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Gene Note

Effects of the plant growth regulators on expression of *MdTFL1* promoter fused β-glucuronidase (GUS) reporter gene in apple (*Malus* spp.) tissues *in vitro*

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Abstract Apple has a relatively long juvenile growth period, taking approximately 8 years to reach the flowering stage. This period has impeded breeding programs. Since *MdTFL1* (an ortholog of *TERMINAL FLOWER1*) is strongly related to juvenility/vegetative and flower initiation in apple, its studies will provide insight into relationship between phytohormones and juvenile/vegetative growth and flower bud formation. In this study, we monitored expression of the *MdTFL1* promoter-β-glucuronidase (GUS) fusion construct in apple tissues *in vitro*, to determine which plant growth regulators affect its expression. Expression of *MdTFL1* was induced strongly throughout newly generated young shoots of cytokinin-treated plants and in apices of shoots on medium containing cytokinin+auxin. These results suggest that cytokinin may induce *MdTFL1* expression to maintain the juvenile form at the base of plant, and that cytokinin and auxin determine whether the shoot apical meristem will transit from the vegetative to the reproductive state.

Key words: Auxin, cytokinin, juvenility, *Malus* spp., *TERMINAL FLOWER1* (*TFL1*).

Apple (*Malus* spp.) is a major fruit crop worldwide, and can be cultivated in cold areas. It has a relatively long juvenile period, in that it takes approximately 8 years to reach the flowering stage (Way 1971). This period limits the efficiency of breeding programs, hampering the development of new cultivars. Therefore, the development and utilization of flower induction techniques are important to accelerate breeding cycles. In apple, seedling juvenility can be shortened by some techniques, for example, ringing of the truck bark, root pruning, and grafting onto dwarfing rootstock (Visser 1964; Way 1971). However, apple shows considerable genetic variation in juvenile growth traits, and even when the above-mentioned techniques are used, the time to reach flowering can only be shortened to 4 years (Way 1971; Zimmerman 1972). In another pome fruit plant, Japanese pear, it is known that certain treatments, such as maleic hydrazide (MH, 1,2-dihydro-3,6-pyridazinedione; coline salt), 2,3,5-triiodobenzoic acid (TIBA), or bending the lateral branch to a horizontal position, can up-regulate cytokinin, but down-regulate auxin in the lateral buds, and then may induce the increase of flower bud production on the shoots (Ito et al. 1999, 2000, 2001), but not the methods for shortening juvenile period.

In the last 10 years, genetic and molecular studies have considerably advanced our understanding of the apple flowering process. It is noteworthy that molecular evidence have clarified the transition process from vegetative to reproductive phase in the apical bud. Among these studies, two apple flowering genes, the *TERMINAL FLOWER1* orthologs (plant phosphatidyl ethanolamine-binding protein homologs) *MdTFL1-1* (*MdTFL1*) and *MdTFL1-2* (*MdTFL1a*), have been isolated and characterized (Esumi et al. 2005; Kotoda et al. 2005, 2006; Mimida et al. 2009, 2011). The temporal and spatial expression patterns of *MdTFL1-1* and *MdTFL1-2* are similar, and both are strongly expressed in the rib meristem zone of the shoot apex throughout the vegetative phase in adult plants. Subsequently, their expression is drastically decreased when the vegetative

Abbreviations: ABA, abscisic acid; BAP, 6-benzylaminopurine; GA4, gibberellin 4; GUS, β-glucuronidase; IBA, indole-3-butyric acid; JM2, Japan Morioka 2; PCR, polymerase chain reaction; PGRs, plant growth regulators; *TFL1*, *TERMINAL FLOWER1*; X-Gluc, 5-bromo-4-chloro-3-indolyl β-D-glucuronide

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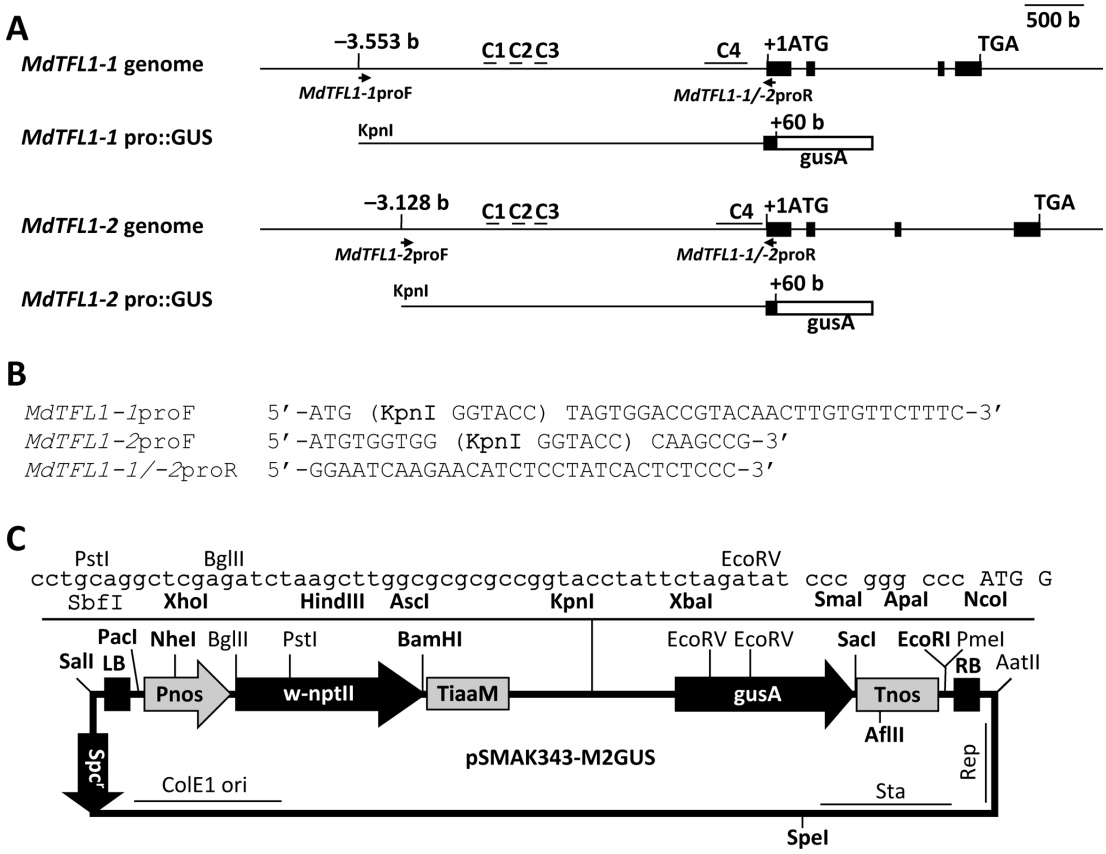

Figure 1. Constructs for *MdTFL1-1* or *MdTFL1-2* promoter fused to β-glucuronidase (GUS) reporter gene (*MdTFL1-1*pro::GUS/pSMAK343-M2GUS and *MdTFL1-2*pro::GUS/pSMAK343-M2GUS). (A) Schematic representation of the gene structures and GUS gene constructs for expression analyses of *MdTFL1-1* and *MdTFL1-2*. C1, C2, C3, and C4 indicate highly conserved regions between *MdTFL1-1* and *MdTFL1-2* (Mimida et al. 2009). Black boxes on bars show exons of *MdTFL1-1* and *MdTFL1-2*, white boxes show gusA genes. Scale bar=5500 bases. (B) Primer sequences used for constructs in this study. (C) Restriction map of pSMAK343-M2GUS. Abbreviations; LB: left T-DNA border, RB: right T-DNA border, Pnos: promoter of nopaline synthase gene, w-nptII: wild-type neomycin phosphotransferase II gene, TiaaM: terminator of *Agrobacterium* indole-3-acetic acid (IAA) monooxygenase gene, gusA: gusA: β-glucuronidase gene, Tnos: terminator of nopaline synthase gene, ColE1 ori: replication origin for ColE1 from pBR322, Rep and sta: replication and stability regions, respectively, of *Pseudomonas* plasmid pVS1, Spc^r: spectinomycin resistance gene from Tn7. Unique restriction site shown in bold font.

meristem begins to change into the inflorescence meristem. High expression of *MdTFL1-1* and *MdTFL1-2* was also observed in the shoot apex of 1-month-old seedling (Mimida et al. 2009, 2011). In addition, transgenic apple plants expressing antisense *MdTFL1-1* bear round serrated leaves and show precocious flowering, with flower initiation occurring 8 months after grafting onto rootstock (Kotoda et al. 2006). These results suggest that *MdTFL1-1* and *MdTFL1-2* repress transition from the vegetative to reproductive meristem at the shoot apex, maintaining the juvenile and vegetative phases (Kotoda et al. 2006; Mimida et al. 2009, 2011).

The use of plant growth regulators (PGRs) is one candidate method for shortening the breeding cycle. However, the effects of phytohormones and PGRs on flower induction and flowering-related genes still remain unclear in apple. Here, we studied expression of the *MdTFL1* promoter-β-glucuronidase (GUS) fusion construct in apple tissues *in vitro*, to determine which PGR affect spatial patterns of its gene expression. Hereby, we clarified relationship between *MdTFL1* expression profiles in response to PGRs and vegetative growth *in vitro* and that guessed relationship among juvenile/vegetative growth and phytohormones in an entire apple tree.

To examine the expression patterns of *MdTFL1* in response to PGRs, we generated two types of transgenic apple plants with the GUS reporter gene driven by the *MdTFL1-1* or *MdTFL1-2* promoter. The regions from −3,553 to +60 b of *MdTFL1-1* and −3,128 to +60 b of *MdTFL1-2* were amplified from the genome DNA of the apple cultivar 'Fuji' (*Malus*×*domestica* Borkh.) by polymerase chain reaction (PCR) (Figure 1). The PCR products were digested with *Kpn*I, and each was then cloned in-frame into the *Kpn*I-*Sma*I sites of the pSMAK343-M2GUS binary vector (modified pSMAK341-M2GUS) upstream of GUS, yielding two constructs; *MdTFL1-1*pro::GUS/pSMAK343-M2GUS and *MdTFL1-2*pro::

GUS/pSMAK343-M2GUS (Figure 1). Transformation of apple was performed according to the method described in Wada et al. (2009). The root stock cultivar 'Japan Morioka 2: JM2' (*M. prunifolia* (Wild.) Borkh. 'Seishi' × *M. pumila* Mill. var. *paradisiacal* Schneid. 'M.9') and the *Agrobacterium tumefaciens* strain LBA4404 harboring the construct *MdTFL1-1*pro::GUS/pSMAK343-M2GUS or *MdTFL1-2*pro::GUS/pSMAK343-M2GUS were used for transformation experiments. We obtained over 50 transgenic lines for each GUS fusion transgene. The GUS staining patterns were similar in the two types of transgenic plants (data not shown). Among them, we selected three representative individuals from the *MdTFL1-1*pro::GUS transgenic plants (#14, #18 and #57) and three from the *MdTFL1-2*pro::GUS transgenic plants (#46, #49 and #69) for further analyses.

In general, we have maintained 'JM2' cultured shoots on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 3% sucrose, 7% bacto-agar, adjusted to pH 5.7, and supplemented with 0.1 mg l^{-1} IBA and 0.5 mg l^{-1} BAP. This medium promotes budding of new shoots, but not roots. The plants were grown at 25°C with a 16-h photoperiod (30 μmol m^{-2} s^{-1}) in the culture room. First, to understand GUS activities of these transgenic plants in the absence of PGRs, we transferred them onto MS medium without any PGR. After 33 days, no GUS staining was observed in any tissues of either *MdTFL1-1*pro::GUS or *MdTFL1-2*pro::GUS (Figure 2A, C). The result suggested that activity levels for GUS expression of these transgenes are too low under the nonhormone-treated condition. On the other hand, in transgenic plants cultured on medium containing IBA+BAP, GUS staining was observed in the vascular tissues of the leaves, shoot apices, and throughout primary shoots that were less than approximately 2 cm in length (Figure 2B, D).

To determine what types and concentrations of PGRs affected GUS staining patterns in transgenic plants, we transferred each line onto MS medium containing abscisic acid (ABA), gibberellin A4 (GA4), auxin (IBA) or cytokinin (BAP), and stained them after 33 days. On MS medium containing ABA or GA4, the GUS staining patterns in both transgenic plants of *MdTFL1-1*pro::GUS and *MdTFL1-2*pro::GUS were not affected (data not shown). On MS medium containing low concentrations of IBA, we observed emergence of roots from the cut edges of shoots. On MS medium containing high concentrations of IBA, calli and roots formed along cut edges (Figure 3A, C, E, G). In these conditions, GUS staining in lines of *MdTFL1-1*pro::GUS and *MdTFL1-2*pro::GUS was observed in roots, calli, and stems in regions located above the medium, and the intensity of the staining was dependent on the IBA concentration (Figure 3B, D, F, H). On MS medium containing BAP, we observed hypertrophy of stems and emergence of new shoots. The more severe phenotypes were associated with higher concentrations of BAP (Figure 3I, K, M, O). In these conditions, GUS staining in lines of *MdTFL1-1*pro::GUS and *MdTFL1-2*pro::GUS was observed throughout young shoots that were less than approximately 2 cm long, but only weakly in shoot apices (Figure 3J, L, N, P).

Unlike field condition, *in vitro* system, is not influenced by the weather, can provide stable and accurate data of the effects of PGRs, and can be applied widely. The present study is designed to investigate the effects of PGRs on the apple shoot growth by monitoring vegetative growth-related gene, *MdTFL1*. It is shown that the continuing vegetative growth of apple shoots cultured *in vitro* is due to cytokinin and auxin inducing *MdTFL1* expression.

The expression patterns of *MdTFL1* on MS medium with IBA+BAP were consistent with those reported for *AtTFL1*pro::GUS in transgenic *Arabidopsis* (Guan et al. 2006). In *Arabidopsis*, inflorescence could be regenerated from callus on medium containing cytokinin and auxin, and the GUS gene driven by the *AtTFL1* promoter was expressed in inflorescence primordium emerging from callus, and in the inflorescence shoot apex (Guan et al. 2006). However, the expression of *AtTFL1*pro::GUS in the reproductive shoot apex of *Arabidopsis* differs from that of *MdTFL1*pro::GUS in the vegetative shoot apex of apple. *AtTFL1* has two functions in *Arabidopsis*; maintaining vegetative growth within the vegetative meristem to delay flowering, and reproductive growth within inflorescence meristems for indeterminate growth (Bradley et al. 1997; Ratcliffe et al. 1998). On the contrary, *MdTFL1* may have only one function in apple; maintaining vegetative growth in both the juvenile and mature plant, because apple produces sympodial inflorescences terminated by a primary flower (Foster et al. 2003; Kotoda et al. 2006; Mimida et al. 2009, 2011). Expression of *MdTFL1* decreased to undetectable levels after the vegetative meristem converted into an inflorescence meristem (Mimida et al. 2011). Taken together, these results imply that the two phytohormones cytokinin and auxin are important for maintaining shoot meristem fate by inducing the *TFL1* gene in *Arabidopsis* (a herbaceous plant) and apple (a fruit tree).

In the model of juvenile characteristics of trees described by Kester (1976), there are different localizations of maturation states throughout the plant; the juvenile form may be preserved at the base of plant, while mature forms occur at the periphery of the plant, even though these parts are chronologically younger. Our results showed that expression of *MdTFL1* was induced strongly by cytokinin throughout newly generated young shoots, and also enhanced by the combination of cytokinin and auxin in shoot apices (Figures 2, 3). In apple trees, cytokinin is synthesized in the apical region

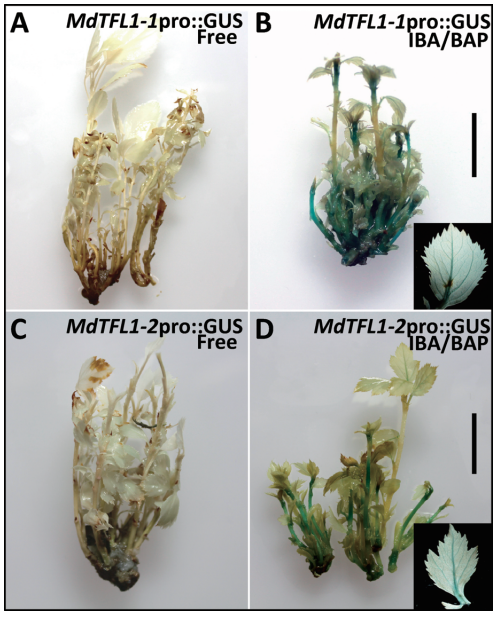

Figure 2. Expression analyses of *MdTFL1-1*pro::GUS and *MdTFL1-2*pro::GUS reporter genes on the plant growth regulator (PGR)-free medium or on *in vitro* culture medium containing IBA+BAP for shoot induction. (A) GUS staining of *MdTFL1-1*pro::GUS/transgenic plant on PGR-free culture medium. (B) GUS staining of transgenic plant with *MdTFL1-1*pro::GUS on medium containing IBA+BAP (0.1 mg l^{-1} and 0.5 mg l^{-1}). (C) GUS staining of *MdTFL1-2*pro::GUS/transgenic plant on PGR-free culture medium. (D) GUS staining of *MdTFL1-1*pro::GUS/transgenic plant on medium containing IBA+BAP (0.1 mg l^{-1} and 0.5 mg l^{-1}). GUS staining analyses were performed at 33 days after transferring onto each medium. Representative transgenic lines of *MdTFL1-1*pro::GUS (#18) and *MdTFL1-2*pro::GUS (#49) are shown, and were stained for 24 hours at 37°C with the following solution: 0.5 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc), 12.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$, 50 mM NaH_2PO_4, 5% methanol, and 0.01% Triton X-100. Scale bars show approximately 1 cm.

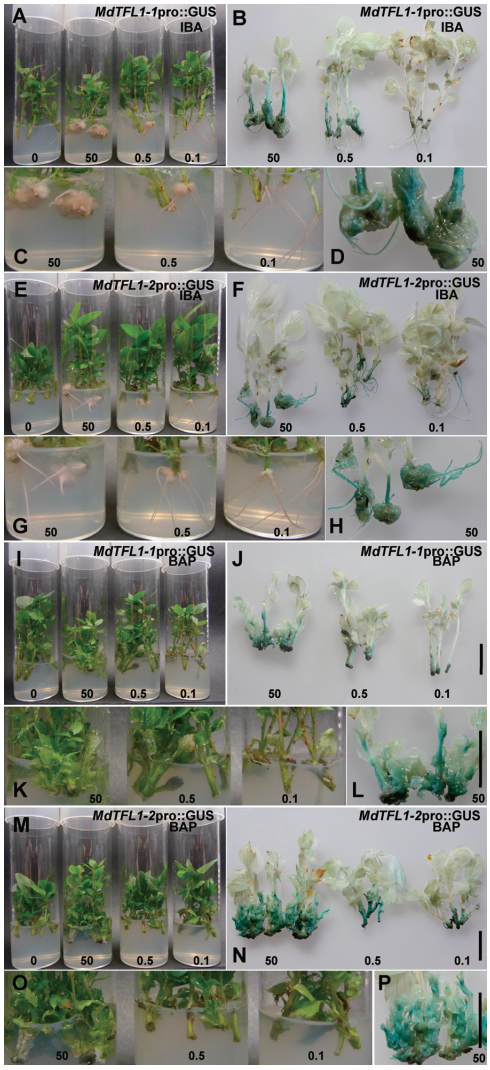

Figure 3. Expression analyses of *MdTFL1-1*pro::GUS or *MdTFL1-2*pro::GUS reporter genes on media containing IBA or BAP. (A) Morphology of *MdTFL1-1*pro::GUS/transgenic plant on medium containing IBA (0, 50, 0.5, 0.1 mg l^{-1}). (B) GUS staining of *MdTFL1-1*pro::GUS/transgenic plant on medium containing IBA (50, 0.5, 0.1 mg l^{-1}). (C) Close-up of pruning position in A (50, 0.5, 0.1 mg l^{-1}). (D) Close-up of pruning position in B (50 mg l^{-1}). (E) Morphology of *MdTFL1-2*pro::GUS/transgenic plant on medium containing IBA (0, 50, 0.5, 0.1 mg l^{-1}). (F) GUS staining of *MdTFL1-2*pro::GUS/transgenic plant on medium containing IBA (0, 50, 0.5, 0.1 mg l^{-1}). (G) Close-up of pruning position in E (50, 0.5, 0.1 mg l^{-1}). (H) Close-up of pruning position in F (50 mg l^{-1}). (I) Morphology of *MdTFL1-1*pro::GUS/transgenic plant on medium containing BAP (0, 50, 0.5, 0.1 mg l^{-1}). (J) GUS staining of *MdTFL1-1*pro::GUS/transgenic plant on medium containing BAP (50, 0.5, 0.1 mg l^{-1}). (K) Close-up of pruning position in I (50, 0.5, 0.1 mg l^{-1}). (L) Close-up of pruning position in J (50 mg l^{-1}). (M) Morphology of *MdTFL1-2*pro::GUS/transgenic plant on medium containing BAP (0, 50, 0.5, 0.1 mg l^{-1}). (N) GUS staining of *MdTFL1-2*pro::GUS/transgenic plant on medium containing BAP (50, 0.5, 0.1 mg l^{-1}). (O) Close-up of pruning position in M (50, 0.5, 0.1 mv). (P) Close-up of pruning position in N (50 mg l^{-1}). Representative transgenic lines of *MdTFL1-1*pro::GUS (#18) and *MdTFL1-2*pro::GUS (#49) are shown. GUS staining analyses were performed at 33 days after transferring to each medium. GUS staining was performed in the same manner as described in Figure 2. Concentration of IBA or BAP (mg l^{-1}) in each medium is indicated at the bottom of each photograph. Scale bars show approximately 2 cm.

of roots, while auxin is produced in the apical region of young shoots (Lockard and Schneider 1981). These observations and our results suggest that the cytokinin produced in the roots induces *MdTFL1* expression to maintain high juvenility at the base of plant. Assuming that the intensity of juvenility is dependent on the cytokinin concentration, there may be a high concentration of cytokinin around the base of the plant above the root. This may explain why *MdTFL1* was strongly expressed in 1-month-old seedlings, as determined by *in situ* hybridization (Mimida et al. 2011). Moreover, cytokinin is translocated to the periphery of the plant, where it may regulate *MdTFL1* expression. Both of cytokinin and auxin (which is produced in the young shoot) are important for expression of *MdTFL1*, which determines whether the shoot apical meristem is in the vegetative or reproductive state. Therefore, the techniques for flower bud induction in Rosaceae tree plants, such as root pruning and ring-girdling the tree trunk (Hackett 1985; Tsukahara et al. 2009; Visser 1964; Way 1971), may decrease the cytokinin content, thereby

down-regulating expression of *MdTFL1* or its orthologs. This would then decrease the juvenility of the seedling, resulting in precocious flowering. On the other hand, removing the shoot apex or bending branch into a horizontal position on the tree also induce flower bud formation in Japanese pear. These procedures may decrease the auxin content, since auxin is mainly produced at the young shoot apices, and thereby maintain apical dominance (Asami and Ito 1933; Ito et al. 1999). Interestingly, the Japanese pear cultivar 'Shinsui', which bears few flower buds on extension shoots, has a high auxin to cytokinin ratio compared with that in 'Hosui', which bears many flower buds (Banno et al. 1985). Thus, there is conflicting data on whether a decrease in cytokinin or auxin (e.g., via ring-girdling the trunk or bending branch etc.) is more important for flower induction. However, our results show that both cytokinin and auxin are required for expression of *MdTFL1* in shoot apices. Therefore, when one or the other is in limited supply at the periphery of the plant, flower initiation will be induced.

There are complex processes for repressing flower initiation and ending the juvenile phase in trees. These processes are controlled by various internal and external factors, such as flowering pathway genes (Hanke et al. 2007), sugar metabolism (Ito et al. 2002), SQUAMOSA Promoter-Binding Protein-Like (SPL)-family genes and microRNAs (Chen et al. 2010; Poethig 2010; Wang et al. 2011), phytohormones (Banno et al. 1985; Buban and Faust 1982; Looney et al. 1985) and environmental conditions, such as CO_2 concentration, temperature, day-length and nutrition (Sachs and Hackett 1983; Stahly and Piringer 1962; Zimmerman 1971). Here, we clarified one part of these complex processes in apple; that is, the relationships among vegetative growth, phytohormones, and expression of *MdTFL1*.

In the future, we will try to integrate above described phase control mechanism and our results in this study, and will determine the effects of PGRs regulating endogenous phytohormones such as cytokinin and auxin on juvenile growth and flower bud formation.

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References

Asami Y, Ito H (1933) A preliminary report on the effects of summer pinching upon the carbohydrate and nitrogen contents of the Japanese pear shoots. *J Hort Assoc Jpn* 4: 185–192

Banno K, Hayashi S, Tanabe K (1985) Relationships between flower bud formation and endogenous growth regulators in Japanese pear cultivars (*Pyrus serotina* Rehd.). *J Jpn Soc Hort Sci* 55: 258–265

Bradley D, Ratcliffe O, Vincent C, Carpenter R, Coen E (1997) Inflorescence commitment and architecture in *Arabidopsis*. *Science* 275: 80–83

Buban T, Faust M (1982) Flower bud induction in apple trees: Internal control and differentiation. *Hort Rev* 4: 174–203

Chen X, Zhang Z, Liu D, Zhang K, Li A, Mao L (2010) SQUAMOSA promoter-binding protein-like transcription factors: star players for plant growth and development. *J Integr Plant Biol* 52: 946–951

Esumi T, Tao R, Yonemori K (2005) Isolation of *LEAFY* and *TERMINAL FLOWER 1* homologues from six fruit tree species in the subfamily Maloideae of the Rosaceae. *Sex Plant Reprod* 17: 277–287

Foster T, Johnston R, Seleznyova A (2003) A morphological and quantitative characterization of early floral development in apple (*Malus*×*domestica* Borkh.). *Ann Bot* (*Lond*) 92: 199–206

Guan CM, Zhu SS, Li XG, Zhang XS (2006) Hormone-regulated inflorescence induction and *TFL1* expression in Arabidopsis callus in vitro. *Plant Cell Rep* 25: 1133–1137

Hackett WP (1985) Juvenility, maturation, and rejuvenility in woody plants. *Hortic Rev* 7: 109–155

Hanke M-V, Flachowsky H, Peil A, Hättasch C (2007) No flower no fruit–genetic potentials to trigger flowering in fruit trees. *Genes Genomes Genomics* 1: 1–20

Ito A, Yaegaki H, Hayama H, Kusaba S, Yamaguchi I, Yoshioka H (1999) Bending shoots stimulates flowering and influences hormone levels in lateral buds of Japanese pear. *Hortscience* 34: 1224–1228

Ito A, Hayama H, Yoshioka H (2000) Effects of plant growth regulators and their time of application on flower bud formation of Japanese pear 'Kosui'. *J Jpn Soc Hort Sci* 69: 529–535

Ito A, Hayama H, Kashimura Y, Yoshioka H (2001) Effect of maleic hydrazide on endogenous cytokinin contents in lateral buds, and its possible role in flower bud formation on the Japanese pear shoot. *Scientia Hort* 87: 199–205

Ito A, Hayama H, Yoshioka H (2002) Sugar metabolism in buds during flower bud formation: a comparison of two Japanese pear [*Pyrus pyrifolia* (Burm.) Nak.] cultivars possessing different flowering habits. *Scientia Hort* 96: 163–175

Kester DE (1976) The relationship of juvenility to plant propagation. *Comb Proc Int Plant Prop Soc* 26: 71–84

Kotoda N, Iwanami H, Takahashi S, Abe K (2006) Antisense expression of *MdTFL1*, a *TFL1*-like gene, reduces the juvenile phase in apple. *J Am Soc Hortic Sci* 131: 74–81

Kotoda N, Wada M (2005) *MdTFL1*, a *TFL1*-like gene of apple, retards the transition from the vegetative to reproductive phase in transgenic *Arabidopsis*. *Plant Sci* 168: 95–104

Looney NE, Pharis RP, Noma M (1985) Promotion of flowering in apple trees with gibberellin A4 and C-3 epi gibberellin A4. *Planta* 165: 292–294

Lockard RG, Schneider GW (1981) Stock and scion growth relationships and the dwarfing mechanism in apple. *Hortic Rev* 3: 315–375

Mimida N, Kotoda N, Ueda T, Igarashi M, Hatsuyama Y, Iwanami H, Moriya S, Abe K (2009) Four *TFL1*/*CEN*-like genes on distinct linkage groups show different expression patterns to regulate vegetative and reproductive development in apple (*Malus*×*domestica* Borkh.). *Plant Cell Physiol* 50: 394–412

Mimida N, Ureshino A, Tanaka N, Shigeta N, Sato N, Moriya-

Tanaka Y, Iwanami H, Honda C, Suzuki A, Komori S, et al. (2011) Expression patterns of several floral genes during flower initiation in the apical buds of apple (*Malus*×*domestica* Borkh.) revealed by in situ hybridization. *Plant Cell Rep* 30: 1485–1492

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol Plant* 15: 473–497

Poethig RS (2010) The past, present, and future of vegetative phase change. *Plant Physiol* 154: 541–544

Ratcliffe OJ, Amaya I, Vincent CA, Rothstein S, Carpenter R, Coen ES, Bradley DJ (1998) A common mechanism controls the life cycle and architecture of plants. *Development* 125: 1609–1615

Sachs RM, Hackett WP (1983) Source–sink relationships and flowering. In: Meudt WJ (ed) *Beltsville symposia in agricultural research. 6. Strategies of plant reproduction.* Osmun Publishing, Totowa, New Jersey, pp 263–272

Stahly EA, Piringer AA (1962) Effects of photoperiod, light quality, and two plant regulators on growth and flowering of Jonathan apple trees. *Proc Am Soc Hortic Sci* 81: 12–17

Tsukahara K, Yamane K, Yamaki Y, Fujishige N, Honjo H (2009) Effects of root pruning and uniconazole treatments on flower bud induction in peach seedlings of the current year. *Hort Res* (Japan) 8: 341–346 (In Japanese)

Visser T (1964) Juvenile phase and growth of apple and pear seedlings. *Euphytica* 13: 119–129

Wang JW, Park MY, Wang LJ, Koo Y, Chen XY, Weigel D, Poethig RS (2011) miRNA control of vegetative phase transition in trees. *PLoS Genet* 7: e1002012

Way RD (1971) Hastening the fruiting of apple seedlings. *J Amer Soc Hort Sci* 96: 384–389

Wada M, Ureshino A, Tanaka N, Komori S, Takahashi S, Kudo K, Bessho H (2009) Anatomical analysis by two approaches ensure the promoter activities of apple *AFL* genes. *J Jpn Soc Hort Sci* 78: 32–39

Zimmerman RH (1971) Flowering in crabapple seedlings: methods of shortening the juvenile phase. *J Amer Soc Hort Sci* 96: 404–411

Zimmerman RH (1972) Juvenility and flowering in woody plants: a review. *HortScience* 7: 447–455