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RESEARCH PAPER

Expressional regulation of *PpDAM5* and *PpDAM6*, peach (*Prunus persica*) dormancy-associated MADS-box genes, by low temperature and dormancy-breaking reagent treatment

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Abstract

The present study investigated the expressional regulation of *PpDAM5* and *PpDAM6*, two of the six peach (*Prunus persica*) dormancy-associated MADS-box genes, in relation to lateral bud endodormancy. *PpDAM5* and *PpDAM6* were originally identified as homologues of *Arabidopsis SHORT VEGETATIVE PHASE/AGAMOUS-LIKE 24* identified in the *EVERGROWING* locus of peach. Furthermore, *PpDAM5* and *PpDAM6* have recently been suggested to be involved in terminal bud dormancy. In this study, seasonal expression analyses using leaves, stems, and lateral buds of high-chill and low-chill peaches in field conditions indicated that both genes were up-regulated during the endodormancy period and down-regulated with endodormancy release. Controlled environment experiments showed that the expression of both *PpDAM5* and *PpDAM6* were up-regulated by ambient cool temperatures in autumn, while they were down-regulated by the prolonged period of cold temperatures in winter. A negative correlation between expression levels of *PpDAM5* and *PpDAM6* and bud burst percentage was found in the prolonged cold temperature treatment. Application of the dormancy-breaking reagent cyanamide to endo/ecodormant lateral buds induced early bud break and down-regulation of *PpDAM5* and *PpDAM6* expression at the same time. These results collectively suggest that *PpDAM5* and *PpDAM6* may function in the chilling requirement of peach lateral buds through growth-inhibiting functions for bud break.

Key words: bud dormancy, chilling requirement, cyanamide, low-chill peaches, *Prunus*, *StMADS11*.

Introduction

Bud dormancy allows perennial plants of temperate and boreal zones to survive low winter temperatures. Lateral buds are formed in early summer and enter a paradormant state that is mainly caused by apical dominance. By mid- to late autumn, the timing varies depending on species, the inhibitory control of bud growth shifts to the bud itself; these buds are referred to as being endodormant (Lang, 1987). Plants are incapable of emerging from this type of dormancy despite removal of terminal buds or defoliation. Endodormant buds require a certain amount of chilling to make the transition to an ecodormant state, in which buds are capable of resuming growth in favourable environments (Crabbe and Barnola, 1996; Faust *et al.*, 1997). In contrast to endodormancy, ecodormancy is imposed by external environmental factors such as cold or drought stress, which induce critical signals that prevent bud growth (Crabbe and Barnola, 1996; Lang, 1987; Horvath *et al.*, 2003).

Endodormancy is the result of physiological changes in response to an internal signal that inhibits the continuous rapid growth towards bud break. Certain signals that mediate the induction of endodormancy have been identified and characterized. The role of plant hormones such as abscisic acid, gibberellic acid, and ethylene in endodormancy is well established (Rohde *et al.*, 2002; Rohde and Bhalerao, 2007). Although a complex set of overlapping hormonal signals is known to be responsive to various environmental and physiological conditions related to dormancy (Horvath *et al.*, 2003; Rohde and

Abbreviations: CBF, C-repeat/DRE binding factor; CR, chilling requirement; SSH/MOS, suppression subtractive hybridization/mirror orientation selection.

Bhalerao, 2007), the manner in which environmental and hormonal signals are involved in dormancy remains to be elucidated.

Studies using both genetic and molecular approaches to examine the mechanisms that regulate bud endodormancy and dormancy release have recently been conducted for various plant species (for reviews, see Rohde and Bhalerao, 2007; Horvath, 2009). Endogenous processes that induce growth cessation and terminal bud set have been partly characterized. Böhlenius *et al.* (2006) suggested that homologues of *CONSTANS* and *FLOWERING LOCUS T*, which are flowering regulators in *Arabidopsis thaliana*, are involved in short-day-induced seasonal growth cessation of *Populus trichocarpa*. Recently, *CENTRORADIALIS (CEN)/TERMINAL FLOWER 1* was also shown to be involved in the endodormancy release of *Populus* (Mohamed *et al.*, 2010). However, genes that are involved in lateral bud endodormancy induction and release in temperate fruit tree species are poorly understood.

Suppression subtractive hybridization supplemented with mirror orientation selection (SSH/MOS) and differential screening were performed to identify genes that are preferentially expressed in endodormant buds of the Japanese apricot (*Prunus mume* Sieb. et Zucc.), a temperate fruit tree species (Yamane *et al.*, 2008). A MADS-box gene that shows endodormancy-associated expression was obtained. Seasonal expression analysis suggested that the MADS-box gene was up-regulated during endodormancy induction and down-regulated during endodormancy release. Sequence and phylogenic analyses of the full-length cDNA clone revealed a similarity between this gene and *StMADS11*-clade MADS-box genes such as *SHORT VEGETATIVE PHASE* and *AGAMOUS-LIKE 24* of *Arabidopsis* (Yamane *et al.*, 2008). In addition, Bielenberg *et al.* (2008) identified six *StMADS11*-clade MADS-box genes as candidate genes associated with terminal bud formation in peach [*Prunus persica* (L.) Batsch] and named them *DORMANCY ASSOCIATED MADS* (*DAM*) genes. Six *PpDAM* genes showed distinct seasonal expression patterns in the terminal buds of peach, and *PpDAM1*, *PpDAM2*, and *PpDAM4* were suggested to be closely associated with terminal bud formation (Li *et al.*, 2009). Horvath *et al.* (2008) also indicated a possible involvement of the *DAM* homologues *EeDAM1* and *EeDAM2* with endodormancy induction in leafy spurge (*Euphorbia esula*). Recently, Fan *et al.* (2010) showed the localization of a strong quantitative trait locus associated with the chilling requirement (CR) and a blooming date of peach *PpDAM6* locus. Moreover, Jimenez *et al.* (2010) showed that expression levels of *PpDAM5* and *PpDAM6* in shoot tips were negatively correlated with the time required for bud break in forcing conditions. These results suggested that *StMADS11*-clade MADS-box genes are candidates for internal factors controlling the endodormancy of perennial plants. However, no direct evidence has been presented for the involvement of *DAM* in bud dormancy through its growth inhibitory effects.

Currently, uniform flowering, which is indispensable for stable fruit production, is difficult to achieve in either protected or orchard cultivation in the southern areas of Japan. This situation is caused by irregular lateral bud endodormancy release, possibly due to global warming (Sugiura *et al.*, 2007). Therefore, the genetic factors that control endodormancy were investigated in order to understand the molecular basis of endodormancy regulation in temperate fruit tree species, which could lead to the artificial control of endodormancy through cultural practices and/or rapid breeding such as marker-assisted selection. As a first step, the investigation focused on *PpDAM5* and *PpDAM6*, the most likely candidate genes for peach lateral bud endodormancy release (Fan *et al.*, 2010; Jimenez *et al.*, 2010; R. Sassaki, H. Yamane, T. Ooka, H. Jotatsu, T. Akagi, and R. Tao, unpublished data).

In this study, the different seasonal expression patterns of *PpDAM5* and *PpDAM6* were demonstrated in peach cultivars with different CR for bud break. Subsequently, temperature-regulated changes in the expression of *PpDAM5* and *PpDAM6* were shown, suggesting their association with temperature-mediated dormancy regulation. Finally, the effects of dormancy-breaking stimuli, such as a prolonged period of low temperature and the effect of the dormancy-breaking chemical cyanamide, on dormancy status and expression of *PpDAM5* and *PpDAM6*, were investigated. Based on the results obtained, the biological functions of *PpDAM5* and *PpDAM6* are discussed.

Materials and methods

Plant materials

The peach cultivars Akatsuki, Shimizu Hakuto, and Tsukuba Ichigo were used in this study. Shimizu Hakuto and Akatsuki, grafted on Ohatsumomo rootstock, were obtained from a commercial nursery and planted at the Kyoto University experimental farm in 2002. Cuttings of Tsukuba Ichigo were kindly provided by Dr Kataoka from Kagawa University in 2002, and these cuttings were planted at the Kyoto University experimental farm in 2005. Tsukuba Ichigo is the F_2 offspring of Akame (high-chill)×Okinawa (Tsukuba strain). Okinawa is a rootstock cultivar with a low CR for bud break (low-chill) and Tsukuba Ichigo was bred as a low-chill rootstock cultivar at the National Institute of Fruit Tree Science, Tsukuba, Japan. The chilling requirements for bud break of Akatsuki, Shimizu Hakuto, and Tsukuba Ichigo are ~900 h CR, 1000 h CR, and 400–450 h CR, respectively. Akatsuki and Shimizu Hakuto are categorized as high-chill cultivars whereas Tsukuba Ichigo is a low-chill cultivar.

Long 1-year-old branches (current season's growth) of Shimizu Hakuto and Tsukuba Ichigo were collected monthly from June to March in 2007 or from June to February in 2008. Shimizu Hakuto and Tsukuba Ichigo trees were aged 7 and 6 years, respectively, and were considered to be in the adult phase. Air temperature was recorded at 15-min intervals and chilling accumulation was calculated according to the 'chill hours' (<7.2 °C). Pot-grown adult trees of Akatsuki were used for the ambient cool temperature (15–18 °C) treatment experiment. For the cold temperature (6–12 °C) treatment experiment, long 1-year-old branches of Akatsuki collected on 10 December 2008 were used. For the cyanamide treatment experiment, long 1-year-old branches of adult trees of Shimizu Hakuto collected on 25 December 2009 were used.

Seasonal endodormancy status and expression analysis

The timing of endodormancy induction and endodormancy release of lateral vegetative buds of Shimizu Hakuto and Tsukuba Ichigo

were investigated by two different methods. From June to March, three 1-year-old branches of ~50 cm length were cut from trees and the basal parts were placed in water containing Misakifarm™ (Otsuka Kagaku, Tokushima, Japan), a commercial preparation containing nutrients and fungicides for the prolonged life of flower cuttings. At the same time, the basal parts of 10 single-node cuttings obtained from the middle portions of each of three branches were placed in water containing Misakifarm™. The branches and single node cuttings were maintained at 22 °C under cool white fluorescent light with a 16-h light/8-h dark photoperiod. The water containing Misakifarm™ was replaced every 2 weeks. Lateral vegetative buds showing leaves were considered to have burst.

Vegetative buds of Shimizu Hakuto and Tsukuba Ichigo and leaves and stems of Shimizu Hakuto were collected monthly and immediately frozen in liquid nitrogen and stored at −80 °C until use. Total RNA was isolated from the collected tissues as described in Yamane *et al.* (2008). After DNase I treatment (Takara Bio, Inc., Ohtsu, Japan), 1 μg of total RNA was used for cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Based on the genomic DNA sequence for *PpDAM5* and *PpDAM6* from the NCBI nucleotide database, gene-specific TaqMan® probes and primers for specifically detecting transcripts of *PpDAM5* and *PpDAM6*, were designed and synthesized. The primer pairs DAM5F and DAM5R and PpDAM6F and PpDAM6R were used with the TaqMan® probes DAM5-T and DAM6-T, respectively, to detect transcripts of *PpDAM5* and *PpDAM6*, respectively (Table 1). Real-time PCR analysis with TaqMan® probes was performed using LightCycler 480 (F. Hoffmann-La Roche AG, Basel, Switzerland) and probe master mix (F. Hoffmann-La Roche AG). The reaction mixture consists of 1× probe master mix, 500 nM each of forward and reverse primers, 200 nM TaqMan® probe, and cDNA equivalent to the amount synthesized from 4 ng of total RNA in a 20-μl reaction volume. As a control, *UBIQUITIN* (*UBQ*) transcript accumulation was monitored by real-time PCR using SYBR Green Master Mix (Roche). *UBQ* was selected as a reference because the transcript level of *UBQ* appeared to be most stable across tissue types and sampling dates among the transcript levels of three genes tested, *UBQ*, *ACTIN*, and *Elongation Factor 1* (*EF-1*), although expression of both *UBQ* and *ACTIN* was far more stable than *EF-1*. A primer pair PaubiF and PaubiR was used for *UBQ*-specific real-time PCR (Table 1). PCR was performed using a programme of 45 cycles at 95 °C for 10 s, 56 °C for 20 s, and 72 °C for 1 s with an initial heating at 95 °C for 5 min. For *UBQ*-specific real-time PCR, dissociation curve analysis was performed to confirm that the fluorescence was only derived from the gene-specific amplification. Three technical replicates were performed for each gene and mesurement. The quantities of the transcripts of *PpDAM5* and *PpDAM6* in each sample were normalized using the *UBQ* transcripts.

Table 1. Sequences of primers and TaqMan® probes used in this study

Primer or TaqMan® probe	Sequence (fluorescent probe)	T_m (°C)
DAM5F	5′-ATCTCCACCACCTGCAACAGT-3′	61.9
DAM5R	5′-CTTCTTAACGCCCCAGTTTGAG-3′	62.3
PpDAM6F	5′-TAATGTTGGAGGTGGAGGAGAA-3′	60.8
PpDAM6R	5′-GGGAAGCCCCAGTTTGAGA-3′	62.5
DAM5-T (probe)	5′-(VIC)TCTTGAAGATGACTCCTCCGA(MGB)-3′	69.3
DAM6-T (probe)	5′-(FAM)TGAAGATGACTGCTCCGATGT CACTTTATC-(TAMRA)-3′	69.0
PaubiF	5′-CGAACCCTAGCCGATTACAA-3′	56.5
PaubiR	5′-AGTGGTTCGCCATGAAAGTC-3′	56.3

Ambient cool temperature and prolonged low-temperature treatments in controlled environment conditions

The experiment was conducted on 8 September 2008; 10 pot-grown Akatsuki trees with five 1-year-old branches were used. Four pots each were placed in two different growth chambers with different growth conditions. The two growth conditions used were under a 12-h light/12-h dark photoperiod (300 μmol/m^2/s) at 18 °C light/15 °C dark [cool(+)] and at 28 °C light/23 °C dark [cool(−)]. Buds consisting of both flower and vegetative buds were excised from the middle portions of branches at 0, 10, and 20 d after treatment and immediately frozen in liquid nitrogen and kept at −80 °C until use. Total RNA was isolated and used for the expression analysis of *PpDAM5* and *PpDAM6*, as described above.

In prolonged low-temperature treatment, 1-year-old branches were cut from Akatsuki trees on 10 December 2008, and placed in a growth chamber under a 9-h light/15-h dark photoperiod cycle at 12 °C light/6 °C dark [cold(+)] or 24 °C light/22 °C dark [cold(−)]. To investigate the endodormancy status changes by treatment, three branches were transferred to forcing conditions (9-h light/15-h dark photoperiod cycle at 24 °C light/22 °C dark) at 0 d and 60 d after treatment and their bud burst was recorded for 60 d. Vegetative buds were excised from the middle portions of branches at 0 d and 60 d after treatment and immediately frozen in liquid nitrogen and stored at −80 °C until use. Total RNA was isolated from the buds stored at −80 °C and used for expression analysis of *PpDAM5* and *PpDAM6*, as described above.

Dormancy-breaking reagent (cyanamide) treatment

On 25 December 2009, long 1-year-old branches were cut from Shimizu Hakuto trees. Their basal parts were placed in water containing Misakifarm™ and incubated in a greenhouse for a natural day length and under controlled temperature (18–22 °C). Subsequently, diluted CX-10™ (Nippon Carbide industries Co., Inc., Tokyo, Japan) solution (containing 0.6% effective cyanamide) with 0.05% Tween 20 (Wako Pure Chemical Industries, Ltd, Osaka, Japan) added as a surfactant was sprayed onto the branches. In the control treatment, 0.05% Tween 20 was used as a spray. Vegetative buds were excised from the middle portions of branches at 0, 2, and 4 weeks after treatment and immediately frozen in liquid nitrogen and stored at −80 °C until use. Total RNA was isolated and used for the expression analysis of *PpDAM5* and *PpDAM6*, as described above. The percentages of the branches with bud break were recorded using 13–15 branches 5 weeks after treatment.

Results

Seasonal endodormancy status and expression patterns of PpDAM5 and PpDAM6 in lateral buds

Table 2 shows the seasonal changes in the percentage bud break of vegetative buds of the high-chill cultivar Shimizu Hakuto and the low-chill cultivar Tsukuba Ichigo in forcing conditions. In Shimizu Hakuto, no bud burst was observed in the branches sampled in July [chilling hours (CH), 0 h], August (CH, 0 h), September (CH, 0 h), October (CH, 2 h), November (CH, 64 h), and December (CH, 414 h). In January (CH, 1053 h), 32.7% of the vegetative buds had opened. In February (CH, 1831 h), >60% of the vegetative buds had burst. No bud burst was observed in Tsukuba Ichigo from September to November. In December, 31% of vegetative buds of Tsukuba Ichigo had burst. In January and February, the number of Tsukuba Ichigo buds that opened was higher than those of high-chill Shimizu Hakuto. In the case when single node cuttings were used instead of whole

branches to examine dormancy status, Tsukuba Ichigo buds opened in all the months tested, while no Shimizu Hakuto buds opened from August to December (Table 2).

The seasonal expression changes of *PpDAM5* and *PpDAM6* in the lateral vegetative buds were monitored by real-time RT-PCR analysis using gene-specific TaqMan® probe and primers. From June to November, the expression levels of *PpDAM5* and *PpDAM6* in high-chill Shimizu Hakuto steadily increased. The highest transcript levels of *PpDAM5* and *PpDAM6* were observed in November. Subsequently, their transcript levels in vegetative buds steadily decreased towards spring (Fig. 1). From June to September, the expression levels of *PpDAM5* and *PpDAM6* in low-chill Tsukuba Ichigo were similar to or little bit lower than those in high-chill Shimizu Hakuto. However, their expression levels showed distinct patterns from October, with the transcript levels in low-chill Tsukuba Ichigo being much lower than those in Shimizu Hakuto. Transcript levels of *PpDAM5* and *PpDAM6* in Tsukuba Ichigo in January and February and those in Shimizu Hakuto in March were lowest (Fig. 1).

Seasonal expression changes of PpDAM5 and PpDAM6 in leaves and stems

In leaves of Shimizu Hakuto, the seasonal expression patterns of *PpDAM5* and *PpDAM6* steadily increased from early summer to autumn until the peak in October just before defoliation in the field (Fig. 2). Both *PpDAM5* and *PpDAM6* expression in stems peaked in November and December and drastically decreased in January and continued decreasing until spring. After December, the *PpDAM5* and *PpDAM6* expression patterns in stems were similar to those in lateral vegetative buds (Fig. 1).

Up-regulation of PpDAM5 and PpDAM6 in lateral buds by autumn ambient cool temperature (15–18 °C)

Seasonal expression analysis demonstrated that the transcript levels of both *PpDAM5* and *PpDAM6* increased in autumn when the ambient temperature decreased. It was assumed that changes in their expression level were controlled by alterations in the ambient temperature. To test this hypothesis, the effect of ambient cool temperature on the expression of *PpDAM5* and *PpDAM6* was investigated in a controlled environment experiment. The expression of both *PpDAM5* and *PpDAM6* increased in the cool(+) treatment, but remained constant in the cool(−) treatment (Fig. 3).

Down-regulation of PpDAM5 and PpDAM6 through exposure to prolonged winter cold temperature (6–12 °C) treatment

Seasonal expression analysis (Fig. 1) demonstrated that the transcript level of both *PpDAM5* and *PpDAM6* steadily decreased from November to March when the plants were exposed to a prolonged cold temperature period over autumn and winter, and subsequently to warm temperatures in early spring. To examine the changes in expression of *PpDAM5* and *PpDAM6* in response to the prolonged cold temperature in early winter, branches excised in December were subjected to cold(+) or cold(−) temperature treatment, and dormancy status and *PpDAM5* and *PpDAM6* transcript levels in lateral vegetative buds were

Table 2. Seasonal endodormancy depth of lateral vegetative buds of two peach cultivars using whole branches (A) or branch parts (B)

A. Whole branches

	Bud burst percentage by month									
Cultivar	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.
Shimizu Hakuto	73.3	0	0	0	0	0	0	32.7	62.7	100
Tsukuba Ichigo	86.7	31.7	3.3	0	0	0	31	54	95.5	NT

B. Single node cuttings

	Days to bud burst by month[a]									
Cultivar	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.
Shimizu Hakuto	++	++	−	−	−	−	−	+	++	NT
Tsukuba Ichigo	++	++	++	++	+	+	++	++	++	NT

[a] First bud burst was observed within 15 d (++), 30 d (+), or not observed within 30 d (−).
NT, not tested.

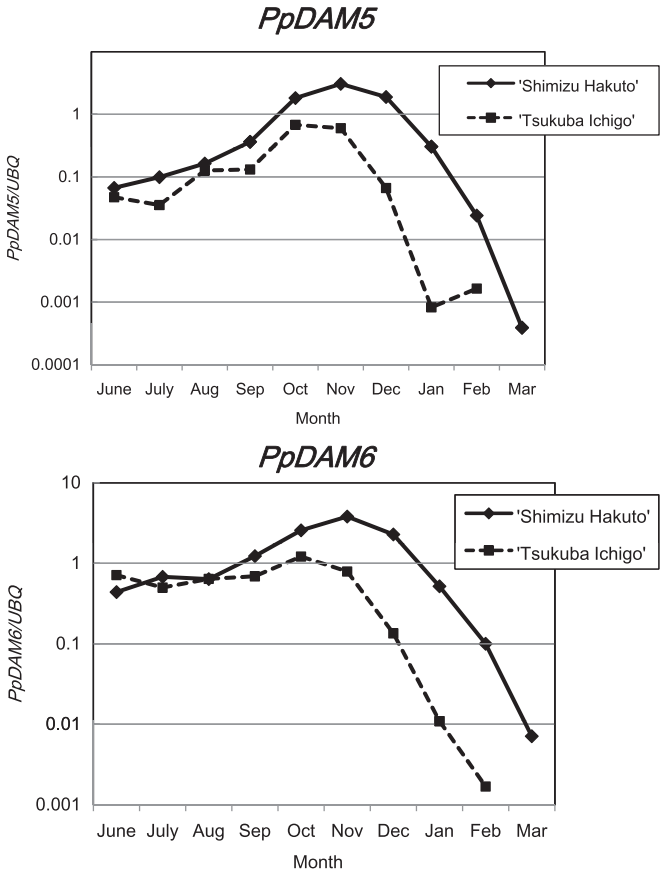

Fig. 1. Seasonal expression changes of *PpDAM5* and *PpDAM6* in lateral vegetative buds of two peach cultivars, high-chill cultivar Shimizu Hakuto and low-chill cultivar Tsukuba Ichigo. Each data point represents the mean value of three technical replicates.

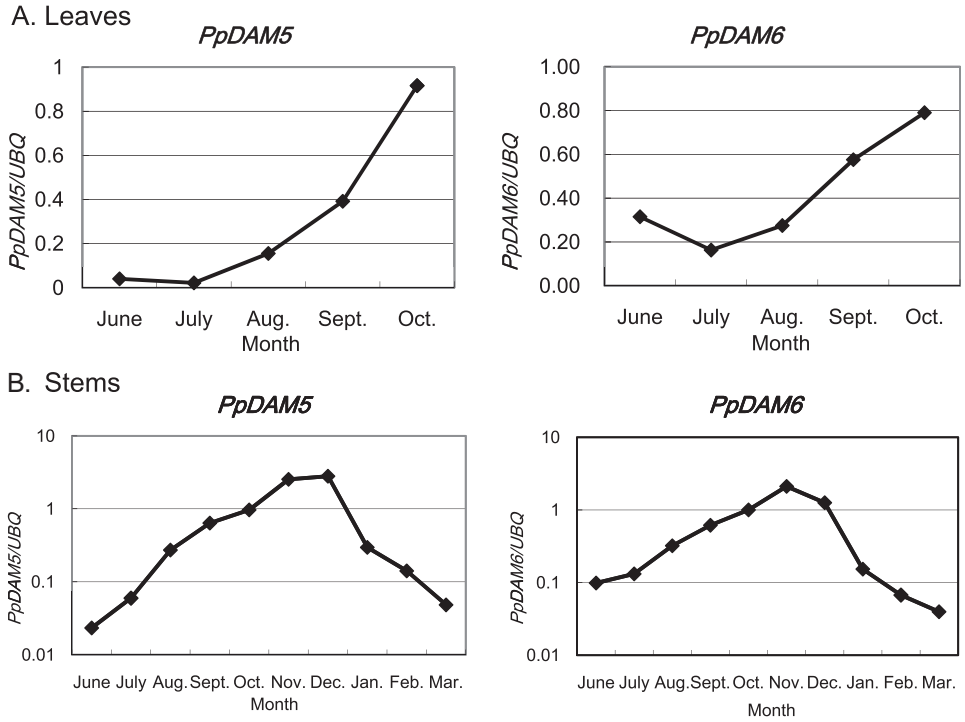

Fig. 2. Seasonal expression changes of *PpDAM5* and *PpDAM6* in leaves (A) and stems (B) of peach (cultivar Shimizu Hakuto). Each data point represents the mean value of three technical replicates.

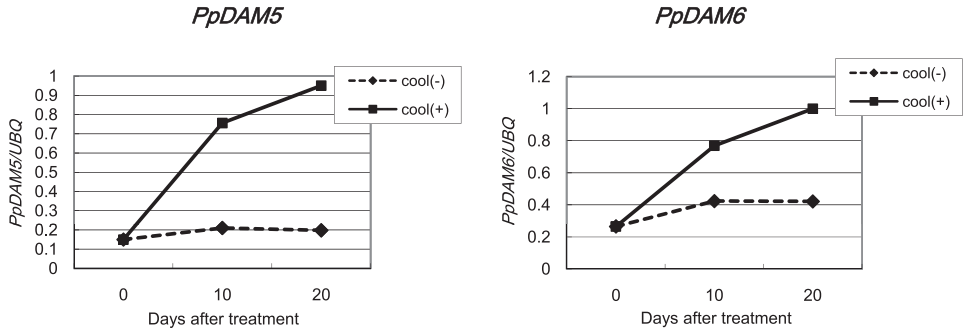

Fig. 3. Effects of ambient cool temperature (15–18 °C) in autumn (September) on the expression of *PpDAM5* (left) and *PpDAM6* (right) in lateral vegetative buds of peach (cultivar Akatsuki). Each data point represents the mean value of three technical replicates.

investigated. Cold temperature treatment for 60 d enhanced bud break. In forcing conditions, no Akatsuki buds opened after the cold(−) treatment, while most buds opened within 9 d after the cold(+) treatment. *PpDAM5* and *PpDAM6* transcript levels were constant after the cold(−) treatment, while they decreased after the cold(+) treatment. As a result, *PpDAM5* and *PpDAM6* transcript levels were lower in the cold(+) treatment than in the cold(−) treatment (Fig. 4).

Down-regulation of PpDAM5 and PpDAM6 by cyanamide treatment

Cyanamide, a dormancy-breaking reagent, treatment in late December (at ~500 CH) effectively promoted Shimizu Hakuto bud break. Five weeks after treatment, at least one bud on eight of 13 (61.5%) cyanamide-treated branches had burst, whereas none of the buds on 13 untreated branches burst. Although *PpDAM5* and *PpDAM6* transcript levels decreased in both cyanamide-treated and -untreated branches, they were much lower in treated branches than in untreated ones (Fig. 5). Changes in the expression patterns of *PpDAM5* and *PpDAM6* differed, *PpDAM6* showed a greater difference in expression level between treated and untreated branches than that observed for *PpDAM5*.

Discussion

It was previously reported that the putative homologue of *PpDAM6* in Japanese apricot, *PmDAM6*, showed endodormancy-associated expression in lateral buds (Yamane *et al.*, 2008). Further comprehensive expression and transgenic analyses suggested an association between *PmDAM6* with lateral bud endodormancy induction and release in Japanese apricot through its putative growth inhibitory functions against bud break and shoot growth (R. Sassaki,

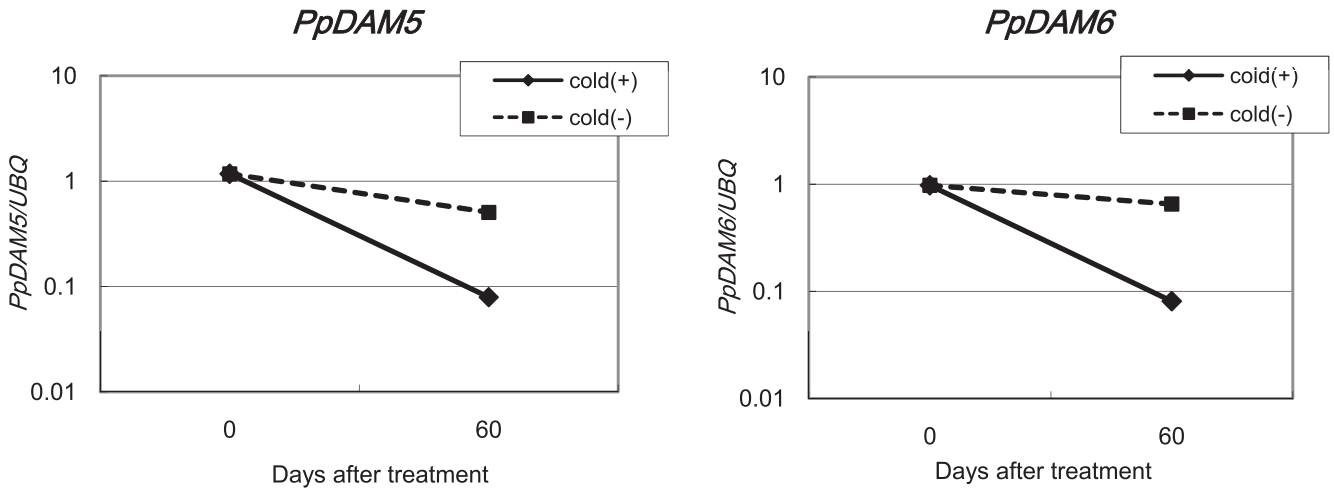

Fig. 4. Effects of prolonged low temperature (6–12 °C) in winter (December) on the expression of *PpDAM5* (left) and *PpDAM6* (right) in lateral vegetative buds of peach (cultivar Akatsuki). Each data point represents the mean value of three technical replicates.

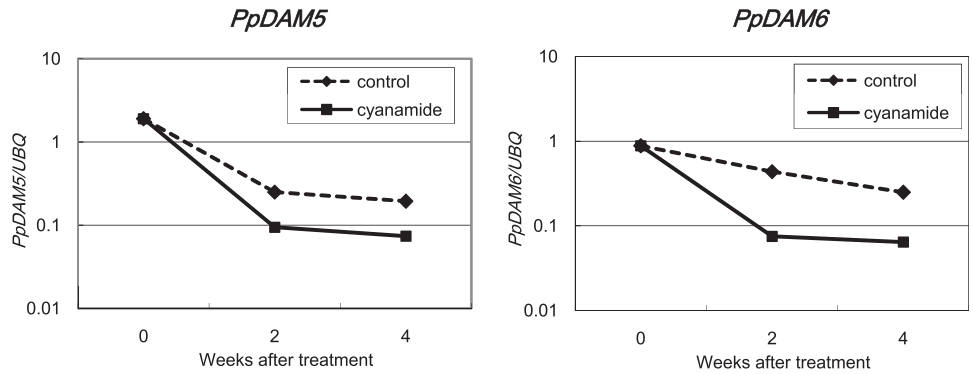

Fig. 5. The effects of cyanamide treatment on the expression of *PpDAM5* (left) and *PpDAM6* (right) in lateral vegetative buds of peach (cultivar Shimizu Hakuto). Each data point represents the mean value of three technical replicates.

H. Yamane, T. Ooka, H. Jotatsu, T. Akagi, and R. Tao, unpublished data).

PpDAM genes were initially identified as candidate genes for growth cessation and terminal bud formation in peach using the peach *evergrowing* mutant (Bielenberg *et al.*, 2008). However, Li *et al.* (2009) suggested that three peach *PpDAM* genes, namely *PpDAM1*, *PpDAM2*, and *PpDAM4*, were likely candidates to regulate terminal bud formation. Later, Jimenez *et al.* (2010) showed that *PpDAM5* and *PpDAM6* had functions other than terminal bud formation. Namely, they demonstrated negative correlation of the expression of *PpDAM5* and *PpDAM6* with peach terminal bud break. Furthermore, Horvath *et al.* (2010) reported the association of putative homologues of *PpDAM*s, *EeDAM1*, and *EeDAM2* with endodormancy induction in leafy spurge crown buds. With regard to lateral buds, *PpDAM5* and *PpDAM6* have been shown to be co-localized with a quantitative trait locus associated with the chilling requirement and bloom date of peach (Fan *et al.*, 2010). Although this suggests that *PpDAM5* and *PpDAM6* are responsible for regulating the chilling requirement and/or bloom date in peach lateral buds, comprehensive expressional analyses of *PpDAMs* with lateral bud endodormancy have not been conducted. This study focused on the relationship between the expression of *PpDAM*s and lateral bud endodormancy status with different environmental and chemical stimuli.

The effects of environmental signals on the expression of *DAM*s have been studied in perennial plants. A reduction in photoperiod and a short period of cold exposure induces the expression of peach *PpDAM*s (Li *et al.*, 2009) and leafy spurge *EeDAM*s (Horvath *et al.*, 2010), respectively. The effects of prolonged cold exposure on *PpDAM*s (Jimenez *et al.*, 2010) and *EeDAM*s (Horvath *et al.*, 2010) were also investigated. Although down-regulation of *DAM*s was recorded following prolonged cold exposure in both plants, lack of a control treatment made it difficult to interpret the results conclusively.

In this study, the association of *PpDAM5* and *PpDAM6* with phenological changes in the endodormancy status of lateral vegetative buds of two peach cultivars with different chilling requirements for bud break were first validated. The effects of temperature and dormancy-breaking reagent stimuli on the expression of *PpDAM5* and *PpDAM6* were then characterized, with viable control treatments. The results further substantiated previous studies that have

demonstrated *PpDAM5* and *PpDAM6* to have significant roles in endodormancy regulation in peach.

The low-chill cultivar entered endodormancy later than the high-chill cultivar, probably because of the prolonged period of shoot growth in low-chill cultivars in summer. Based on the bud burst percentage, the endodormancy of the high-chill cultivar appeared to be released after January, whereas that of the low-chill cultivar was released after November/December. Thus, the endodormancy period of the low-chill cultivar was shorter than that of the high-chill cultivar. Endodormancy was induced in July/August in Shimizu Hakuto and in September/October in Tsukuba Ichigo. However, prominent increases in the expression of *PpDAM5* and *PpDAM6* were not found in the respective period in either cultivar. This observation indicates that *PpDAM5* and *PpDAM6* are unlikely to trigger lateral bud endodormancy, although they seemed to be involved in endodormancy maintenance and release in late fall to early spring because their expression was subsequently up-regulated and down-regulated in coordination with endodormancy progression and release in the lateral buds of both cultivars. The same trends in dormancy-coordinated expression pattern were observed in *PpDAM5* and *PpDAM6* in stems and leaves. Dormancy-associated expression patterns of *PpDAM5* and *PpDAM6* were also found in peach terminal buds (Li *et al.*, 2009; Jimenez *et al.*, 2010). These observations collectively indicate that the expression of *PpDAM5* and *PpDAM6* is regulated in an endodormancy-coordinated manner in whole shoots of peach, contrasting with the dormancy-associated, but bud-specific, expression of *EeDAM1* and *EeDAM2* (Horvath *et al.*, 2010).

Lateral bud dormancy status is conceptually divided into the following five phases: paradormant, endodormant, ecodormant, and the two transition phases para–endo and endo–eco phases (Lang, 1987). Based on the present authors' survey of seasonal endodormancy status, Shimizu Hakuto peach was paradormant in June, in the para–endo transition phase in July, endodormant from August to December, in the endo–eco transition phase in January, and ecodormant in February and March. Transcript levels of paradormant buds were similar to those of endodormant buds. In addition, when the bud burst percentage in forcing conditions and transcripts levels of *PpDAM5* and *PpDAM6* between February and March were compared, a higher bud burst percentage and lower transcript levels were found in March than in February. These results suggested that *PpDAM5* and *PpDAM6* could function as a dose-dependent growth inhibitor not only in endodormant buds but also in paradormant and ecodormant buds.

Li *et al.* (2009) demonstrated that *PpDAM5* and *PpDAM6* were up-regulated by a reduction in photoperiod in controlled environment experiments. As the shortening of photoperiod in autumn coincides with a fall in temperature, the effects of temperature changes on *PpDAM5* and *PpDAM6* expression under a 12-h light/12-h dark photoperiod cycle in September were tested. Expression of both *PpDAM5* and *PpDAM6* was up-regulated only by cool(+) treatment, indicating that ambient cool temperature in September stimulates *PpDAM5* and *PpDAM6* expression. In July, similar experiments were conducted under a 14-h light/10-h dark photoperiod cycle with the same cool temperature regime (data not shown); however, this trend was only observed with *PpDAM5* and not with *PpDAM6*. This suggests that the up-regulation of *PpDAM5* and *PpDAM6* expression by ambient cool temperature depends on the bud stage and/or photoperiod condition. Horvath *et al.* (2010) found that *EeDAM1* was cold stress (11 °C) responsive and contains putative C-repeat/DRE-binding factor (CBF) sites, which are known to be *cis*-regulating motifs targeted by the cold/drought stress CBF regulon, within the first 2000-base upstream region from the *EeDAM1* translation initiation codon. Since CBF sites were found in both *PpDAM5* and *PpDAM6* (data not shown), it is possible that their cold-responsive expression was controlled by CBF protein as suggested for cold-responsive *EeDAM1* expression.

The controlled low-temperature treatment experiment indicates that a prolonged period of low temperature (6–12 °C) is required for both the steady down-regulation of *PpDAM5* and *PpDAM6* and the increase in bud burst percentage; thus, further substantiating the observations made in peach shoot terminals by Jimenez *et al.* (2010). Horvath *et al.* (2010) suggested that environmentally regulated down-regulation of *DAM1* appeared to be controlled, at least in part, by chromatin remodelling. Since *FLOWERING LOCUS C* (*FLC*), a MADS-box gene involved in vernalization of *Arabidopsis*, was also repressed by chromatin remodelling triggered by a prolonged period of low temperature (Amasino, 2010), it would be intriguing to see whether the down-regulation of *PpDAM5* and *PpDAM6* is mediated by the same mechanisms.

Various chemicals and culture methods have been tested for their dormancy-breaking effects in several fruit tree species (Chang and Lin, 1989; Nee, 1991; Bound and Jones, 2004). One of the best known dormancy-breaking chemicals is cyanamide (hydrogen cyanamide), which has been used for dormancy studies and has been incorporated into cultural practices (Or *et al.*, 2000, 2002; Walton *et al.*, 2009). Application of cyanamide at the end of the endodormant or endo–ecodormant stage in late winter/early spring is known to induce synchronized bud burst in grapevines. However, cyanamide is ineffective in the middle of an endodormant stage. Although the effects of cyanamide on peach dormancy have been poorly studied, probably because of its potential toxicity to peach buds, these application methods successfully enhanced Shimizu Hakuto peach bud burst with simultaneous down-regulation of *PpDAM5* and *PpDAM6*. This result strongly supported the idea that *PpDAM5* and *PpDAM6* could act as a dose-dependent inhibitor to peach bud break not only at the endodormant stage but also at the ecodormant stage.

Endodormant buds differ from other dormant buds (paradormant and ecodormant buds), in their inability to resume growth under favourable conditions. This suggests that internal factors inhibiting bud burst and subsequent shoot growth should be present in endodormant buds

(Faust *et al.*, 1997). Although *PpDAM5* and *PpDAM6* could be good candidates for this function in peach endodormant buds, the present study indicates that they act not only in endodormant buds but also in ecodormant buds. Therefore, *PpDAM5* and *PpDAM6* are different from the previously presumed internal inhibitors that are assumed to be specifically present in endodormant buds. Transgenic approaches are currently underway to completely elucidate the functions of *PpDAM*s.

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References

Amasino R. 2010. Seasonal and developmental timing of flowering. *The Plant Journal* **61,** 1001–1013.

Bielenberg WY, Li Z, Zhebentyayeva T, Fan S, Reighard GL, Scorza R, Abbott AG. 2008. Sequencing and annotation of the evergrowing locus in peach [*Prunus persica* (L.) Batsch] reveals a cluster of six MADS-box transcription factors as candidate genes for regulation of terminal bud formation. *Tree Genetics and Genomes* **4,** 495–507.

Böhlenius H, Huang T, Charbonnel-Campaa L, Brunner AM, Jansson S, Strauss SH, Nilsson O. 2006. CO/FT regulatory module controls timing of flowering and seasonal growth cessation in trees. *Science* **312,** 1040–1043.

Bound SA, Jones KM. 2004. Hydrogen cyanamide impacts on flowering, crop load, and fruit quality of red 'Fuji' apple (*Malus domestica*). *New Zealand Journal of Crop and Horticultural Science* **32,** 227–234.

Chang HS, Lin CH. 1989. The application of cyanamide for termination of dormancy in Syhjou persimmon with *in vitro* cutting test and field-test. *Gartenbauwissenschaft* **54,** 30–33.

Crabbe J, Barnola P. 1996. A new conceptual approach to bud dormancy in woody plants. In: Lang GA, ed. *Plant dormancy: physiology, biochemistry and molecular biology*. CAB International, Wallingford, UK, 83–113.

Fan S, Bielenberg DG, Zhebentyayeva TN, Reighard GL, Okie WR, Holland D, Abbott AG. 2010. Mapping quantitative trait loci associated with chilling requirement, heat requirement and bloom date in peach (*Prunus persica*). *New Phytologist* **185,** 917–930.

Faust M, Erez A, Rowland LJ, Wang SY, Norman HA. 1997. Bud dormancy in perennial fruit trees: physiological basis for dormancy induction, maintenance, and release. *HortScience* **32,** 623–629.

Horvath DP. 2009. Common mechanisms regulate flowering and dormancy. *Plant Science* **177,** 523–531.

Horvath DP, Anderson JV, Chao WS, Foley ME. 2003. Knowing when to grow: signals regulating bud dormancy. *Trends in Plant Science* **8,** 534–540.

Horvath DP, Chao WS, Suttle JC, Thimmapuram J, Anderson JV. 2008. Transcriptome analysis identifies novel responses and potential regulatory genes involved in seasonal dormancy transitions of leafy spurge (*Euphorbia esula* L.). *BMC Genomics* **9,** 536.

Horvath DP, Sung S, Kim D, Chao W, Anderson J. 2010. Characterization, expression and function of *DORMANCY ASSOCIATED MADS-BOX* genes from leafy spurge. *Plant Molecular Biology* **73,** 169–179.

Jimenez S, Reighard GL, Bielenberg DG. 2010. Gene expression of *DAM5* and *DAM6* is suppressed by chilling temperatures and inversely correlated with bud break rate. *Plant Molecular Biology* **73,** 157–167.

Lang GA. 1987. Dormancy - a new universal terminology. *HortScience* **22,** 817–820.

Li Z, Reighard GL, Abbott AG, Bielenberg DG. 2009. Dormancy-associated MADS genes from the *EVG* locus of peach [*Prunus persica* (L.) Batsch] have distinct seasonal and photoperiodic expression patterns. *Journal of Experimental Botany* **60,** 3521–3530.

Mohamed R, Wang CT, Ma C, *et al*. 2010. *Populus CEN/TFL1*regulates first onset of flowering, axillary meristem identity and dormancy release in *Populus. The Plant Journal* **62,** 674–688.

Nee CC. 1991. Breaking dormancy in kiwifruit with chilling or chemical treatments. *New Zealand Journal of Crop and Horticultural Science* **19,** 419–421.

Or E, Vilozny I, Eyal Y, Ogrodovitch A. 2000. The transduction of the signal for grape bud dormancy breaking induced by hydrogen cyanamide may involve the SNF-like protein kinase GDBRPK. *Plant Molecular Biology* **43,** 483–494.

Or E, Vilozny I, Fennell A, Eyal Y, Ogrodvitch A. 2002. Dormancy in grape buds: isolation and characterization of catalase cDNA and analysis of its expression following chemical induction of bud dormancy release. *Plant Science* **162,** 121–130.

Rohde A, Bhalerao RP. 2007. Plant dormancy in the perennial context. *Trends in Plant Science* **12,** 217–223.

Rohde A, Prinsen E, De Rycke R, Engler G, Van Montagu M, Boerjan W. 2002. PtABI3 impinges on the growth and differentiation of embryonic leaves during bud set in poplar. *The Plant Cell* **14,** 1885–1901.

Sugiura T, Kuroda H, Sugiura H. 2007. Influence of the current state of global warming on fruit tree growth in Japan. *Horticultural Research (Japan)* **6,** 257–263 [In Japanese with English summary].

Walton EF, Wu RM, Richardson AC, *et al*. 2009. A rapid transcriptional activation is induced by the dormancy-breaking chemical hydrogen cyanamide in kiwifruit (*Actinidia deliciosa*) buds. *Journal of Experimental Botany* **60,** 3835–3848.

Yamane H, Kashiwa Y, Ooka T, Tao R, Yonemori K. 2008. Suppression subtractive hybridization and differential screening reveals endodormancy-associated expression of an *SVP/AGL24*-type MADS-box gene in lateral vegetative buds of Japanese apricot. *Journal of American Society for Horticultural Science* **133,** 708–716.