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Comparative Analyses of Dormancy-associated MADS-box Genes, *PpDAM5* and *PpDAM6*, in Low- and High-chill Peaches (*Prunus persica* L.)

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This study investigated the regulation of the seasonal expression of *PpDAM5* and *PpDAM6*, two of the six peach (*Prunus persica*) dormancy-associated MADS-box genes, in relation to the endodormancy and development of lateral vegetative and flower buds of low- and high-chill peach cultivars. *PpDAM5* and *PpDAM6* were originally found as homologs of *Arabidopsis SVP/AGL24* at the *EVERGROWING* (*EVG*) locus of peach and have been recently shown to be involved in lateral bud endodormancy. Seasonal expression analyses in this study indicated that *PpDAM5* and *PpDAM6* transcript levels in lateral vegetative buds of both low- and high-chill cultivars in the field negatively correlated with bud burst percentages determined under forcing conditions. Negative correlation was also found between their transcript levels and the flower organ enlargement rate. These results suggest that distinct seasonal expression patterns of *PpDAM5* and *PpDAM6* are correlated with a distinct chilling requirement for bud break and flowering of low- and high-chill cultivars. Characterization of the genomic structure of *PpDAM5* and *PpDAM6* revealed the presence of large insertions in the first introns of both *PpDAM5* and *PpDAM6* in low-chill peach. Alteration of the genomic structure is discussed with respect to the low-chill character.

Key Words: bud dormancy, chilling requirement, marker-assisted selection, *Prunus*.

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 mainly because of apical dominance. By mid- to late fall, with the exact timing depending on the species, the inhibitory control of bud growth shifts to the bud itself; these buds are referred to as being endodormant (Lang, 1987). Plants do not emerge from this type of dormancy by removal of terminal buds or defoliation. Endodormant buds require a certain amount of chilling accumulation for transition to an ecodormant state from which buds can resume growth in favorable environments (Crabbe and Barnola, 1996; Faust et al., 1997; Horvath et al., 2003; Lang, 1987). Although a certain chilling exposure period leads the endodormant buds to an ecodormant state, the length and intensity of this chilling requirement (CR) vary with species and even among the genotypes of a given species; however, the molecular basis of the difference in CRs is yet to be clarified.

The peach *evergrowing* (*evg*) mutant has been used as a genetic tool to investigate growth cessation and terminal bud formation (Rodriguez et al., 1994). A genomic deletion was recently detected with the loss of expression of six tandem-arrayed MADS-box genes (*PpDAM1-6* for *Prunus persica DORMANCY-ASSOCIATED MADS-BOX*) at the *EVG* locus, indicating that *PpDAM*s could be major candidates for controlling growth cessation (Bielenberg et al., 2004, 2008). Later, Li et al. (2009) demonstrated that *PpDAM1*, *PpDAM2*, and *PpDAM4* are more closely associated with terminal bud formation than the other three *PpDAM*s, although all six *PpDAM*s showed distinct changes in seasonal expression in shoot apex of peach. Furthermore, Jimenez et al. (2010) reported that the expression levels of *PpDAM5* and *PpDAM6* are inversely correlated with the bud break rate of peach terminal buds. Taken together, these results could indicate that *PpDAM*s are associated not only with endodormancy induction but also with

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endodormancy release. *DAM*-like genes reported in Japanese apricot (*P. mume* Sieb. et Zucc.) (Yamane et al., 2008) and raspberry (Mazzitelli et al., 2007) are also associated with lateral bud endodormancy release. The recent finding of the co-localization of a strong QTL for CR and blooming time within the region of the *EVG* locus where *PpDAM5* and *PpDAM6* reside (Fan et al., 2010) further supports the idea that *PpDAM5* and *PpDAM6* could control CR of peach bud.

Synchronized and uniform flowering as well as vegetative bud burst are essential for stable production of temperate fruit; however, irregular and non-uniform flowering as well as vegetative bud burst are often observed in both protected and normal orchard cultivations in southern areas of Japan, probably because of the irregular release of lateral bud endodormancy caused by recent global warming (Sugiura et al., 2007, 2010). This led us to investigate the genetic factors controlling CR and endodormancy regulation, with a final goal of artificially controlling endodormancy in temperate fruit trees through the development of novel cultural practices and/or marker-assisted selection (MAS), rapid breeding procedures.

In this study, we first compared seasonal expression patterns of *PpDAM5* and *PpDAM6* in peach cultivars with different CRs for bud break to confirm their involvement in endodormancy. We then performed an experiment to investigate the possible involvement of *PpDAM5* and *PpDAM6* in the phenological regulation of flower bud development. Finally, we compared *PpDAM5* and *PpDAM6* gene structures in low- and high-chill peaches to determine whether any genotype-dependent alterations can be utilized for MAS breeding.

Materials and Methods

Peach cultivars used in this study

A peach cultivar 'Akatsuki' and a seedling of 'Okinawa' [hereafter 'Okinawa (Tsukuba)'] were used in this study. 'Akatsuki' grafted on 'Ohatsumomo' rootstock was obtained from a commercial nursery and planted at the experimental farm of Kyoto University in 2002. Cuttings of 'Okinawa (Tsukuba)' were kindly provided by Dr. Kataoka at Kagawa University in 2002 and also planted at the experimental farm of Kyoto University in 2005. 'Okinawa (Tsukuba)' is a rootstock cultivar with a low CR for bud break (low-chill). CR for bud break of 'Akatsuki' and 'Okinawa (Tsukuba)' is approximately 1,000 h and 100 h, respectively. As shown in Figure 1, the endodormancy status of pot-grown trees of low-chill 'Okinawa (Tsukuba)' and high-chill 'Akatsuki' was distinct in November in our environmental conditions, in which the former was released from endodormancy while the latter was still in the endodormant state. Adult trees of high-chill 'Shimizu Hakuto' with 1000-h CR were also used for genomic DNA blot analysis.

Fig. 1. Different endodormancy status between two peach cultivars. Endodormancy of 'Okinawa (Tsukuba)' (left) was released in mid-November, while 'Akatsuki' (right) was still endodormant. Each plant was transferred to under forcing conditions (greenhouse controlled at 25 ± 3°C under natural daylength) in mid-November (CH: 64 h). Only 'Okinawa (Tsukuba)' (left) flowered and resumed vegetative growth.

Endodormancy status and flower bud development

The timing of endodormancy release of 'Akatsuki' and 'Okinawa (Tsukuba)' was investigated. Three branches of approximately 50 cm in length of the current season's growth were cut in the middle of each month from October 2007 to March 2008 in 'Akatsuki' and from October 2007 to February 2008 in 'Okinawa (Tsukuba)'. The basal parts of these branches were placed in water containing Misakifarm™ (Otsuka Kagaku, Tokushima, Japan), a commercial preparation containing nutrients and fungicides for the prolonged life of flower cuttings, and maintained at 22–28°C under natural daylength. The water was replaced every 2 weeks. Vegetative and flower buds showing leaves or white petals, respectively, were considered to have burst. Bud burst was recorded for one month after treatment. Field air temperature was recorded at 15-min intervals and chilling accumulation was calculated according to the "chill hours (CH)" (<7.2°C).

Flower buds of 'Akatsuki' and 'Okinawa (Tsukuba)' were collected and observed monthly from November to February. They were detached from the branches and fixed in FAA (3.7% formaldehyde, 5% acetic acid, and 50% ethanol). After the scales were removed from the fixed buds, samples were dehydrated in an ethanol series, soaked in isoamyl acetate, and critical-point dried in liquid CO_2. The dried samples were mounted on stubs and sputter-coated with gold (Hitachi, Tokyo, Japan).

Specimens were observed using a scanning electron microscope (VE-8800 real surface view, Keyence, Tokyo, Japan).

PpDAM5 and PpDAM6 expression analysis

Vegetative and flower buds of 'Akatsuki' and 'Okinawa (Tsukuba)' were collected monthly and immediately frozen in liquid nitrogen and stored at −80°C until used. Total RNA was isolated from the collected tissues, as described by Yamane et al. (2008). After DNaseI treatment (Takara Bio Inc., Ohtsu, Japan), 1 μg total RNA was used for cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, USA). Gene-specific TaqMan® probes and primers were designed and synthesized for the specific detection of *PpDAM5* and *PpDAM6*, based on the genomic DNA sequences of *PpDAM5* or *PpDAM6* in the NCBI nucleotide database. The primer pair DAM5F and DAM5R was used with the TaqMan® probe DAM5-T to detect transcripts of *PpDAM5*, while PpDAM6F and PpDAM6R were used with the DAM6-T probe to detect *PpDAM6* (Table 1). Real-time PCR analysis with the TaqMan® probe was performed using a Lightcycler 480 (Roche Diagnostics GmbH, Mannheim, Germany) and probe master mix (Roche Diagnostics GmbH). The reaction mixture consisted 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 total RNA in a 20-μL reaction volume. As a reference, *ACTIN* transcript accumulation was monitored by real-time PCR using SYBR green master mix (Roche Diagnostics GmbH). The primer pair qp-ActF and qp-ActR was used for *ACTIN*-specific real-time PCR (Table 1). PCR was performed using a program of 45 cycles at 95°C for 10 s, 56°C for 20 s, and 72°C for 1 s, with initial heating at 95°C for 5 min. For *ACTIN* gene-specific real-time PCR, dissociation curve analysis was performed to confirm that the fluorescence was only derived from the gene-specific amplification. Three replicates were performed for each gene. The quantities of the *PpDAM5* and *PpDAM6* transcripts in each sample were normalized using the *ACTIN* transcripts.

Genomic DNA blot analysis

Genomic DNA was isolated from young leaves of 'Akatsuki', 'Shimizu Hakuto', and 'Okinawa (Tsukuba)' using the Nucleon PhytoPure plant and fungal DNA extraction kit (GE Healthcare UK Ltd., Buckinghamshire, UK) with some modifications, as described previously (Yamane et al., 2008). In brief, 1.5 g frozen leaves were ground to a powder with a Multi-beads shocker (Yasui Kikai, Osaka, Japan), suspended in washing buffer [10 mM Tris-HCl (pH 9.0), 0.5 M sucrose, 10 mM EDTA (pH 8.0), and 80 mM KCl], and mixed thoroughly. The mixture was centrifuged (6500 × *g* at 4°C for 15 min) to collect the pellet. The pellet was resuspended in washing buffer and centrifuged again. Genomic DNA was isolated from the pellet using the Nucleon PhytoPure plant and fungal DNA extraction kit according to the manufacturer's instructions, and further purified by phenol/chloroform extraction.

Genomic DNA (5 μg) was digested with *Eco*RI, run on a 0.8% (w/v) agarose gel, and transferred to a Biodyne PLUS nylon filter (Pall, Port Washington, USA). The membrane was probed with a DIG-labeled *PmDAM6* probe (Yamane et al., 2008) containing nucleotide sequences corresponding to the MADS domain of *PmDAM6* in order to detect all *PpDAM*s. After hybridization at 60°C, the membrane was washed under low stringency conditions, as described previously (Watari et al., 2007). The hybridized signals were visualized using LAS-3000 mini (Fujifilm, Tokyo, Japan).

Genomic library construction, screening, and sequencing

A fosmid library was constructed from genomic DNA of the peach cultivar 'Okinawa (Tsukuba)' using the CopyControl fosmid library production kit (Epicenter, Madison, USA). The library was screened with a DIG-dUTP-labeled probe synthesized from a *PmDAM6* partial fragment corresponding to the K-box region (Yamane et al., 2008). Positive clones were subsequently used as templates to confirm the presence of *PpDAM5* and *PpDAM6* by gene-specific PCR. Nucleotide sequences of the selected fosmid clones were determined by the partial digestion and shotgun sequencing technique using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Port City, USA) and an ABI 3730xl capillary sequencer (Applied Biosystems).

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

Primer or Taqman® probe	Sequence (fluorescent probe)	Tm (°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)TGAAGATGACTGCTCCGATGTCACTTTATC-(TAMRA)-3'	69.0
qp-ActF	5'-CAACTGGTATTGTGCTGGATTCTG-3'	59.5
qp-ActR	5'-CAGCAAGGTCAAGACGAAGGAT-3'	59.4

Results

Phenological changes in lateral bud endodormancy status and flower organ development

Seasonal changes in the percentage bud break of vegetative and flower buds of high-chill 'Akatsuki' and low-chill 'Okinawa (Tsukuba)' under forcing conditions are shown in Table 2. No 'Akatsuki' bud burst was recorded in October (CH 2 h), November (CH 64 h), or December (CH 414 h). In January (CH 1,053 h), 16.2% and 27.8% of the 'Akatsuki' flower and vegetative buds, respectively, opened. More than 30% opened in February (CH 1,831 h). As with 'Akatsuki', none of the 'Okinawa (Tsukuba)' buds opened in October; however, in November, 6.7% of both 'Okinawa (Tsukuba)' vegetative and flower buds opened. Their bud burst percentages in January and February were higher than those of 'Akatsuki'.

Phenological changes in flower bud development of high-chill 'Akatsuki' and low-chill 'Okinawa (Tsukuba)' were observed using SEM. The formation of flower organs, including sepals, petals, stamens, and pistils, had completed in both 'Akatsuki' and 'Okinawa (Tsukuba)' in November (Fig. 2). After December, these organs enlarged and elongated inside the flower buds faster in 'Okinawa (Tsukuba)' than in 'Akatsuki' (Fig. 2). The flower buds finally opened under field conditions at the end of March and the beginning of April in 'Okinawa (Tsukuba)' and 'Akatsuki', respectively (data not shown).

Seasonal expression patterns of PpDAM5 and PpDAM6

Figure 3 shows the seasonal expression changes of *PmDAM* genes in vegetative and flower buds of two peach cultivars as determined by real-time reverse transcription (RT)-PCR using gene-specific TaqMan® probes and primers. Sequencing of genomic fragments and cDNAs cloned from 'Akatsuki' and 'Okinawa (Tsukuba)' revealed that the designed probe and primer sequences were conserved in these cultivars (data not shown). The highest transcript levels of *PpDAM5* and *PpDAM6* were observed in October and November in low-chill 'Okinawa (Tsukuba)' and high-chill 'Akatsuki', respectively. Although *PpDAM5* and *PpDAM6* transcript levels steadily decreased toward spring, regardless of bud types or cultivar, they were significantly lower in 'Okinawa (Tsukuba)' than in 'Akatsuki' in all months tested (Fig. 3). The transcript levels of 'Okinawa (Tsukuba)' in vegetative buds in December were almost the same as those of 'Akatsuki' in February. Similarly, in flower buds, the transcript levels of 'Okinawa (Tsukuba)' in December were almost the same as those of 'Akatsuki' in March (Fig. 3).

Structure of PpDAM5 and PpDAM6 in 'Okinawa (Tsukuba)'

Genomic DNA blot analysis detected several *DAM*-specific hybridization signals on the blot (Fig. 4). 'Okinawa (Tsukuba)' yielded hybridization patterns different from the high-chill cultivars. Six hybridization signals observed in 'Akatsuki' and 'Shimizu Hakuto' corresponded to the expected hybridization signals estimated from the nucleotide sequence information of the peach genomic region containing *PpDAM1* to *PpDAM6* (Bielenberg et al., 2008) (NCBI/DDBJ accession number: DQ863257). Although 'Okinawa (Tsukuba)' yielded five bands corresponding to *PpDAM1* to *PpDAM5*, the band corresponding to *PpDAM6* was longer. We designated *PpDAM6* found in 'Okinawa (Tsukuba)' as *PpDAM6-a1*. Based on the hybridization pattern, 'Okinawa (Tsukuba)' appeared to be homozygous for *PpDAM6-a1*.

Six fosmid clones putatively containing *PpDAM6-a1* were obtained by screening the 'Okinawa (Tsukuba)' genomic library. One of the clones appeared to contain the genomic region (about 40 kbp) containing *PpDAM4*, *PpDAM5*, and *PpDAM6-a1*. The DNA sequence of this clone was compared with the genomic region that contained *PpDAM*s of 'Nemaguard' [Bielenberg et al. (2008) described this cultivar as a wild-type dormant cultivar] (NCBI/DDBJ accession number: DQ863257). Although several indels and SNPs were found in the

Table 2. Seasonal endodormancy status of lateral vegetative buds and flower buds of two peach cultivars.

Cultivar	Month (bud burst %)					
	Oct. (CH: 2 h)	Nov. (64 h)	Dec. (414 h)	Jan. (1053 h)	Feb. (1831 h)	Mar. (2143 h)
	Vegetative buds					
'Akatsuki'	0	0	0	27.8	34.4	81.7
'Okinawa (Tsukuba)'	0	6.7	38.3	48.6	100	n.t.[z]
	Flower buds					
'Akatsuki'	0	0	0	16.2	52.3	100
'Okinawa (Tsukuba)'	0	6.7	12.5	94.8	93.3	n.t.[z]

Three branches of approximately 50 cm in length of the current season's growth were cut in the middle of each month. Vegetative and flower buds showing leaves or white petals, respectively, were considered to have burst. Bud burst was observed for one month after treatment.

[z] n.t. is "not tested".

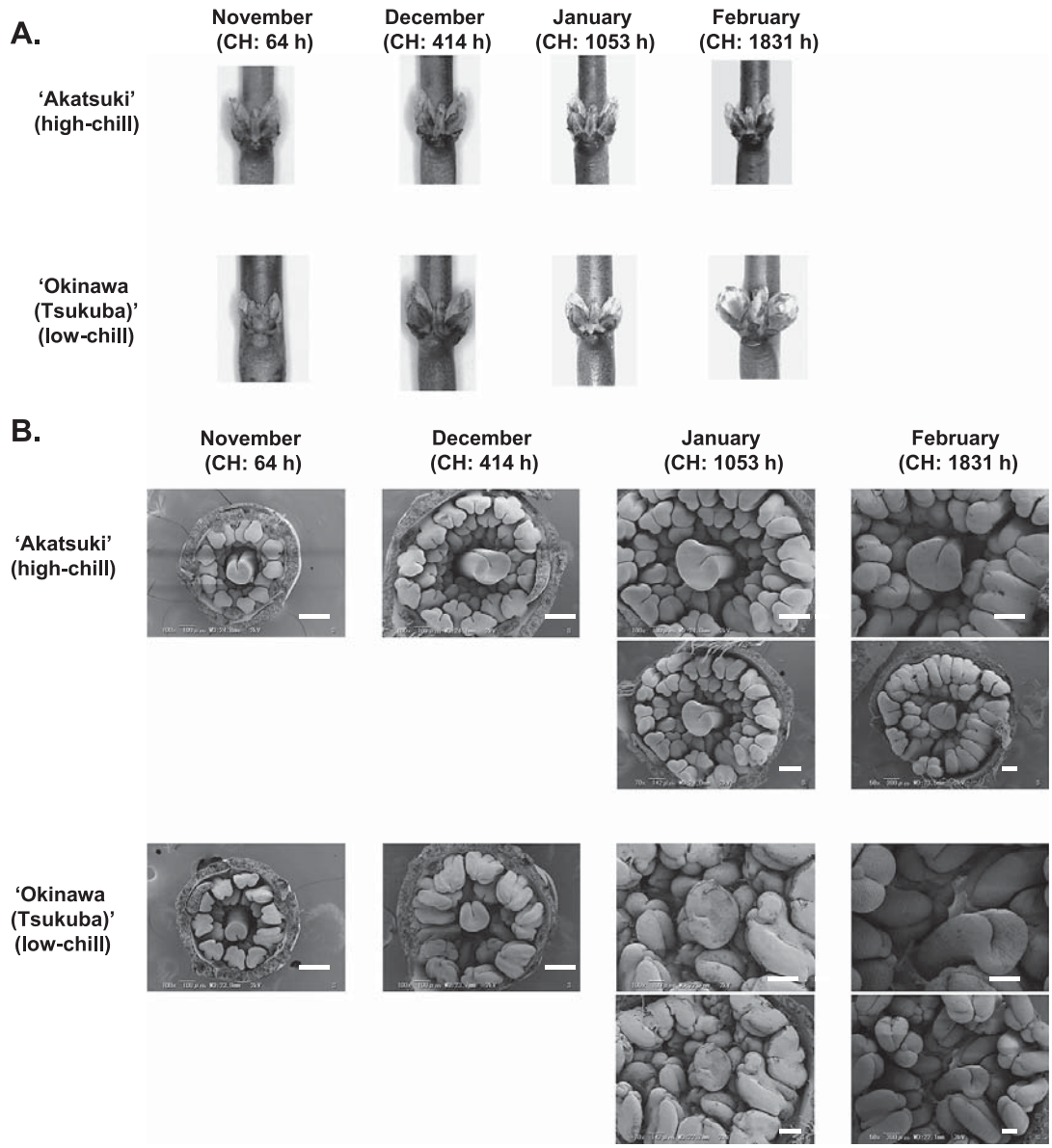

Fig. 2. Development of flower buds during dormancy in peach cultivars. Flower buds of 'Akatsuki' (high-chill) and 'Okinawa (Tsukuba)' (low-chill) are shown. Approximately ten buds are observed in the middle of each month and pictures taken from the average sample at sampling month are shown. The hours of chilling accumulation of each sampling month are indicated. A. Enlarged bud pictures of both cultivars. B. SEM pictures of internal flower buds in each sampling month are shown. Note that the same magnification is used to show pictures of both cultivars in the upper rows. In the lower rows, although the same magnification is used to show pictures of both cultivars in the same month, different magnification is used for January and February. Bars = 200 μm.

'Okinawa (Tsukuba)' genomic region that contained *PpDAM6-a1*, the most prominent differences were found in the first introns of *PpDAM5* and *PpDAM6-a1*. Namely, 598 bp and 2,604 bp of inserted fragments were found in the first introns of *PpDAM5* and *PpDAM6-a1*, respectively (Fig. 5). Since there appeared to be a difference in *PpDAM5* of 'Okinawa (Tsukuba)' and 'Nemaguard', we designated the 'Okinawa (Tsukuba)' *PpDAM5* linked to *PpDAM6-a1* as *PpDAM5-a1*.

Discussion

This study demonstrated negative correlations between the level of *PpDAM5* and *PpDAM6* expression and that of endodormancy of lateral buds. Based on the experimental results obtained with Japanese apricot (Yamane et al., 2008), we proposed that *PmDAM6*, an ortholog of *PpDAM6*, could be one of the internal growth inhibitors that are localized in endodormant buds to prevent buds from resuming growth. The results obtained in this study not only supported our hypothesis that *Prunus DAM*s could encode dose-dependent negative regulators against bud break but also confirmed the previous indications that *PpDAM*s were associated with peach terminal bud endodormancy (Jimenez et al., 2010). Furthermore, this study suggested that threshold transcript levels of *PpDAM5* and *PpDAM6* for inhibiting

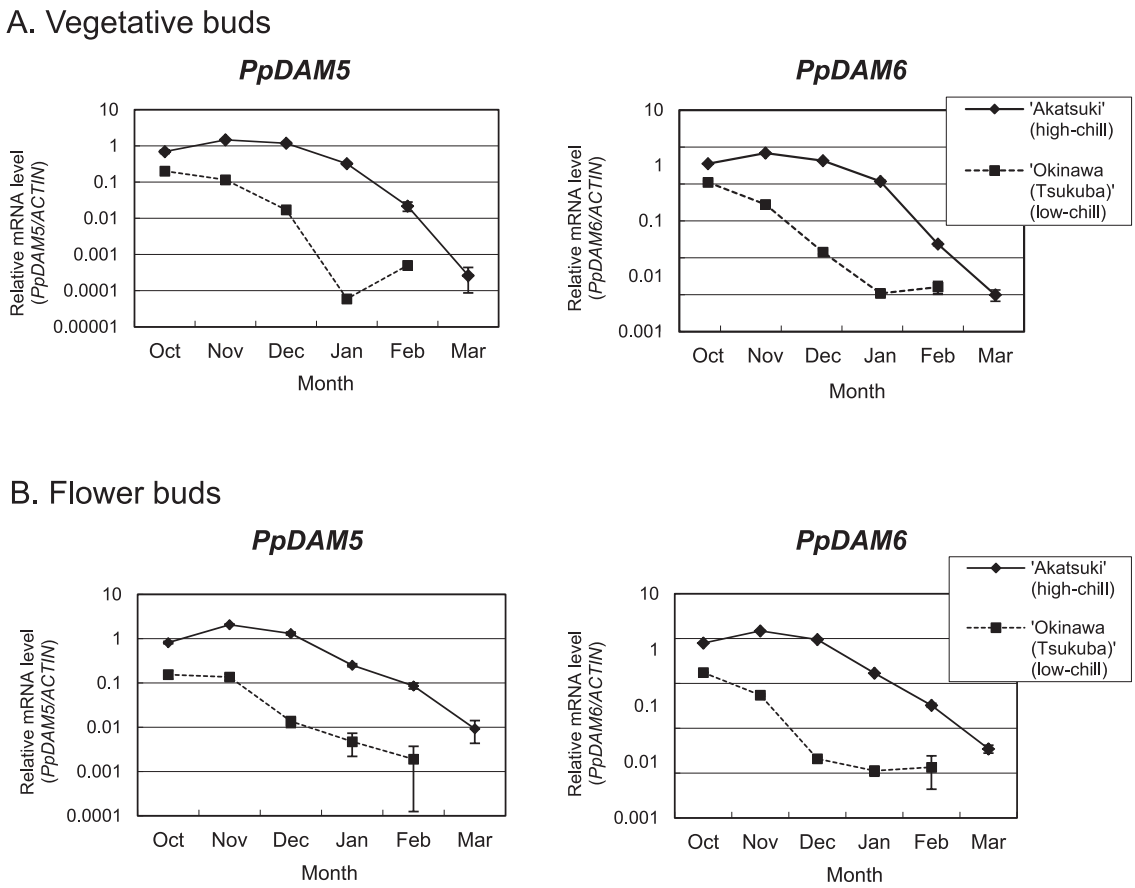

Fig. 3. Seasonal expression changes of *PpDAM5* and *PpDAM6* in lateral vegetative (A) and flower (B) buds of two peach cultivars differing in chilling requirement, high-chill 'Akatsuki' and low-chill 'Okinawa (Tsukuba)'. The means of three replicates are shown with error bar (SD).

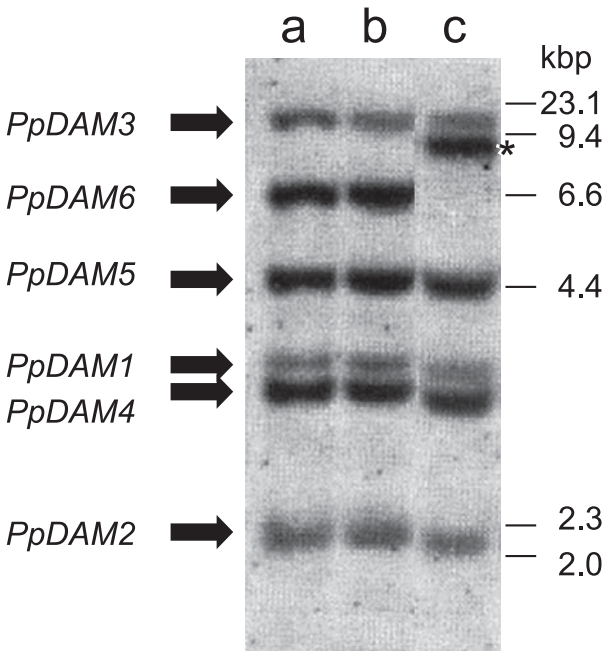

Fig. 4. Genomic DNA blot analysis of peach cultivars. Genomic DNA was digested with *Eco*RI and probed with partial fragment of *PmDAM6*. Lanes, (a) 'Shimizu Hakuto' (high-chill), (b) 'Akatsuki' (high-chill), (c) 'Okinawa (Tsukuba)' (low-chill). The band corresponding to *PpDAM6-a1* allele is indicated by *asterisk* (see text).

bud burst differ depending on the cultivars because it appeared that higher *PpDAM5* and *PpDAM6* transcription levels were required to inhibit bud burst of 'Akatsuki' than 'Okinawa (Tsukuba)'.

A typical peach leaf axil has three separate axillary buds: a single vegetative and two flower buds. A peach vegetative bud contains an undifferentiated shoot apical meristem that generates next season's annual shoot growth, whereas a flower bud contains a single flower meristem that develops into a solitary flower. We investigated whether flower meristem initiation and flower organ differentiation would progress during the endodormancy period. Phenological changes in flower bud development were observed from the deep endodormant stage until the ecodormant stage, using SEM both in high-chill 'Akatsuki' and low-chill 'Okinawa (Tsukuba)'. Although flower organ differentiation was completed in 'Akatsuki' buds by November, the following flower organ enlargement was arrested temporarily from November to January when high transcript levels of *PpDAM5* and *PpDAM6* were found. In 'Okinawa (Tsukuba)' in contrast, flower organs steadily enlarged from November to February. Low transcript levels of *PpDAM5* and *PpDAM6* in 'Okinawa (Tsukuba)' may have been insufficient to inhibit flower organ enlargement. The finding that the endodormancy

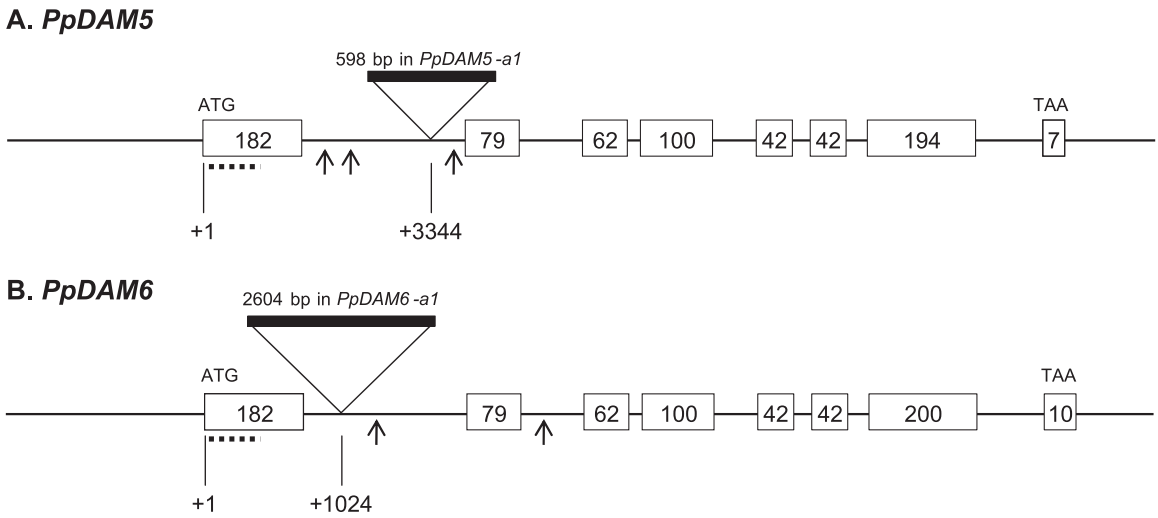

Fig. 5. Schematic representation of genomic structure of *PpDAM5-a1* and *PpDAM6-a1* in 'Okinawa (Tsukuba)'. Relatively large fragments were inserted in the first introns of *PpDAM5* and *PpDAM6*. Exons and introns are designated as boxes and lines, respectively. 'A' of the start codon of each gene is positioned at +1. Probe regions used for genomic DNA blot analysis (Fig. 4) are designated by horizontal dotted lines. *Eco*RI cut sites are indicated by *arrow*s.

break coincided well with the timing of the initiation of flower organ enlargement both in 'Okinawa (Tsukuba)' and 'Akatsuki' could indicate that peach flower bud endodormancy is defined by the temporal arrest of flower organ enlargement. Again, the negative correlation of *PpDAM5* and *PpDAM6* transcript levels with flower organ enlargement suggested that these gene products could act as dose-dependent growth inhibitors in buds.

In *Arabidopsis*, although many studies suggest the involvement of several homeotic genes, such as class-A, B, C, E MADS-box genes, in flower organ differentiation (Liu et al., 2009), fewer studies have focused on the molecular basis of flower organ enlargement, which is possibly modulated by plant hormone synthesis and response. This study suggests that flower bud dormancy in peach could be controlled by the arrest of flower organ enlargement rather than flower organ differentiation. Further studies to elucidate flower organ enlargement in plants could lead to a more comprehensive understanding of flower bud dormancy not only in peach but also in other temperate fruit tree species.

Accumulation of *PpDAM5* and *PpDAM6* transcripts in buds of low-chill 'Okinawa (Tsukuba)' was lower than that in high-chill 'Akatsuki' at all stages investigated. Furthermore, *PpDAM5* and *PpDAM6* transcript levels decreased earlier in 'Okinawa (Tsukuba)' than in 'Akatsuki'. We assumed that these different expression patterns in *PpDAM5* and *PpDAM6* could confer different CRs on 'Okinawa (Tsukuba)' and 'Akatsuki'. As it has been reported that one QTL for CR overlapped the *EVG* locus where *PpDAM5* and *PpDAM6* are located (Fan et al., 2010), we suspected that the different gene structure that was demonstrated by genomic DNA blot analysis may contribute to the different expression patterns of *PpDAM5* and *PpDAM6* observed between low- and high-chill cultivars. Sequencing of the genomic region where *PpDAM5* and *PpDAM6* are located revealed the presence of relatively large insertions in the first intron of both *PpDAM5-a1* and *PpDAM6-a1* in low-chill 'Okinawa (Tsukuba)'. It is known that the insertion of transposons into the intron could affect a reduced level of mRNA by putatively perturbing normal gene expression in a number of different ways, particularly by disrupting or reducing the efficiency of normal splicing events or by affecting transcription (Weil and Wessler, 1990). In fact, it was reported that the transposon insertion in one accession in *Arabidopsis* could lead to reduced transcription of *FLOWERING LOCUS C* (Gazzani et al., 2003). Because large fragments found in both *PpDAM5-a1* and *PpDAM6-a1* in low-chill 'Okinawa (Tsukuba)' did not resemble any known transposons, whether these inserted fragments were responsible for the low transcription level of *PpDAM5-a1* and *PpDAM6-a1* is still unclear. We are currently performing linkage analyses using F_2 segregating populations to determine whether this gene modification is responsible for the different expression levels in *PpDAM5* and *PpDAM6* and whether it is utilized for MAS of low-chill peaches. Recent reports showing the adverse effects of global warming on temperate fruit and nut tree cultivation (Honjo, 2007; Luedeling et al., 2009; Sugiura et al., 2007, 2010) highlight the urgent need for new cultivars that can adapt to climate changes (Lyrene, 2005; Yamaguchi et al., 2007).

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