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Molecular Cloning of Dormancy-associated MADS-box Gene Homologs and Their Characterization during Seasonal Endodormancy Transitional Phases of Japanese Pear

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Abstract. To understand the role of the MIKC-type *dormancy-associated MADS-box* (*DAM*) genes in the regulation of endodormancy in japanese pear (*Pyrus pyrifolia*), we isolated two *DAM* genes from 'Kosui' and characterized their expression throughout the seasonal endodormancy phases in 'Kosui', as well as in TP-85–119 taiwanese pear (*P. pyrifolia*), which is a less dormant type. Several copies of the corresponding *DAM* genes are present in the *P. pyrifolia* genome. Rapid amplification of cDNA ends enabled the isolation of two full-length cDNAs, designated as *PpMADS13–1* and *PpMADS13–2*, with complete open reading frames encoding 227 and 234 amino acids, respectively. Multialignment of the two 'Kosui' and the database DAM genes (based on the deduced amino acid sequences) showed that PpMADS13–1 and PpMADS13–2 were highly identical to the Rosaceae DAM genes and encoded the conserved domains characteristic of other MIKC-type MADS-box genes. The phylogenetic relationships showed that PpMADS13–1 and PpMADS13–2 were more closely related to the *Prunus* DAM, though they formed a unique subclade. The specific expression analysis of *PpMADS13–1* and *PpMADS13–2* by real-time polymerase chain reaction showed that both *DAM* genes are gradually down-regulated concomitant with endodormancy breaking. *PpMADS13–1* and *PpMADS13–2* showed similar fluctuations in expression patterns, although *PpMADS13–2* was more highly expressed relative to *PpMADS13–1*. The expression of *PpMADS13–1* and *PpMADS13–2* in the less dormant taiwanese pear, TP-85–119, was quite low (nearly zero level), which is consistent with a down-regulated pattern of expression of the *DAM* genes in japanese pear, peach (*Prunus persica*), and japanese apricot (*Prunus mume*). Differential genomic DNA methylation patterns detected in *PpMADS13–1* and *PpMADS13–2* were not concomitant with seasonal endodormancy transition phases, suggesting that DNA methylation in these loci under investigation may not be linked to endodormancy progression in 'Kosui'.

Perennial deciduous plants possess a distinct feature of being able to recurrently suspend and resume growth in response to seasonal and environmental conditions such as photoperiod and low temperatures. This inability to initiate growth from meristematic structure (and other organs and cells with the capacity for growth resumption), referred to as dormancy, is a complex process that is necessary for plant survival in an unfavorable environment (Rohde and Bhalerao, 2007).

Lateral buds formed in the early summer season enter a paradormant state due mainly to apical dominance. Toward autumn, the control of bud growth inhibition shifts to the bud itself and these dormant structures are referred to as being endodormant (Lang, 1987). The removal of terminal buds or defoliation does not restore the plants' capacity to emerge from endodormancy. A certain amount of chilling accumulation is required for the transition of endodormant buds to an ecodormant state from which buds are capable of growth resumption (dormancy breaking) under favorable environmental conditions

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(Faust et al., 1997; Rohde and Bhalerao, 2007; Yamane et al., 2008). Unlike endodormancy, which is genetically controlled, ecodormancy is provoked by limitations in environmental factors (mainly cold stress) that induce critical signals for bud growth inhibition (Crabbe and Barnola, 1996; Horvath et al., 2003; Yamane et al., 2008).

The commercial production of temperate fruit trees in warmer areas is hindered by a lack of sufficient chilling hours. This has often necessitated the use of chemicals or other physical means whose efficacy and level of phytotoxicity varies with the depth of endodormancy (Keilin et al., 2007). Furthermore, the chilling requirement for endodormancy breaking is potentially hampered by the recent environmental perturbations associated with global warming, leading to serious problems such as trees with barren branches or slow-growing shoots, and irregular or loss of flowering with its resultant effect on productivity (Arora et al., 2003; Sugiura et al., 2007). A search for internal factors or environmental cues controlling endodormancy is therefore of the utmost importance to overcome the problem associated with low chilling requirement for fruit production.

Mutants exhibiting continuous growth in winter have been identified by genetic studies in woody perennial species such as *Corylus avallana* (Thompson et al., 1985) and peach (Rodriguez et al., 1994). Of these, the *evergrowing* peach mutant (previously known as *evergreen*, USDA PI442380), a nondormant genotype identified from southern Mexico, is the best-described mutant (Rodriguez et al., 1994; Thompson et al., 1985; Werner and Okie, 1998). The *evergrowing* peach trees fails to respond to winter dormancy cues and maintains continuous growth of the terminal apices and a lack of leaf abscission during the prevailing short days and low temperatures until killed by freezing temperatures (Bielenberg et al., 2004; Wang et al., 2002). Inheritance studies have indicated that the nondormant *evg* trait is controlled by a single recessive nuclear locus (Rodriguez et al., 1994; Wang et al., 2002). Genetic and molecular approaches have led to the identification of putative genes regulating bud endodormancy, among which MIKC-type *MADS box* genes were identified as strong candidate genes for regulating endodormancy breaking and were designated as *dormancy-associated MADS-box* (*DAM*) genes (Bielenberg et al., 2008). It has been reported that the expression of *DAM* genes is lost in the *evergrowing* peach mutant and decreased toward endodormancy breaking in wild peach (Bielenberg et al., 2004, 2008; Jiménez et al., 2009). Recent studies have also shown that *DAM* genes are differentially expressed in response to seasonal dormancy transitions in other plant systems, including raspberry (*Rubus idaeus*), japanese apricot, leafy spurge (*Euphorbia esula*), and peach (Horvath et al., 2008; Li et al., 2009; Mazzitelli et al., 2007; Yamane et al., 2008).

Knowledge of regulation of endodormancy in japanese pear is of the utmost importance to provide the basis for manipulation of the genes controlling endodormancy through genetic and/or transgenic approaches toward a more stable and economic production of this important fruit tree. In this study, we report the molecular cloning of two *DAM* genes, *PpMADS13–1* and *PpMADS13–2*, in the *SVP/AGL24* gene family in japanese pear and we analyzed their expression patterns in the lateral leaf buds through seasonal dormancy transitions. In addition, DNA methylation has been reported to play an important regulatory role in floral differentiation of plants (Dennis and Peacock, 2007; Henderson and Dean, 2004). In particular, it was reported that vernalization and demethylation of DNA are associated with promotion of flowering in *Arabidopsis thaliana* (Finnegan et al., 2000; Genger et al., 2003). Therefore, we further investigated the role of epigenetic control in the *PpMADS13–1* and *PpMADS13–2* loci involving differential genomic DNA methylation through the seasonal dormancy transitional phases. This study suggested the involvement of *DAM* genes in endodormancy breaking through a down-regulated expression pattern concomitant with the seasonal endodormancy transition phases in the lateral leaf buds. Epigenetic control involving differential genomic DNA methylation of *PpMADS13–1* and *PpMADS13–2* is not associated with the seasonal endodormancy phases in japanese pear. To the best of our knowledge, this is the first report on the cloning and characterization of *DAM* genes in pear.

Materials and Methods

Plant materials. Samples were collected from 34-year-old trees of 'Kosui' japanese pear and 20-year-old trees of TP-85–119, a less-dormant taiwanese pear type (included to confirm the physiological role of the pear *DAM* genes) grown at the orchard of the National Institute of Fruit Tree Science, Tsukuba, Japan (lat. 36°N, long. 140°E). Lateral leaf buds collected at intervals throughout the seasonal dormancy transitional phases, from early Sept. 2008 to mid-Feb. 2009 [i.e., 2008 (9 Sept., 21 Oct., 12 Nov., 9 Dec., and 24 Dec.) and 2009 (8 Jan. and 12 Feb.)], were used in this study to investigate the expression of the DAM genes in 'Kosui'. The lateral leaf buds of TP-85–119 were collected from mid-November to mid-Dec. 2008 (i.e., 12 Nov. and 9 Dec. 2008). In this study, lateral leaf buds were used instead of floral buds to avoid the confounding effect of floral identity genes on the DAM genes as has been highlighted by Yamane et al. (2008). The dormancy status of the field-grown trees during each of these collection dates was estimated based on the evaluation of cut branches. For the evaluation of endodormancy status in the lateral leaf buds, five current elongating branches with lengths of about 60 cm and bearing predominantly leaf buds were taken at each collection date and placed in distilled water contained in 500-mL vials in an incubator kept at 25 ± 1.0 °C under cool white fluorescent light and a 16-h photoperiod for the forcing treatment. The distilled water in the vials was changed at 2- to 3-d intervals. The endodormancy status (as percentage budbreak or sprouting ratio) at each collection date, which is defined as the stage at which green tissue becomes visible under the bud scales, was recorded based on 5 to 9 buds per branch averaged over five branches scored at 21 d after incubation. The collected lateral leaf buds were immediately frozen in liquid nitrogen and stored at –80 °C until needed for RNA and/or DNA extraction.

Isolation of partial DAM gene fragment from 'Kosui'. Total RNA was isolated from 'Kosui' lateral vegetative buds collected toward the end of the 2008 growing season (21 Oct., which coincided with 0% sprouting ratio) using a hot borate extraction procedure (Wan and Wilkins, 1994). First-strand cDNA was synthesized from 1 μg of total RNA using a SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA) and was then used as template for homology-based amplification of the *DAM* gene fragments. The reverse transcriptase (RT)-polymerase chain reaction (PCR) reaction was performed with the forward (5′-GCAGCATCCCAACTCTTTCT-3′) and reverse (5′-ATGGGATTGCAAGGTACAGC-3′) primers

designed on the basis of *Ppdam6* (GenBank accession no. DQ863252) using the Primer 3 software (Rozen and Skaletsky, 2000) and used for the initial amplification. The RT-PCR was performed in a total volume of 50 μL containing 125 ng of cDNA, 500 μM dNTPs, 375 μM $MgSO_4$, 3.125 μM of each primer, 0.4 U of KOD Plus polymerase, and 0.1× KOD buffer (Toyobo, Osaka, Japan). Following a pre-PCR heating at 95 °C for 2 min, a cycling profile of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 4 min was repeated 35 times. A final extension for 10 min at 72 °C was performed. The 609-bp product was cloned into the pCR®-Blunt vector using a Zero Blunt® PCR Cloning Kit (Invitrogen, Foster City, CA), sequenced, and annotated to confirm its homology to *DAM* genes from other plant species (data not shown). This 609-bp fragment was designated as *pPpMADS13#2*.

Southern blot analysis to determine the copy number of the DAM genes in the pear genome. To estimate the copy number of the *DAM* genes in the japanese pear genome, as well as understand whether the DAM genes are present in the taiwanese pear genome, genomic DNA was extracted from fresh flower bud tissues of 'Kosui', 'Gold Nijisseiki' (*P. pyrifolia*), 'Hosui' (*P. pyrifolia*), and TP-85–119 according to a crude nuclear extraction method (Thomas et al., 1993). Genomic DNA (10 μg) was digested at 37 °C overnight with the restriction enzymes (*Eco*RI and *Hin*dIII). These enzymes were selected because they lacked cleavage sites in the probe region. The resulting restricted fragments from *Eco*RI and *Hin*dIII digests were size fractionated in a 0.8% (w/v) SeaKem® GTG® agarose gel (Cambrex BioSciences, Rockland, ME) using 0.8% TAE as an electrophoresis running buffer. The fractionated, digested DNA was blotted onto nylon membranes (Hybond-N; Amersham Biosciences, Piscataway, NJ). The 609-bp *pPpMADS13#2* was used to generate the hybridization probe using a DIG DNA Labeling Kit (Roche, Mannheim, Germany). Prehybridization (1 h) and hybridization (overnight) were done using a high SDS hybridization buffer containing 5× SSC, 50% formamide, 50 mM NaH_2PO_4+Na_2HPO_4 (pH 7.0), 2% blocking reagent (Roche), 0.1% N-lauroylsarcosine, and 7% SDS at 42 °C. After hybridization, the membranes were washed twice at 65 °C for 15 min at moderate stringency (1× SSC and 0.1% SDS). The detection was performed according to the manufacturer's protocol using a DIG-CSPD system (Roche), and the membranes were exposed to X-ray film (Fuji Film, Tokyo).

Isolation of the full-length PpMADS13–1 and PpMADS13–2 clones from 'Kosui'. To isolate the full-length cDNAs of the pear *DAM* genes, 5′- and 3′-rapid amplification of cDNA ends (RACE) were performed. A 1-μg aliquot of total RNA from 'Kosui' japanese pear was used to prepare 5′-and 3′-RACE-Ready cDNAs with a SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions. The 5′- and 3′-RACE were carried out with 5′- and 3′-specific primers (first 5′-RACE MADS13: 5′-CCTCGGGC AGATAACATCACCATCCT-3′, second 5′-RACE MADS13: 5′-ACCTTGCAATCCCATCCTTGGTACTGG-3′, and 3′-RACE MADS13: 5′-ATGGTGGAGAAGAAGGCGTGACAT CTG-3′) designed based on the initial 609-bp *pPpMADS13#2* sequence and the preceding RACE PCR (for the second 5′-RACE). The cDNA ends were ligated into pCR®2.1 (Invitrogen) and sequenced. The resultant sequences were aligned to obtain a full-length cDNA sequence. Based on the full-length cDNA sequence information, forward (5′-GCAGCATCCCAACTCTT TCT-3′) and reverse (5′-CACAGAAATGCCTTCCGATT-3′) primers were designed to clone the full-length *DAM* genes by RT-PCR using the amplification conditions as described for isolation of the partial fragment. The full-length genes were designated as *PpMADS13–1* and *PpMADS13–2*, and have been deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers AB504716 and AB504717, respectively.

Analysis of cDNA sequences and construction of phylogenetic tree. The full-length *PpMADS13–1* and *PpMADS13–2* cDNA sequences were used to search homologous sequences via tBLASTx in National Center for Biotechnology Information (NCBI). Multiple alignments of the predicted full-length amino acid sequences were performed between 'Kosui' *DAM* genes and those of other plant species using ClustalW (Thompson et al., 1994). A phylogenetic relationship tree was then constructed by the neighbor-joining (NJ) method. The NJ method was chosen because it is known to be quite efficient in obtaining reliable trees from large data sets (Zhang and Nei, 1996). Typical motifs of the *DAM* genes that are conserved among 'Kosui' and other plant species were analyzed via ScanProsite (de Castro et al., 2006).

Real-time quantitative RT-PCR. Total RNA was isolated from the lateral leaf bud samples of 'Kosui' and the less dormant taiwanese pear, TP-85–119, was collected as detailed above using a modified hot borate extraction method (Wan and Wilkins, 1994). The synthesis of the first-strand cDNA was made using the SuperScript™ III First Strand Synthesis System (Invitrogen). The 5.0-μg aliquot of total RNA used in the reaction was first treated with DNase (Promega, Madison, WI) and was reverse-transcribed using SuperScript III Oligo (dT) 20 Primers according to the manufacturer's instructions (Invitrogen). For quantitative real-time PCR, specific primers for *PpMADS13–1* [forward: 5′-ACCCACGCTTCATCAGC TACT-3′ and reverse: 5′-CCAATTTTTCTAACTTCTG TAGTTCACC-3′; product size of 149 bp] and PpMADS13–2 [forward: 5′-AAATCACGCTTCACCAACTGC-3′ and reverse: 5′-CACCAATTTTTCTAACTTGTTCAGTTGATA-3′; product size of 152 bp] were designed using the Primer Express software. As an internal control, the pear *actin* gene was used for the amplification of 'Kosui' and taiwanese pear cDNAs using the *actin*-specific primers (forward: 5′-CAGG CATTCACGAGACCACA-3′ and reverse: 5′-TGCCAG GAACATGGTAGAA-3′) to obtain the relative gene expression levels of *PpMADS13–1* and *PpMADS13–2*. The real-time quantification of the first-strand cDNA was performed on the Light Cycler 2.0 System (Roche) and was analyzed with the Light Cycler Software, version 4. The reaction mixture (15 μL) contained 2.0 μL of cDNA sample (equivalent to 118 ng of the total RNA), 3.0 μM of each primer, and 7.5 μL of SYBR® Green Premix ExTaq II (Takara, Shiga, Japan). For a control reaction, no template was added to the reaction mixture, resulting in no detectable fluorescence signal from the reaction. The PCR conditions were set as follows: initial denaturation for 20 s at 95 °C, followed by 55 cycles of denaturation at 95 °C for 5 s, annealing for 20 s at 60 °C, and extension for 72 °C at 15 s. Each reaction was subjected to melting-point analysis to confirm single amplified products. Three replications were carried out for each gene. The specificity of each amplification reaction for a given primer set was verified in three ways: 1) by the melt gradient in which fluorescence decreases at a single discrete temperature, indicating the separation of both strands of a single DNA species; 2) by the specific restriction enzyme digestion analysis of the amplified products using *Xho*I and *Pst*I, which

can restrict only the amplified sequence of PpMADS13–1 and PpMADS13–2, respectively; and 3) by the simultaneous analysis of the plasmids of the respective genes using each primer set resulting in marked differences in the absolute quantification values from the respective standardized plasmids (templates) at a given concentration. Transcript levels were estimated using the established standard curve based on the dilution series of the plasmid and these levels were normalized against the *actin* transcript level in each sample, establishing a relative expression value.

Methylation status of PpMADS13–1 and PpMADS13–2 during endodormancy transition phases. To investigate the genomic DNA methylation status, genomic DNA was extracted as described above from the vegetative buds of 'Kosui' collected at the different seasonal dormancy transition stages from 2008 (21 Oct., 12 Nov., 9 Dec., and 24 Dec.) and 2009 (8 Jan. and 12 Feb.). Genomic DNA (10 µg) was digested with the methylation-sensitive isoschizomer pair *Hpa*II/*Msp*I. *Hpa*II and *Msp*I have multiple cleavage sites in the *DAM* genes. The resulting restricted fragments from *Hpa*II and *Msp*I digests were size fractionated and blotted onto nylon membranes as described above. To understand the methylation status of *PpMADS13–1* and *PpMADS13–2* loci, a common reverse primer (5′-GGAAGCCCCAGTTTGAGAGAC-3′) and gene-specific forward primers for *PpMADS13–1* (5′-ATCAAGAT CAGAAAGATCGAC-3′; 647-bp) and *PpMADS13–2* (5′-ATGGTGAAAAGGATGAATGAG-3′; 701-bp) were used to generate specific *PpMADS13–1* and *PpMADS13–2* DIG-labeled probes from the coding region. Prehybridization (1 h) and hybridization (overnight) were done as described above. After hybridization, the membranes were washed twice at 65 °C for 15 min at high stringency (0.5× SSC and 0.1% SDS). The detection was performed as described above.

Results

Isolation of partial DAM gene fragment from 'Kosui'. To isolate a partial cDNA fragment of the *DAM* genes from 'Kosui', RT-PCR was performed using primers conserved among the *P. persica* DAM family members and were designed on the basis of *Ppdam6* (GenBank accession no. DQ863252). The ≈609-bp PCR product obtained was cloned into the pCR®-Blunt vector using a Zero Blunt® PCR Cloning Kit (Invitrogen), sequenced, and annotated to confirm its homology to *DAM* genes from other plant species (data not shown). The alignment of protein sequences of this initial partial DAM fragment, pPpMADS13#2, revealed high amino acid conservation among the japanese pear and *Prunus* DAM proteins (data not shown). The nucleotide sequences of the *pPpMADS13#2* showed high conservation to the corresponding regions of the later-identified full-length *PpMADS13–1* (86%) and *PpMADS13–2* (99%), but neither was identical to both. This conserved initial PCR fragment, *pPpMADS13#2*, was chosen in the Southern blotting experiment to estimate the copy number of the *DAM* genes in the pear genome.

Southern blotting analysis to determine the distribution of the DAM genes in the genome of japanese pear. Southern blotting analysis was carried out with genomic DNA of 'Kosui'. Hybridization under moderate stringency conditions revealed one to two major bands and a number of weak fragments, suggesting that several copies of the *pPpMADS13#2* may be present in the pear genome (Fig. 1A).

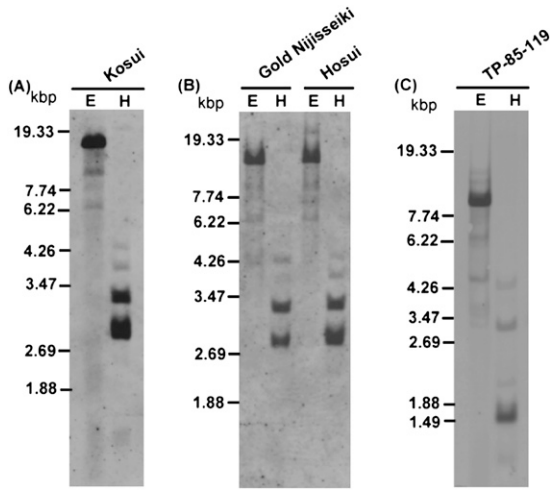

Fig. 1. Southern blotting analysis showing distribution of the *DAM* genes in the pear genome: japanese pear ['Kosui' (**A**), 'Gold Nijisseiki' and 'Hosui' (**B**)] and taiwanese pear [TP-85–119 (**C**)]. Ten micrograms of genomic DNA was digested with *Eco*RI (E) and *Hin*dIII (H). The DIG-labeled partial *DAM* fragment, *pPpMADS13#2* (609 bp) was used as a hybridization probe. Positions of molecular size markers are shown at the left.

Southern blotting analysis involving the genomic DNA of two other japanese pear cultivars (Gold Nijisseiki and Hosui) digested with the same enzymes and probe also yielded the same banding patterns as 'Kosui' (Fig. 1B). In addition, Southern blotting analysis of the taiwanese pear (TP-85–119) showed similar banding patterns, which indicated that the DAM genes are also present in the TP-85–119 genome. However, differences in the lower bands between TP-85–119 and the japanese pear type were observed under similar hybridization conditions (Fig. 1C).

Cloning of full-length cDNAs of pear DAM genes and sequence analysis. To isolate full-length cDNA of japanese pear *DAM* genes from 'Kosui', 3′- and 5′-RACE was performed using the corresponding gene-specific primers designed based on the 609-bp partial fragment (*pPpMADS13#2*). The first and second 5′-RACE produced fragments of 471 and 459 bp, respectively, while the 3′-RACE produced a 411-bp product. The partial 609-bp RT-PCR fragment and the RACE fragments were aligned to obtain a 1191-bp contig containing a 239-bp 5′-noncoding region, a 705-bp open reading frame (ORF), and a 247-bp 3′-non-coding region. Forward and reverse primers were designed based on this contig for the isolation of the full-length cDNAs. Sequencing of 20 randomly selected clones produced two groups of clones that we designated as *PpMADS13–1* and *PpMADS13–2*, with each group of clones possessing 100% nucleotide sequence similarity. The ORF of *PpMADS13–1* encodes a 227-amino acid polypeptide with a predicted molecular mass of 25.1 kDa and an isoelectric point (*pI*) of 5.5, while the *PpMADS13–2* ORF encodes a 234-amino acid polypeptide with a 26.4 kDa predicted molecular mass and a *pI* of 8.4. The alignment of the complete PpMADS13–1 and PpMADS13–2 protein sequences revealed high amino acid conservation among pear and *Prunus* DAM proteins, including the LEDDCSD motif (Jiménez et al., 2009) at amino acid positions 233 to 239 in the C-terminal region (Fig. 2). Moreover, no serine-aspartate change, which exists in peach DAM1 and DAM3, at this potential positively selected site (amino acid

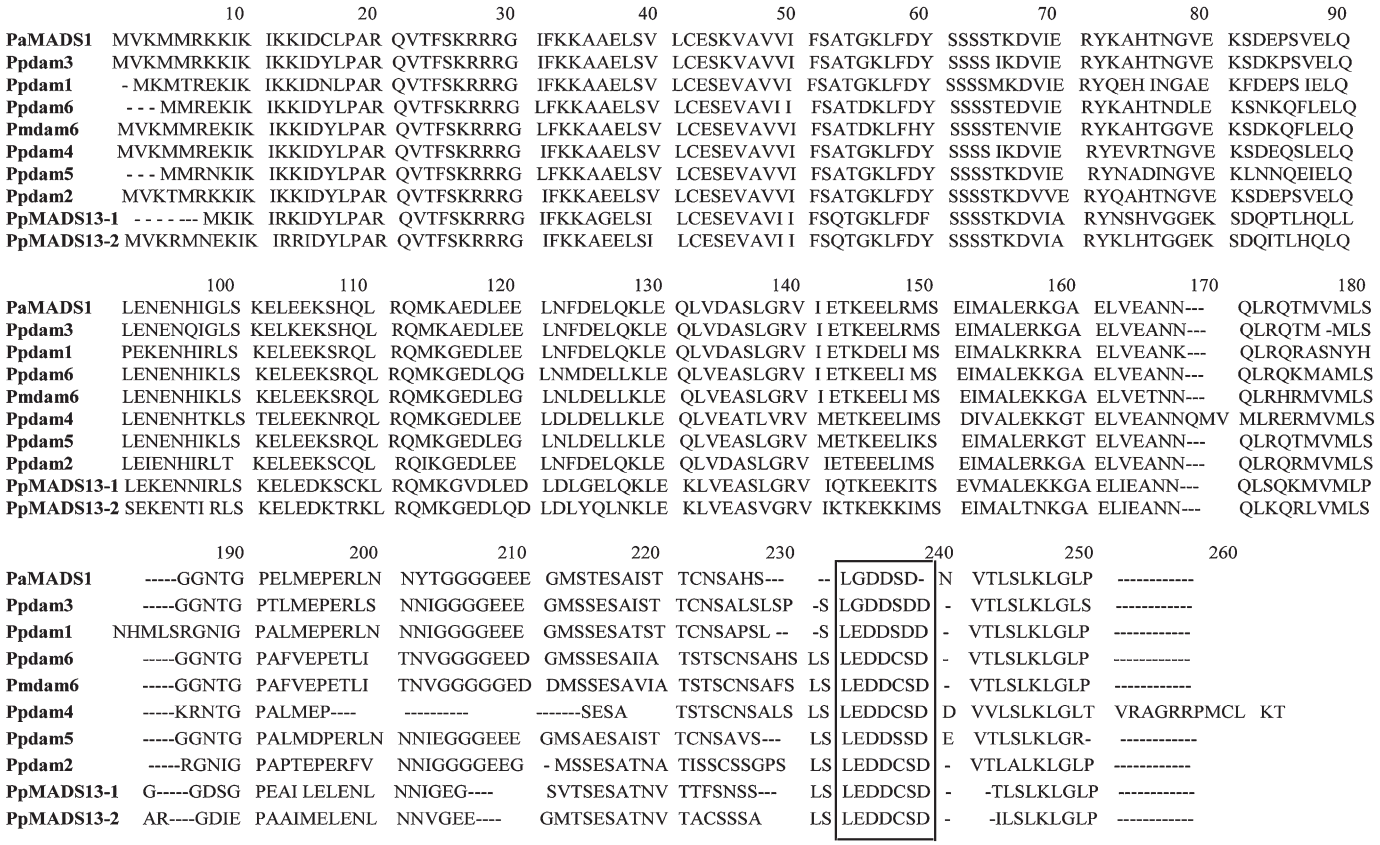

Fig. 2. Multiple alignments of the deduced amino acid sequences of *PpMADS13–1* and *PpMADS13–2* and those of *Prunus persica* (*Ppdam1*, DQ863253; *Ppdam2*, DQ863255; *Ppdam3*, DQ863256; *Ppdam4*, DQ863250; *Ppdam5*, DQ863251; and *Ppdam6*, DQ863252), *Prunus mume* (*Pmdam6*, AB437345), and *Prunus avium* (*PaMADS1*, EU196363). Sequence comparison was obtained using ClustalW and included M, I, K, and C domains. The LEDDCSD motif in the C-terminal region is boxed.

position 238) has occurred in the PpMADS13–1 and PpMADS13–2 (Jiménez et al., 2009). The similarity score for pairwise comparison of the amino acid sequences between PpMADS13–1 and PpMADS13–2 was 81.0%.

Sequence analysis by a tBLASTx similarity search against the NCBI nonredundant database revealed that *PpMADS13–1* and *PpMADS13–2* are highly similar to known *DAM* genes identified in several other species (Table 1), which is consistent with the partial fragment used as hybridization probe for Southern analysis (data not shown). Alignment of the predicted amino acid sequences from these sequences showed that *PpMADS13–1* and *PpMADS13–2* contained the well-conserved MIKC-motifs characteristic of *DAMS* (Fig. 2). The M-domain, which is important in homeotic regulation, is about 60 amino acids long. The variable I-domain, consisting of about 30 amino acids, links the M- and K-domains. The K-domain is about 70 amino acids long and is linked to the most variable and functionally important C-terminal domain. A phylogenetic tree constructed based on the complete amino acid sequences (M, I, K, and C domains) of *PpMADS13–1* and *PpMADS13–2* and other 17 *DAM* genes (Fig. 3) revealed that the pear *DAM* genes are more closely related to those of *Prunus species*. However, the pear *DAM* genes formed an independent subclade.

Seasonal changes in the endodormancy transition phases and expression analysis of PpMADS13–1 and PpMADS13–2 genes. To examine the changes in lateral leaf bud gene expression during the endodormancy transition phases, it was necessary to define the dormancy status of the lateral leaf buds during the endodormancy transitional phases. The seasonal changes in the endodormancy transition phases assessed in the lateral leaf buds from early Sept. 2008 to mid-Feb. 2009 are shown in Fig. 4A. In early September, the sprouting percentage in 'Kosui' was 8% and declined to 0% throughout the 21 Oct., 12 Nov., and 9 Dec. sampling dates when a sharp increase occurred, reaching 56%, 79%, and 93% on 24 Dec., 8 Jan., and 12 Feb., respectively (Fig. 4A). These results suggest that 'Kosui' leaf buds were released from endodormancy about mid-December and progressed to eco-dormant stage by late December. The less dormant TP-85–119 had a sprouting ratio of 82% (12 Nov.) and 95% (9 Dec.) when that of the endodormant 'Kosui' was 0%.

The specific gene expression patterns of the *PpMADS13–1* and *PpMADS13–2* genes were investigated in the lateral leaf buds of 'Kosui' through seasonal dormancy transitions. The quantitative real-time PCR showed that the *PpMADS13–1* and *PpMADS13–2* genes were differentially expressed in relation to the endodormancy status in the lateral leaf buds of 'Kosui' (Fig. 4, B and C). The expression level of both DAM genes fluctuated similarly during the period from early September to mid-February and showed some association with endodormancy phase transitions. However, changes in gene expression levels were relatively slower than changes in endodormancy status, especially toward endodormancy release. Both genes had a low expression level by 9 Sept., gradually increased to a peak

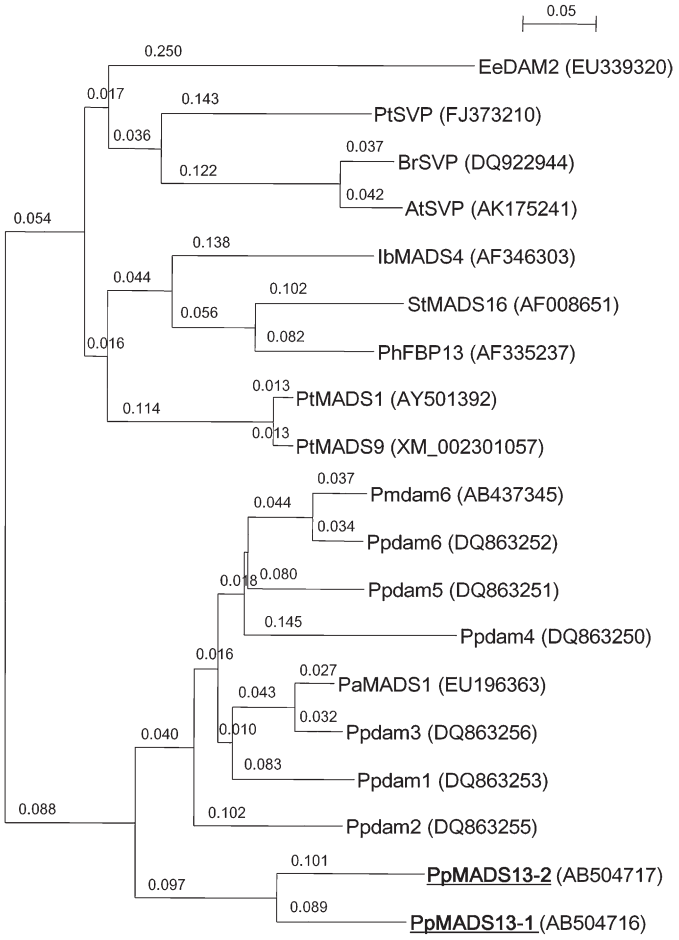

Fig. 3. A phylogenetic tree constructed based on the complete amino acid sequences of the 'Kosui' japanese pear PpMADS13–1 and PpMADS13–2 (underlined), *Prunus persica* (DQ863253, DQ863255, DQ863256, DQ863250, DQ863251, and DQ863252), *Prunus mume* (AB437345), *Prunus avium* (EU196363), *Populus trichocarpa* (XM_002301057), *Populus tomentosa (*AY501392), *Petunia ×hybrida* (AF335237), *Solanum tuberosum* (AF008651), *Ipomoea batatas* (AF346303), *Arabidopsis thaliana* (AK175241), *Brassica rapa* subsp. *campestris* (DQ922944), *Poncirus trifoliata* (FJ373210), and *Euphorbia esula* (EU339320). The values indicate the branch length.

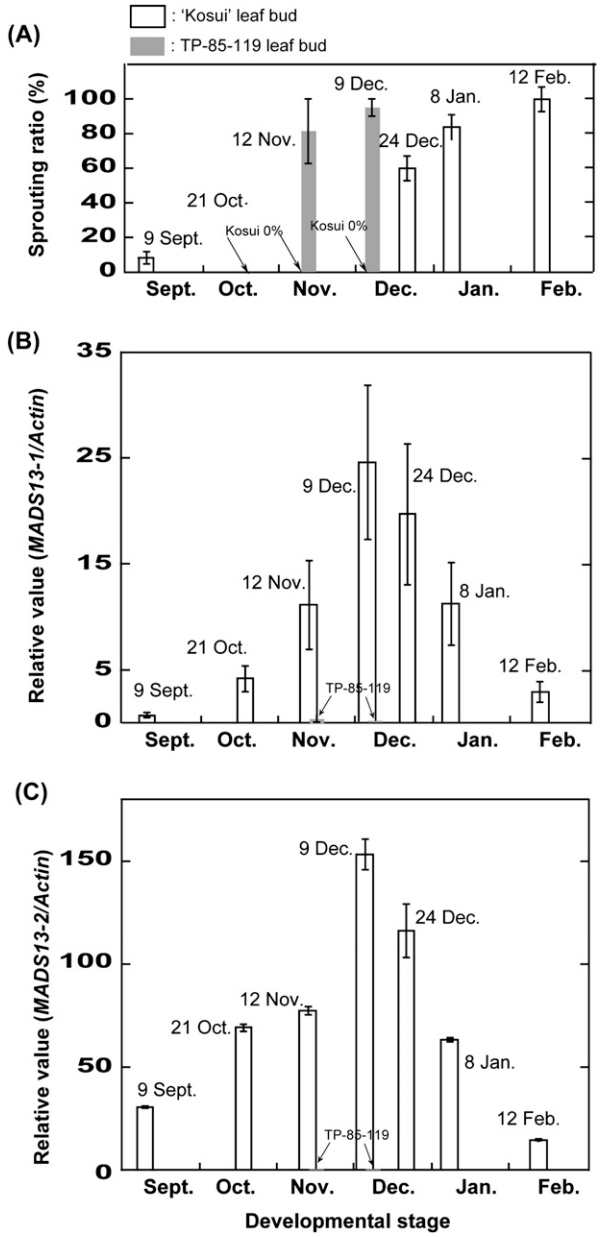

Fig. 4. Sprouting ratio (in percentage) (**A**), relative expression levels of *PpMADS13–1* (**B**), and relative expression levels of *PpMADS13–2* (**C**) in the lateral leaf bud during the endodormancy transition phases in 'Kosui' japanese pear. Data of the less dormant taiwanese pear, TP-85–119 in (**A**–**C**) is shown in gray.

expression level concomitant with endodormancy by 9 Dec., and then gradually decreased to a low level on 12 Feb. The PpMADS13–2 DAM gene was generally more highly expressed than PpMADS13–1 (Fig. 4, B and C). The expression of *PpMADS13–1* was low by 9 Sept., when the sprouting ratio was 8%, increased sharply to a 33-fold maximal level at the peak of endodormancy by 9 Dec. when the sprouting ratio was 0%, and then gradually decreased with increasing sprouting ratio (endodormancy release) to an ≈8-fold lower level by 12 Feb. (when the sprouting ratio was 93%), relative to 9 Dec. (Fig. 4B). It is worth noting that the *DAM* genes not only showed a decreased expression with endodormancy release, but also a build up with the installation of endodormancy. The expression level of *PpMADS13–1* in the less dormant TP-85–119 was very significantly lower (nearly zero level) on 12 Nov. (82% sprouting ratio) and 9 Dec. (95% sprouting ratio), relative to the endodormant 'Kosui' (Fig. 4B). Similarly, the expression of *PpMADS13–2* in 'Kosui' was low by 9 Sept., when the sprouting ratio was 8%, increased sharply to a 5-fold maximal level at the peak of endodormancy by 9 Dec. (when the sprouting ratio was 0%), and then gradually decreased with increasing sprouting ratio (endodormancy release) to a 10-fold lower level by 12 Feb. (when the sprouting ratio was 93%), relative to 9 Dec. (Fig. 4C). The expression level of *PpMADS13–2* in the less dormant TP-85–119 was also very significantly lower (nearly zero level), as with the expression of *PpMADS13–1* (Fig. 4B), on 12 Nov. (82% sprouting ratio) and 9 Dec. (95% sprouting ratio) relative to the endodormant 'Kosui' (Fig. 4C). Unlike *PpMADS13–2,* the expression level of *PpMADS13–1* on 12 Feb. was relatively higher (4-fold) than on 9 Sept. (when the sprouting ratio was just 8%), suggesting the possible role of environmental factors in the level of

expression of these genes in 'Kosui'. Overall, the expression patterns of *PpMADS13–1* and *PpMADS13–2* genes in lateral leaf buds showed a gradual down-regulation of the genes upon endodormancy release.

Methylation status of PpMADS13–1 and PpMADS13–2 during the endodormancy transition phases. Southern blotting analysis using genomic DNA from the lateral vegetative buds from 'Kosui' during the endodormancy transition phases (from mid-October to mid-February) and specific *PpMADS13–1* and *PpMADS13–2* gene probes showed differences in the fragments detected by *Hpa*II and *Msp*I, which reflects methylation at the CpG sites (Fig. 5, A and B). However, DNA methylation in both loci may not be associated with the endodormancy phenomenon, as identical banding patterns were produced in the CCGG allelic sites that were not concomitant with the endodormancy transition phases as shown in Fig. 4A. Several common bands were observed between *PpMADS13–1* and *PpMADS13–2*, suggesting high similarity in the genomic organization between these genes.

Discussion

The six MIKC-type *DAM* genes described in peach (Bielenberg et al., 2004, 2008; Wang et al., 2002) are candidate genes for the regulation of growth cessation and terminal bud formation in peach. The *DAM* genes are down-regulated concomitant with endodormancy release and are not detected in the *evergrowing* mutant (Bielenberg et al., 2008). Since their discovery, interest has been growing in the investigation of these candidate genes in other perennial species, including raspberry, japanese apricot, and leafy spurge (Horvath et al., 2008; Mazzitelli et al., 2007; Yamane et al., 2008). In this study, we isolated two full-length *DAM* genes in 'Kosui' japanese pear, *PpMADS13–1*, and *PpMADS13–2*, investigated their expression, as well as their methylation status during the endodormancy transitional phases in japanese pear.

Southern blotting analysis with *Eco*RI- and *Hin*dIII-digested DNA of three japanese cultivars using a partial *DAM* gene sequence (*pPpMADS13#2*) as a probe was performed. It should be noted that the probe can detect at least *PpMADS13–1* and *PpMADS13–2* genomic regions under moderate stringency because *pPpMADS13#2* share high sequence similarities between *PpMADS13–1* and *PpMADS13–2*. The analysis yielded one or two major bands and a number of weak bands, suggesting that several copies of the *pPpMADS13#2* may be present in the pear genome. Southern blotting analysis of the taiwanese pear (TP-85–119) performed under different electrophoresis conditions also showed similar banding patterns, albeit the observed differences in the lower bands, indicating that taiwanese pear also possesses similar genomic structure for *pPpMADS13#2*. The major hybridizing bands detected in this study were of greater intensity, which may result from being the sequence of highest similarity to the hybridizing probe or, more likely, the possibility of potentially two or more overlapping bands of this gene family. However, these possibilities remain to be clarified. In addition, because only *pPpMADS13#2* was tested in this study, further study is required to confirm the presence of other *DAM* genes in the pear genome like peach (Bielenberg et al., 2004). Jiménez et al. (2009) indicated that the members of the *DAM* genes of the *SVP/StMADS11-like* type in peach (six) follow the trend of increased number as in other perennial species such as poplar [*Populus* spp. (eight)] and grape [*Vitis vinifera* (five)] relative to the decreased number reported in other annual species, including *A. thaliana* (two), tomato [*Solanum lycopersicum* (two)], and rice [*Oryza sativa* (three)].

Two full-length *DAM* genes cloned from japanese pear have high sequence conservation and showed a high degree of identity with *DAM* genes from other plant species at the amino acid level. A conserved motif containing a potential positively selected site (LEDDCSD) of the C-terminal region reported among the peach MIKC-type MADS-box genes was also identified in this study. No serine(S)-aspartate(D) change occurred at this site of the *PpMADS13–1* and *PpMADS13–2* genes, unlike in peach *DAM1* and *DAM3*. This position had been reported to have a posterior possibility of >0.95 and was likely to be functionally significant (Jiménez et al., 2009). As yet, we do not know the number of candidates of this gene family in japanese pear, and their complete identification will confirm whether this single amino

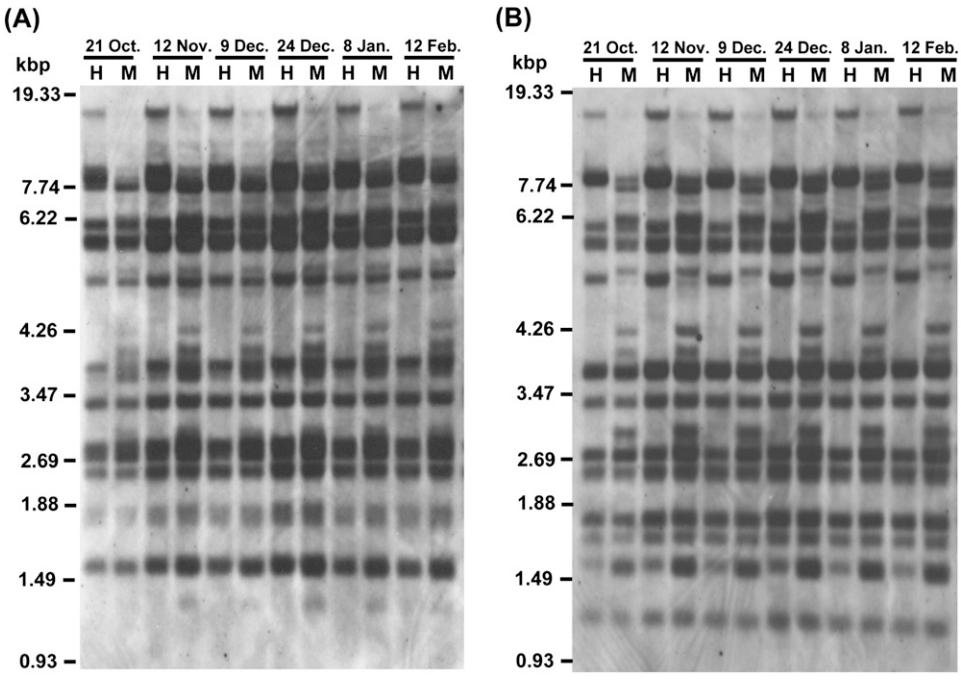

Fig. 5. Methylation analysis of *PpMADS13–1* (**A**) and *PpMADS13–2* (**B**) locus. Lateral leaf bud was collected from 'Kosui' japanese pear at six different seasonal dormancy transition stages from 2008 (21 Oct., 12 Nov., 9 Dec., and 24 Dec.) and 2009 (8 Jan. and 12 Feb.). Genomic DNA (10 µg) was digested with the methylation-sensitive isoschizomer pair *Hpa*II (H) and *Msp*I (M). Specific DIG-labeled coding sequences for *PpMADS13–1*, (**A**, 674 bp) and *PpMADS13–2*, (**B**, 701 bp) were used as hybridization probes. H = *Hpa*II, M = *Msp*I. Positions of molecular size markers are shown at left.

Table 1. Identity of 'Kosui' japanese pear MADS 13–1 and MADS13–2 from a tBlastx similarity search on 16 June 2009 against the NCBI nonredundant (nr) database of 10 top-hit *dam* genes.

	E-value[z]	Identity (%)	Species	Gene	Accession No. (GenBank)
DAM13–1	9 × e-158	77	*Prunus persica*	*dam6*	DQ863252
DAM13–2	5 × e-155	77			
DAM13–1	2 × e-153	77	*Prunus mume*	*dam6*	AB437345
DAM13–2	1 × e-149	76			
DAM13–1	3 × e-151	77	*Prunus persica*	*dam2*	DQ863255
DAM13–2	1 × e-149	76			
DAM13–1	2 × e-148	77	*Prunus persica*	*dams5*	DQ863251
DAM13–2	1 × e-137	75			
DAM13–1	2 × e-147	77	*Prunus avium*	*mads1*	EU196362
DAM13–2	3 × e-138	75			
DAM13–1	2 × e-134	77	*Prunus persica*	*dam3*	DQ863256
DAM13–2	3 × e-126	74			
DAM13–1	4 × e-130	79	*Prunus persica*	*dam4*	DQ863250
DAM13–2	5 × e-129	79			
DAM13–1	1 × e-118	73	*Prunus persica*	*dam1*	DQ863253
DAM13–2	1 × e-123	73			
DAM13–1	4 × e-112	72	*Prunus persica*	*dam2 alpha*	DQ863254
DAM13–2	3 × e-113	72			
DAM13–1	8 × e-51	92	*Prunus persica*	*PpN089G02(BAC)*	DQ863257
DAM13–2	1 × e-55	100			

[z]The E-value from tBlastx that indicated the strongest sequence match.

acid residue is also under significant positive selection among the japanese pear *DAMS*. These results may suggest that the evolutionary divergence in the species does not result in high rates of nucleotide substitutions among the *DAM* genes of japanese pear, peach, and japanese apricot. Relationship analysis based on the phylogenetic analysis constructed using the complete amino acid sequences demonstrated that *PpMADS13–1* and *PpMADS13–2* were more closely related to *Prunus DAM* genes than those of other taxa. However, *PpMADS13–1* and *PpMADS13–2* formed an independent subclade from the *Prunus DAM* genes, suggesting uniqueness based on taxonomic distance. Based on the differences in amino acid sequences, it seems that *PpMADS13–1* and *PpMADS13–2* may likely be derived from different loci, rather than being alleles of the same locus. However, the possibility of an allelic relationship cannot be completely ruled out. In future studies, the possibility of adding new pear *DAM* homologs and phylogenetic analysis will clarify the relationships in the *DAM* genes of *Pyrus* and other members of this gene family. Indeed, work is currently underway to annotate all of the gene candidates of this gene family using a microarray approach to identify other members of this gene cluster in japanese pear.

This study has identified important gene candidates of seasonal endodormancy regulation in japanese pear. The pattern of expression in the two *DAM* genes fluctuated similarly with the seasonal endodormancy phase transitions (induction and release) in 'Kosui' lateral leaf buds, although *PpMADS13–2* was more highly expressed than *PpMADS13–1*. The peak expression in both *DAM* genes occurred on 9 Dec., which coincided with the probable transition phase of deep endodormancy in 'Kosui' before the gradual onset of endodormancy release. Similarly, the expression of these two *DAM* genes in the less dormant TP-85–119 PI was consistently quite low. Although we do not yet know the sequence information of these *DAM* genes in taiwanese pear, our real-time PCR results (based on the analysis of the dissociation curve) indicated reliable amplification by the primers. Therefore, the quite low expression of these genes in the TP-85–119 is consistent with the down-regulation that is associated with the near loss of endodormancy in this taiwanese pear. These results on the seasonal expression of the two *DAM* genes in pear are consistent with the results reported in other species, including *Prunus* species, where the DAM genes were down-regulated with endodormancy release (Bielenberg et al., 2008; Li et al., 2009; Yamane et al., 2008).

To test the hypothesis of epigenetic gene regulation of endodormancy involving genome methylation, we investigated the methylation patterns in the genomic region underlying *PpMADS13–1* and *PpMADS13–2* genes using genomic DNA of 'Kosui' in the course of the endodormancy transition phases (late October to early February). Our results revealed that although DNA methylation occurred in these genes, it is not concomitant with the changes in the gene expression that are associated with endodormancy status, as detected by the isoschizomer pair *Hpa*II/*Msp*I.

Collectively, our results are consistent with the hypothesis of a down-regulated pattern of expression for the *DAM* genes. Our finding in japanese pear is consistent with reports in other deciduous tree species where a gradual down-regulation of the *DAM* gene was correlated with endodormancy release (Horvath et al., 2008; Mazzitelli et al., 2007; Yamane et al., 2008). As has been stated earlier (Bielenberg et al., 2008), a disruption of gene function of these candidate genes, leading to a loss of expression, may be responsible for the nondormant condition in the *evergrowing* mutant types carrying a deletion of the *DAM* genes. As highlighted by Yamane et al. (2008), members of this gene family might function as internal suppressor genes to delay or inhibit bud growth under prevailing favorable conditions for bud burst when none of the external inhibitory effects (e.g., apical dominance or unsuitable environmental conditions) exist. The differential expression patterns of *PpMADS13–1* and *PpMADS13–2* genes at the last stage of sampling (12 Feb.), relative to 9 Sept. (the stage of progression into endodormancy)

suggest a possible effect of different dormancy-inducing environmental cues (such as low temperatures in *Pyrus*) on their seasonal expression. Recent findings have also indicated that *DAM* genes may not only be required for endodormancy induction, but that their induction may be signaled by environmental cues known to regulate endodormancy, such as cold temperatures or short days (Horvath et al., 2008; Li et al., 2009). It is worth noting the fact that although *Pyrus* and *Prunus* are species from the same family, fundamentally different environmental cues trigger their growth cessation, bud formation, and endodormancy induction (Horvath et al., 2008; Li et al., 2009). While short photoperiods are one of the important signals to trigger endodormancy in peach, short photoperiods have no effect in japanese pear, but low temperatures are solely responsible for the induction of bud formation and endodormancy (Heide and Prestrud, 2005). Further studies involving the isolation and functional analysis of the *CONSTANS* gene (*CO*) will be necessary for a clear-cut explanation of this difference between pear and peach. Moreover, a *FLOWERING LOCUS T* (*FT*)-like gene that was hypothesized to interact with DAM proteins to regulate dormancy transitions was shown to be reciprocally regulated with the expression of *DAM* genes in leafy spurge (Horvath et al., 2008). Therefore, investigating the expression of these candidate *DAM* and *FT*-like genes in pear under low temperatures will also be worthwhile. We are currently isolating *TERMINAL FLOWER* (*TFL*)- and *FT*-like genes to confirm this hypothesis in japanese pear.

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