Fig. 3c).

Effect of KODA application on the expression of *MdTFL1* and *MdFT1* genes, rate of flower bud formation, and GA concentration

The expression of *MdTFL1* genes at 21 and 35 DAFB did not differ greatly between the KODA-treated and untreated trees, but the expression of *MdTFL1* at 49 DAFB in KODA treatment was lower than the untreated control (Fig. 4a). In contrast, the expression of *MdFT1* genes was not greatly different between the treatments, and the relationship between the expression levels fluctuated at each date (Fig. 4b). The rate of flower bud formation in buds to which KODA was applied was significantly higher than that in the untreated control (Fig. 4c). KODA application did not significantly influence the total GA concentrations throughout the measuring dates (Fig. 5).

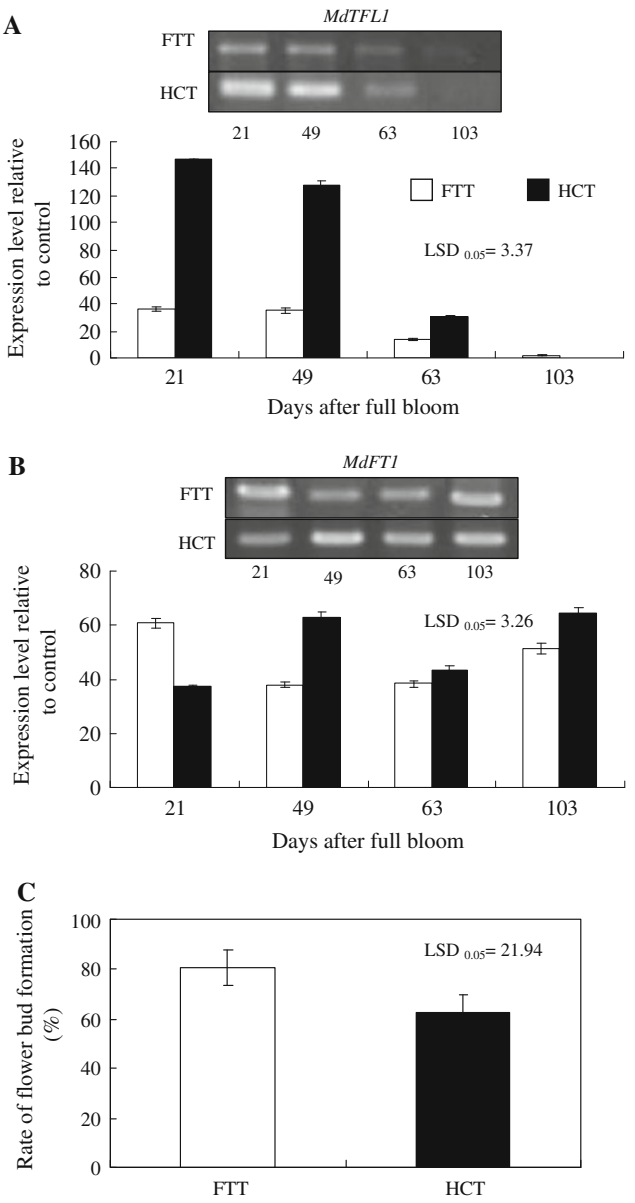

Fig. 2 RT-PCR analysis of *MdTFL1* (**a**) and *MdFT1* (**b**) and the rate of flower bud formation (**c**) in apical buds of 'Fuji' apples subjected to the flower thinning (FTT) and heavy crop treatments (HCT). The experimental values were plotted compared to the control (Actin) value. Data are the means ± SE of three replications

Discussion

The function of *TFL1* in *Arabidopsis* involves the determination of inflorescence meristem identity as well as floral transition (Bradley et al. 1997). It delays flowering and regulates plant growth through the maintenance of indeterminancy of the shoot apex (Ratcliffe et al. 1998).

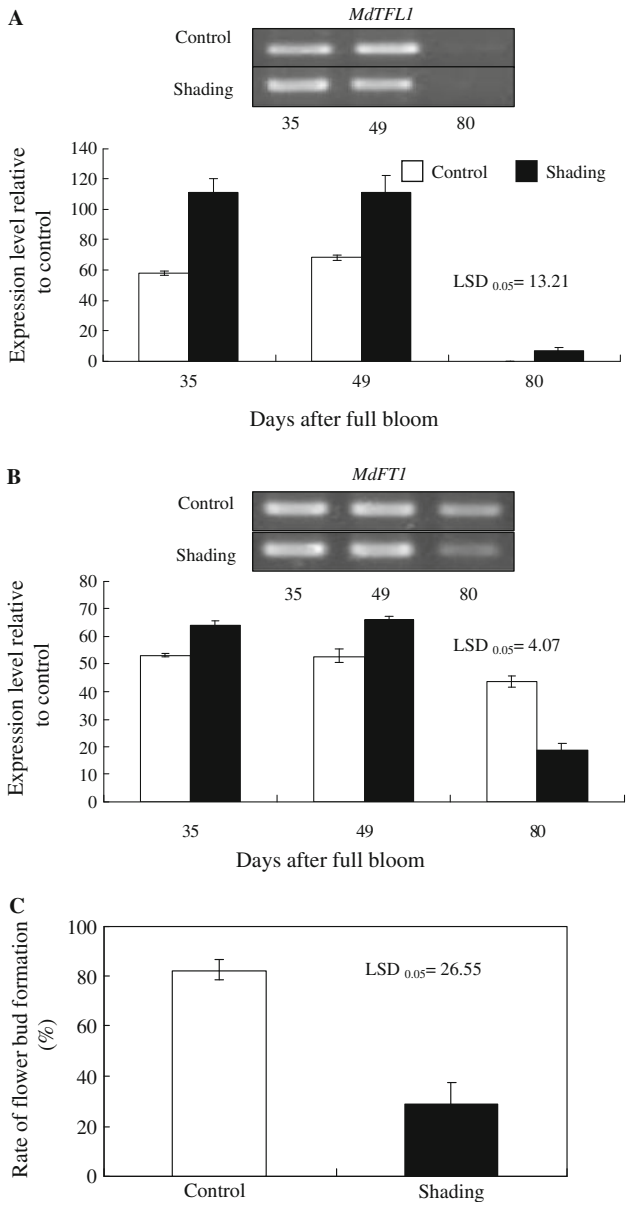

Fig. 3 RT-PCR analysis of *MdTFL1* (**a**) and *MdFT1* (**b**) and the rate of flower bud formation (**c**) in apical buds of 'Fuji' apples subjected to the shading and non-shading treatments. The experimental values were plotted compared to the control (Actin) value. Data are the means ± SE of three replications

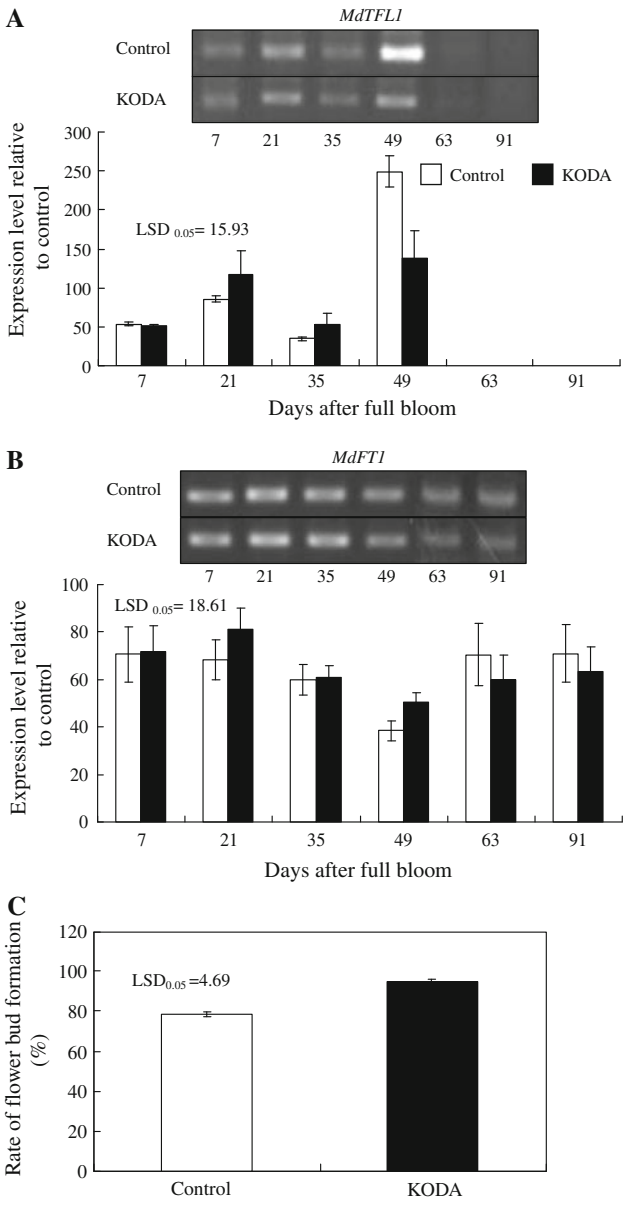

Fig. 4 RT-PCR analysis of *MdTFL1* (**a**) and *MdFT1* (**b**) and the rate of flower bud formation (**c**) in KODA-treated apical buds. We applied100 μM KODA at 7, 21, 35, and 49 days after full bloom. The experimental values were plotted compared to the control (Actin) value. Data are the means ± SE of three replications

A homolog of the *TFL1* gene has been isolated and characterized in fruit trees such as apples (Kotoda and Wada 2005; Kotoda et al. 2006), citrus (Pillitteri et al. 2004), pears (*Pyrus pyrifolia* Nakai) and quinces (*Cydonia oblonga* Mill.) (Esumi et al. 2005). In our previous study, the transition time from vegetative buds to floral buds was 70 DAFB (Kittikorn et al. 2010). The expression of *MdTFL1* decreased sharply after 63 DAFB in our study. This finding is compatible with a role of these genes in the timing from vegetative phase to reproductive phase and in maintaining meristem indeterminacy (lateral and inflorescence meristems) in the bud (Pillitteri et al. 2004; Kotoda and Wada 2005). The induction of flowering and floral development is coincident with the lack of *MdTFL1* expression in the late stages of flower development (Kotoda and Wada 2005).

We demonstrated that endogenous KODA may be associated with flower bud formation that was influenced by fruit load in apples (Kittikorn et al. 2010). That is, the endogenous KODA concentrations in HCT were lower than those in FTT. The presence of fruit inhibits flower initiation in the bourse bud, but this inhibition can be

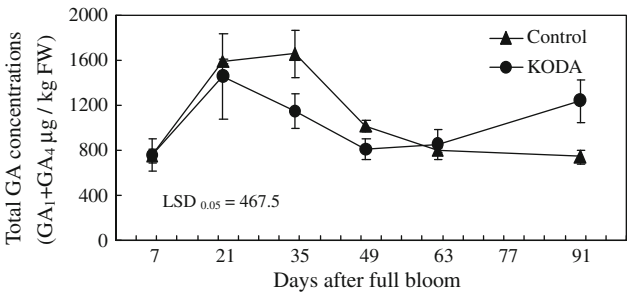

Fig. 5 Effect of KODA application (100 μM) on endogenous GA concentrations in the apical buds. Data are the means ± SE of three replications

relieved if the fruits are removed at an early stage (42–56 DAFB) of development (Dennis and Neilson 1999). The presence of fruit decreases flower induction, and *MdTFL1* is detected strongly in vegetative tissues (Kotoda et al. 2006). Therefore, the high expression of *MdTFL1* in HCT may be associated with the low rate of flower bud formation. Our assumption may be supported by the fact that the expression of *MdTFL1* in shading treatment, during which the rate of flower bud formation was low, was also detected more strongly than that in non-shaded trees.

MdFT1 and *MdFT2* genes have been isolated, and their functions were clarified in apples (Kotoda and Wada 2005; Kotoda et al. 2010). The transcriptional activation of *MdFT1* is closely associated with the regulation of flowering in apples, with expression of genes such as *MdMADS12* (Kotoda et al. 2010). In our study, the expression of *MdFT1* was observed throughout the measuring dates in each treatment. These results agree with the observation that *MdFT1* was highly detected in the apical buds of fruit-bearing shoots from the period of flower induction to the early stage of flower development (Kotoda et al. 2010). However, KODA applied to shoots in the present study did not affect the expression of *MdFT1*. In contrast, the expression of *MdTFL1* at 49 DAFB was decreased by KODA application. We have observed that the degree of shoot elongation was the highest from 35 to 49 DAFB in our study (data not presented). In addition, a previous study showed that the *TFL1* homolog is associated with juvenility in fruit trees (Pillitteri et al. 2004). Therefore, it is possible that KODA application induces flower bud formation in apples through the effect to *MdTFL1* transcription.

GA_4 plays an important role in flower bud physiology in apples (Looney et al. 1985). Exogenous GA_1, GA_4, and iso-GA_7 inhibited flower induction in apples (Ramírez et al. 2004). In our study, KODA application to the buds did not influence endogenous GA concentrations in the buds. However, the KODA application increased the rate of flower bud formation without affecting the GA concentrations. Therefore, KODA and GA may influence the flower bud formation independently. It is unclear where KODA is synthesized in apples. Further analysis will be needed to determine the primary site where KODA that accumulates in the bud is synthesized.

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