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Research paper

Expression and genomic structure of the *dormancy-associated MADS box* genes *MADS13* in Japanese pears (*Pyrus pyrifolia* Nakai) that differ in their chilling requirement for endodormancy release

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We isolated three *dormancy-associated MADS-box* (*DAM*) genes (*MADS13-1*, *MADS13-2* and *MADS13-3*) and showed regulated expression concomitant with endodormancy establishment and release in the leaf buds of Japanese pear 'Kosui'. Comparative analysis between 'Kosui' and Taiwanese pear TP-85-119 ('Hengshanli'), a less dormant pear cultivar, showed reduction of *MADS13-1* expression level in 'Hengshanli' earlier than in 'Kosui' towards endodormancy release, suggesting the possible relationship between chilling requirement and *MADS13-1* expression. Application of hydrogen cyanamide accelerated endodormancy release with a reduction in *MADS13* expression, whereas heat treatment in autumn inhibited endodormancy establishment without induction of *MADS13* expression, indicating a close relationship between the *MADS13* expression pattern and endodormancy phase transitions. Moreover, both the *cis*-acting regulatory elements and the methylation status in the 5′ upstream region of the *MADS13-1* gene were not largely different between 'Kosui' and 'Hengshanli'. Genomic structures of *MADS13-1* from 'Kosui' and 'Hengshanli' revealed a 3218 bp insertion in the first intron of 'Hengshanli' that might be ascribed to the lower expression of *MADS13-1tw*; however, this insertion was also found in pear genotypes with a high chilling requirement. These results indicated that the low expression of *MADS13-1* in 'Hengshanli' towards endodormancy release could not be explained by the identified *cis*-acting regulatory elements, the methylation status of the putative promoter or by intron insertion.

Keywords: dormancy-associated MADS box gene, endodormancy, pear (*Pyrus pyrifolia* Nakai).

Introduction

Perennial woody plants adapt to seasonal environmental changes by modulating their growth rhythm, among which seasonal growth cessation is an important strategy for survival during cold winters (Cooke et al. 2012). Growth cessation during the winter season is a well-studied phenomenon, known as bud dormancy, that is divided into three physiological phases: paradormancy, endodormancy and ecodormancy (Lang 1987, Anderson et al. 2005). Pear bud differentiation occurs during the early summer in Japan (Banno et al. 1986), but outgrowth of these buds is inhibited by shoot tips and/or adjacent leaves possibly due to the balance of phytohormones (Ongaro and Leyser 2008). This growth inhibition is called paradormancy, and the buds then gradually switch to a condition called endodormancy (Saure 1985, Faust 1989). During endodormancy from autumn to winter, bud growth is arrested not by external factors but by internal factors. Once endodormancy is established, buds are incapable of resuming their growth until a chilling requirement is satisfied, whose amount is genetically determined. After endodormancy, buds are gradually shifted to ecodormancy, during which bud release is provoked by environmental factors and buds are able to resume their growth under favourable environmental conditions (Faust et al. 1997).

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Global warming threatens the sustainable production of deciduous fruit trees, including Japanese pear (Sugiura et al. 2012). Warm winter temperature sometimes not only reduces the number of days of low temperature required for endodormancy release but also causes frost damage. In addition, less chilling accumulation results in bud abortion in the following spring season (Yamamoto et al. 2010). All of these phenomena have been recently observed in commercial pear orchards in Japan. Thus, knowledge about the regulation of endodormancy in the Japanese pear is very important to overcome these problems and will also provide the basis for controlling endodormancy through breeding with conventional and/or transgenic approaches towards more stable and economical production of this crop under global warming conditions.

We had previously isolated two full-length *dormancy-associated MADS-box* (*DAM*) genes (*PpMADS13-1* and *PpMADS13-2*) from Japanese pears (*Pyrus pyrifolia* Nakai, 'Kosui') (Ubi et al. 2010). Both *PpMADS13* genes showed similar expression patterns during dormancy depending on the endodormancy status; they are up-regulated towards endodormancy establishment and down-regulated concomitant with endodormancy release, an expression pattern similar to peach *DAM5* and *DAM6* (Li et al. 2009, Yamane et al. 2011*a*). Recent studies have also shown the coincidence of *DAM* expression with the seasonal dormancy phase transition in other plants, including raspberry (Mazzitelli et al. 2007), Japanese apricot (Yamane et al. 2008, Sasaki et al. 2011) and leafy spurge (Horvath et al. 2008). Furthermore, two peach cultivars having high chilling requirements (high-chill), 'Akatsuki' and 'Shimizu Hakuto', had higher expression levels of *DAM6* than two cultivars with low chilling requirements (low-chill), 'Okinawa' and 'Tsukuba Ichigo' (Yamane et al. 2011*a*, 2011*b*). A similar expression pattern was found in a low-chill-type Taiwanese pear TP-85-119 (*P. pyrifolia*, 'Hengshanli') that had a lower level of *DAM* (*MADS13*) expression than 'Kosui', a moderate-chill cultivar (Ubi et al. 2010).

DAM genes belong to the flower regulator clade of MIKC-type MADS-box transcription factor genes that includes *SHORT VEGETATIVE PHASE* (*SVP*) and *AGAMOUS-LIKE 24* (*AGL24*) (Jiménez et al. 2009). *DAM* genes were first identified as candidate genes for terminal bud formation in the *EVERGROWING* (*EVG*) locus of peach (Bielenberg et al. 2008). Six *DAM* genes were found in the peach genome that were tandemly arranged in the *EVG* locus which overlapped with a strong quantitative trait locus (QTL) for chilling requirement and blooming day (Fan et al. 2010). Expression of peach *DAM3* did not show a distinct pattern throughout the annual cycle, but expression of *DAM1*, *DAM2* and *DAM4* was closely related to growth cessation (Li et al. 2009). Interestingly, *DAM5* and *DAM6* were mainly expressed in winter, with expression reaching a maximum concomitant with the acquisition of bud break competence (Li et al. 2009). Four of the six *DAM* genes (*DAM1–DAM4*) are absent in the *evg* non-dormant mutant, and this deletion also causes the loss of transcriptional activity for *DAM5* and *DAM6*. Therefore, the *evg* mutant fails to cease growth and to enter dormancy in the winter season (Bielenberg et al. 2008). All these results suggested that the *DAM* genes could play a crucial role in the endodormancy phase transition and the quantity of chilling requirement for endodormancy release.

In this study, we first report the characterization of three sets of *DAM* genes (*MADS13-1*, *MADS13-2* and *MADS13-3*), from a moderate-chill pear cultivar 'Kosui' and a low-chill Taiwanese pear 'Hengshanli'. We also confirmed that the expression pattern of *MADS13* correlated with the endodormancy phase transitions in the lateral leaf buds of both cultivars. Then, to reconfirm the involvement of *MADS13* in the endodormancy phase transition, the expression of 'Kosui' *PpMADS13-1*, *PpMADS13-2* and *PpMADS13-3* was investigated in lateral leaf buds treated with hydrogen cyanamide (HC) and with heat in autumn. Subsequently, *cis*-acting regulatory elements in the promoter regions, a key component in the regulation of gene expression, were investigated in 'Kosui' and 'Hengshanli'. In addition, since epigenetic mechanisms such as DNA methylation resulted in the null or low expression of target genes by interrupting the binding of transcription factors and other proteins to DNA (Zeisel 2012), the methylation status of the promoter region of *MADS13-1* from 'Kosui' and 'Hengshanli' was compared to gain insight into the potential differences that could foster different chilling requirements among these pears. On the other hand, an insertion in the first intron of *DAM5* and *DAM6* found in low-chill peach cultivars was speculated to be involved in the low expression of the *DAM* genes (Yamane et al. 2011*b*); however, the genomic structure of pear *DAM* genes has not yet been investigated. Thus, we also compared the genomic structures of *MADS13-1* between a moderate-chill cultivar 'Kosui' and a low-chill cultivar 'Hengshanli'. Finally, we reported the relationships between the first intron structures and dormancy characteristics in several pear genotypes that differed in their chill unit (CU).

Materials and methods

Plant materials and endodormancy status

Samples were collected from pear trees grown in the orchard of the NARO Institute of Fruit Tree Science, Tsukuba, Japan (lat. 36°N, long. 140°E). Lateral leaf buds of cultivars 'Kosui' and 'Hengshanli' from annual succulent shoots were collected during the endodormancy transition phases in the 2009/2010 and 2011/2012 growing seasons. The chilling requirement for endodormancy release of 'Kosui' and 'Hengshanli' was 1000–1200 CU and <200 CU, respectively (Tamura et al. 2001, Takemura et al. 2012). Lateral leaf buds were immediately frozen in liquid nitrogen and stored at −80 °C until needed for RNA extraction. The dormancy status of the buds in the field-grown

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trees on each collection date was evaluated as described by Ubi et al. (2010). Briefly, five succulent shoots (length >60 cm) bearing predominantly buds were collected on each date and placed in 500-ml containers of distilled water; the shoots were then maintained in an incubator held at 25 °C under cool white fluorescent light and a 16-h photoperiod to force the buds. The distilled water in the vials was changed at 2 to 3 day intervals. The dormancy status at each collection date, defined as the stage at which green tissue becomes visible under the bud scales, was recorded based on five to nine buds per branch averaged over five branches scored at 21 days after incubation.

Isolation of three MADS13 genes from 'Kosui' and 'Hengshanli'

For 'Kosui', we had already isolated two *DAM* genes, *PpMADS13-1* and *PpMADS13-2* (Ubi et al. 2010), but we found a third gene during the experiment; therefore, we decided to isolate and characterize this gene. Total RNA was isolated from 'Kosui' lateral leaf buds collected on 18 November 2009 using the hot borate extraction procedure of Wan and Wilkins (1994). First-strand cDNA synthesized from 1 μg of total RNA was then used as a template for reverse transcription-polymerase chain reaction (RT-PCR). The RT-PCR was performed in a total volume of 50 μl containing 125 ng of cDNA, 10 mM of dNTPs, 1.25 U of Ex Taq and 1× Ex Taq buffer (TaKaRa, Shiga, Japan) using the primer set (forward: 5′-AAT TTC CCG AAA CTT CAG ACC TA-3′; reverse: 5′-GAA GTG CAA CTC TCC ATC CG-3′). The PCR conditions were as follows: 98 °C for 10 s, 68 °C for 1 min was repeated 35 times. The product was cloned into the pCR®-2.1-TOPO vector (Invitrogen, Foster City, CA, USA). After sequence confirmation, the gene was designated as *PpMADS13-3* and deposited in the DNA Data Bank of Japan (DDBJ) under accession number AB774474.

Similarly, we also isolated the three *MADS13* genes from 'Hengshanli' that corresponded to 'Kosui' *PpMADS13-1*, *PpMADS13-2* and *PpMADS13-3*. Total RNA was isolated from 'Hengshanli' lateral leaf buds collected on 18 November and first-strand cDNA was synthesized and was then used for RT-PCR as described above. Since similar sequences for Japanese and Taiwanese *MADS13* genes were expected, primer sets used for the isolation of 'Kosui' *MADS13* genes were used for amplifying 'Hengshanli' genes (see Supplementary Table S1 available as Supplementary Data at *Tree Physiology* Online). RT-PCR was performed in a total volume of 50 μl containing 125 ng of cDNA, 500 μM of dNTPs, 375 μM of $MgCl_2$, 3.125 μM of each primer, 0.4 U of KOD Plus polymerase and 0.1× KOD buffer (Toyobo, Osaka, Japan). Following a heat denaturation step at 95 °C for 2 min, a cycling profile of 94 °C for 30 s, 50 °C for 30 s, 68 °C for 1 min was repeated 35 times. The product was cloned into the pCR®-Blunt vector (Invitrogen). Sequencing reactions were carried out with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and the products were sequenced on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). After sequence confirmation, the genes were designated as *PpMADS13-1tw*, *PpMADS13-2tw* and *PpMADS13-3tw* and deposited in the DDBJ under accession numbers AB775140, AB774475 and AB775141, respectively.

Southern blot hybridization and phylogenetic tree analysis

To estimate the copy number of *MADS13*, genomic DNA was extracted from fresh young leaves of 'Kosui' using a crude nuclear extraction method (Thomas et al. 1993). Genomic DNA (10 μg) was digested at 37 °C overnight with the restriction enzymes EcoRI or XbaI. These enzymes were selected because they lacked cleavage sites in the probe region. A 379 bp region of *PpMADS13-1* covering the K-box to the stop codon was used to generate the hybridization probe using a DIG DNA Labeling Kit (Roche Diagnostics, Mannheim, Germany). Fractionation, pre-hybridization, hybridization and detection were done according to a previous report (Ubi et al. 2010).

DAM, *DAM*-like genes, *SVP*-like *MADS-box* genes, apple *MADS-box* genes deposited in the National Center for Biotechnology Information (NCBI) database and putative apple *DAM* genes (MDP0000322567, MDP0000527190 and MDP0000259294) deposited in the Genome Database for Rosaceae (GDR) were retrieved (see Supplementary Table S2 available as Supplementary Data at *Tree Physiology* Online) to assemble a phylogenetic tree. Sequences were aligned using the ClustalW algorithm (Thompson et al. 1994) and were manually edited in Geneious (Biomatters Ltd, Auckland, New Zealand). Maximum likelihood and neighbour-joining methods were carried out using the PHYML plugin and the provided tree builder in Geneious v. 5.3.6. For testing the robustness of the tree, 1000 bootstrap replicates were carried out.

Acceleration of endodormancy release by HC and inhibition of endodormancy establishment by heat treatment

To accelerate endodormancy release by HC (CX-10, Nippon Carbide Industries, Tokyo, Japan) treatment, 'Kosui' succulent shoots (length >60 cm) were collected on 19 December 2011 from the orchard and sprayed with distilled water (control) or 1% (w/v) (0.24 M) HC (Sakamoto et al. 2010). On the collection date the shoots had nearly completed endodormancy establishment. After treatment, all shoots were incubated at 25 °C and lateral leaf buds were collected at 0, 10 and 21 days after treatment. To inhibit endodormancy establishment, potted 'Kosui' trees were transferred to the phytotron (25 °C) on 20 September 2011 and maintained for 128 days under a natural photoperiod. Lateral leaf buds were collected at 85 and 128

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days after transfer to the phytotron and used for expression analyses.

Expression analysis with reverse transcription-quantitative PCR

Total RNA was isolated from the 'Kosui' and 'Hengshanli' lateral leaf buds collected during the endodormancy stage and from the 'Kosui' lateral leaf buds subjected to acceleration and inhibition conditions using the modified hot borate extraction method of Wan and Wilkins (1994). The first-strand cDNA was synthesized using the SuperScript™ III First-Strand Synthesis System (Invitrogen). A 5-µg aliquot of total RNA used in the reaction was first treated with DNase I (Promega, Madison, WI, USA) and reverse transcribed using SuperScript III oligo (dT) 20 primers according to the manufacturer's instructions (Invitrogen). For reverse transcription-quantitative PCR (RT-qPCR), specific primers were designed (see Supplementary Table S1 available as Supplementary Data at *Tree Physiology* Online). Although the previous primer set (Ubi et al. 2010) was designed based on 'Kosui' *PpMADS13-1* and *PpMADS13-2* sequences, these primer sets could not perfectly correspond to 'Hengshanli' because Ubi et al. (2010) did not isolate any *MADS13* from this cultivar at that time. Thus, we designed a new common primer set for *MADS13-1* that works for both 'Kosui' and 'Hengshanli'. RT-qPCR was performed using a 7500 Real Time PCR System (Applied Biosystems) and the results were analysed with the 7500 System Sequence Detection Software ver. 1.4. The reaction mixture (10 µl) contained 1.0 µl of the cDNA sample (equivalent to ~20 ng of the total RNA), 0.8 µM of each primer, 0.2 µl of ROX Reference Dye and 50 µl of SYBR® Green Premix ExTaq II (TaKaRa). For the control reaction, no template was added to the reaction mixture, resulting in no detectable fluorescence signal. The PCR conditions were as follows: initial denaturation for 10 s at 95 °C, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing and extension for 34 s at 62 °C. Each reaction was subjected to melting-point analysis to confirm the amplification of single products. RT-qPCR was carried out three times using different cDNA templates derived from different total RNA pools, in which each run contained at least two repetitions for each sample. Transcript levels were estimated using a standard curve based on a dilution series of the plasmid, and these levels were normalized against the *SAND* (Chao et al. 2012) or *HistoneH3* (Kotoda et al. 2010) transcript level in each sample, establishing a relative expression value. Pear *SAND* and *HistoneH3a* genes are deposited in DDBJ under accession numbers AB795982 and AB623164, respectively.

Isolation of the 5′ upstream region of MADS13-1 from 'Kosui' and 'Hengshanli'

To isolate the 5′ upstream region of *MADS13-1* from 'Kosui' and 'Hengshanli', we used a Universal Genome Walker Kit (Clontech, Palo Alto, CA, USA) according to the supplier's protocol. Gene-specific primers were designed (see Supplementary Table S1 available as Supplementary Data at *Tree Physiology* Online), and the PCR products (~3–5 kb in length) were cloned into the pCR®-XL-TOPO vector (Invitrogen) via the TA cloning method. Based on the 5′ upstream sequence, forward and reverse primers were designed to clone *DAM* promoters with their respective coding regions (~–2500 to +200 bp). PCR was performed in a total volume of 50 µl containing 50 ng of genomic DNA, 0.2 mM of dNTPs, 1.5 mM of $MgCl_2$, 0.3 µM of each primer, 1 U of KOD Plus Neo polymerase and 1× KOD Plus Neo buffer (Toyobo). Following a denaturation step at 94 °C for 2 min, a cycling profile of 98 °C for 10 s and 68 °C for 4 min was repeated 35 times. The product was cloned into the pCR®-Blunt vector (Invitrogen). Specific primers for isolation of the 5′ upstream region of *MADS13-1* are shown in Supplementary Table S1 available as Supplementary Data at *Tree Physiology* Online. After DNA sequencing, the promoter fragments were scanned using the PLACE database (www.dna.affrc.go.jp/PLACE, Higo et al. 1999) to identify known *cis*-acting regulatory elements.

Methylation status of the 5′ upstream region of MADS13-1 in 'Kosui' and 'Hengshanli'

The methylation status of the 5′ upstream region of *MADS13-1* was determined by quantitative analysis of DNA methylation using real-time PCR (qAMP) as described by Molnar et al. (2010). Briefly, 100 ng of genomic DNA from endodormant buds (14 December 2011) and ecodormant buds (26 January 2012) of 'Kosui' and 'Hengshanli' were incubated for 2 h with 3 U McrBC (TaKaRa) at 37 °C; buffer-only reactions were also incubated as negative controls. The reaction mixture (10 µl) contained 1.0 µl of digested sample (equivalent to ~5 ng of digested or undigested genomic DNA), 2.0 µM of each primer, 0.4 µl of ROX Reference Dye and 10 µl of SYBR® Green Premix ExTaq II (TaKaRa). Target regions were amplified by the following conditions: initial denaturation for 3 min at 94 °C, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension for 34 s at 72 °C. Specific primers for qAMP are shown in Supplementary Table S1 available as Supplementary Data at *Tree Physiology* Online. The percentage of methylation was determined according to the method of Oakes et al. (2006). DNA methylation is often found in inactive transposons; thus, the methylation level of retrotransposon *Ppcrt1* (AB550651) was evaluated as a control.

Isolation of a genomic clone of MADS13-1 from 'Kosui' and 'Hengshanli'

Since the linkage map of Japanese pear showed co-linearity with that of apple based on an SSR marker study (Yamamoto et al. 2002, 2007), the exon/intron structures of *DAM* homologues were assumed to resemble each other. Therefore,

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we designed primers to isolate genomic clones of 'Kosui' and 'Hengshanli' *MADS13-1* using putative *DAM* sequences from the apple draft sequence deposited in the GDR. Primer sets were first designed at each exon (see Supplementary Table S1 available as Supplementary Data at *Tree Physiology* Online). The PCRs for amplification of the second–fourth and fourth–seventh exons were performed in a total volume of 50 µl containing 50 ng of genomic DNA, 0.2 mM of dNTPs, 1.5 mM of $MgCl_2$, 0.3 µM of each primer, 1 U of KOD Plus Neo polymerase and 1× KOD Plus Neo buffer (Toyobo). Following a denaturation step at 94 °C for 2 min, a cycling profile of 98 °C for 10 s and 68 °C for 8 min was repeated 35 times. The product was cloned into the pCR®-Blunt vector (Invitrogen). The PCRs for amplification of the first–second and seventh–eighth exons were performed in a total volume of 50 µl containing 50 ng of genomic DNA, 10 mM of dNTPs, 1.25 U of Ex Taq and 1× Ex Taq buffer (TaKaRa). The PCR conditions were as follows: 98 °C for 10 s and 68 °C for 8 min was repeated 35 times. The product was cloned into the pCR®-XL-TOPO vector (Invitrogen).

Amplification of the first intron in pear genotypes

Amplification of the insertion in the first intron of *MADS13-1* genes was performed on genomic DNA from 10 pear genotypes ('Kosui', 'Hengshanli', 'Xiangshuili', *Pyrus calleryana*, 'Nijisseki', 'Niitaka', 'Kikusui', 'Imamuraaki', 'Beijing Baili' and 'Okusankichi'). Primer sets were designed based on the sequences found for cultivars 'Kosui' and 'Hengshanli' (see Supplementary Table S1 available as Supplementary Data at *Tree Physiology* Online). The PCR was performed in a total volume of 50 µl containing 50 ng of genomic DNA, 0.2 mM of dNTPs, 1.5 mM of $MgCl_2$, 0.3 µM of each primer, 1 U of KOD Plus Neo polymerase and 1× KOD Plus Neo buffer (Toyobo). Following a denaturation step at 94 °C for 2 min, a cycling profile of 98 °C for 10 s and 68 °C for 2 min was repeated 35 times. Similarity of the fragments amplified in four cultivars ('Kosui', 'Hengshanli', 'Niitaka' and 'Beijing Baili') was confirmed by sequencing.

Statistical analysis

The data were subjected to the one-way analysis of variance test or the *t*-test using R (2.9.0) (R Development Core Team 2009).

Results

Isolation of three MADS13 cDNAs from 'Kosui' and 'Hengshanli'

We have successfully isolated *PpMADS13-1*, *PpMADS13-2* and *PpMADS13-3* from 'Kosui' and *PpMADS13-1tw*, *PpMADS13-2tw* and *PpMADS13-3tw* from 'Hengshanli'. The nucleotide sequence similarity was 98% for *PpMADS13-1* and *PpMADS13-1tw*, 100% for *PpMADS13-2* and *PpMADS13-2tw*, and 98% for *PpMADS13-3* and *PpMADS13-3tw* (data not shown). The chromosome of Japanese pear was known to show co-linearity with the apple (Yamamoto et al. 2002, 2007). Thus, when putative apple *DAM* genes were first screened in the predicted CDS library provided by GDR, three candidate CDSs were identified, i.e., MDP0000322567, MDP0000527190 and MDP0000259294 in chromosomes 8 and 16 (Figure 1a; see Supplementary Table S3 available as Supplementary Data at *Tree Physiology* Online). High similarity (89%) was obtained between MDP0000322567 and *PpMADS13-1*. High similarity (88%) was observed between MDP000052790 and *PpMADS13-2*/*PpMADS13-3*. MDP0000259294 also showed relatively high similarity to *PpMADS13-1* (84%), *PpMADS13-2* (85%) and *PpMADS13-3* (85%), respectively, but we could not precisely annotate its relationship with pear *MADS13* because we have isolated only three pear *MADS13* genes at present. In fact, Southern blot hybridization showed 3–5 strong signals (Figure 1b). From the results of a phylogenetic study, the tribes Pyreae and Amygdaleae were classified into different clusters (Figure 1c), with the pear *MADS13* genes being more closely related to the putative *DAM* genes from apple rather than peach.

Expression of MADS13 during endodormancy of 'Kosui' and 'Hengshanli'

We investigated the dormancy status of 'Kosui' lateral leaf buds in terms of its sprouting ratio under forcing conditions at 25 °C. The sprouting ratio declined to 0% on 30 September, and no sprouting was observed until 18 November, followed by a sharp increase to 33, 91 and 100% on 15 December, 14 January and 16 February, respectively (Figure 2a). Expression of *PpMADS13-1* and *PpMADS13-2* began to increase on 9 September, reaching peak levels around 15 December (Figure 2b and c). By contrast, obvious induction of *PpMADS13-3* was not observed upon endodormancy establishment with expression maintained at a fairly constant level during the endodormancy stage (Figure 2d). After a short time of endodormancy release, the expression of all three genes decreased; therefore, the repression of *PpMADS13* genes nearly coincided with the growth resumption (Figure 2a–c). On the other hand, the sprouting ratio of 'Hengshanli' decreased slightly to 75% on 21 October but soon recovered to 89% on 18 November, reflecting the lower endodormancy trait of this cultivar (Figure 3a). Unfortunately, a primer set for RT-qPCR of *MADS13-2* and *MADS13-3* in 'Kosui' could not discriminate those in 'Hengshanli'. Here, we only investigated the expression of *PpMADS13-1tw* in 'Hengshanli'. The expression pattern of *PpMADS13-1tw* (Figure 3b) was similar to that of *PpMADS13-1* in 'Kosui' (Figure 2b); the expression level of *PpMADS13-1tw* peaked on 18 November, which lagged 1 month behind from the phase transition of growth resumption. Interestingly, the expression level of *PpMADS13-1tw* in 'Hengshanli' declined earlier

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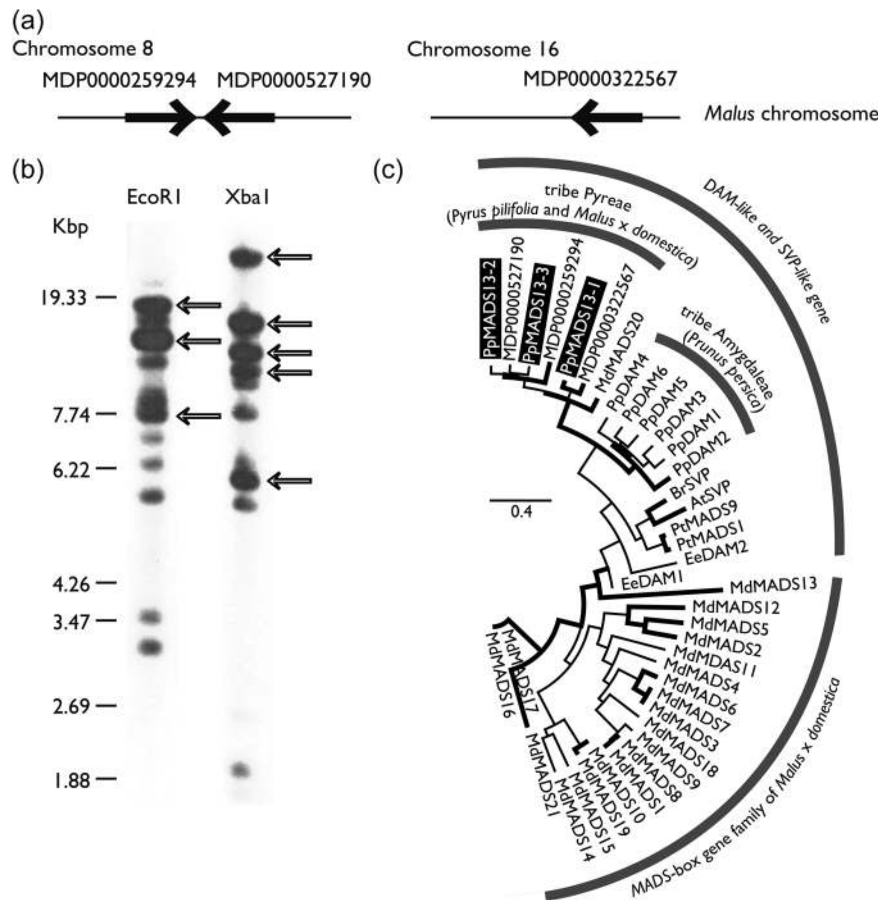

Figure 1. (a) Model of the *Malus MADS13*-like sequences annotated by GDR on their chromosomes. Arrows on the line (chromosome) represent *MADS13*-like genes; arrows pointing right indicate the plus strand and pointing left indicate the minus strand. (b) Southern blot hybridization showing the distribution of *MADS13* genes in the 'Kosui' genome. Ten micrograms of genomic DNA were digested with EcoRI and XbaI. The DIG-labelled partial *MADS13* fragment, *PpMADS13-1* (379 bp), was used as a hybridization probe. Positions of the molecular size markers are shown at the left. Strong bands are indicated with arrows. (c) Phylogenetic tree constructed based on the complete amino acid sequences of Japanese pear 'Kosui' PpMADS13-1, PpMADS13-2 and PpMADS13-3. A maximum likelihood rooted tree is shown. Thick lines indicate high support from both the maximum likelihood (>95) and neighbour-joining methods. *DAM*, *DAM*-like genes, *SVP*-like *MADS-box* genes, apple *MADS-box* genes deposited in the NCBI database and putative apple *DAM* genes deposited in GDR were used to assemble a phylogenetic tree (see Supplementary Table S2 available as Supplementary Data at *Tree Physiology* Online). At, *Arabidopsis thaliana* (L.) Heynh.; Br, *Brassica rapa* subsp. *campestris* (L.) A.R. Clapham; Ee, *Euphorbia esula* L.; Pt (MADS1), *Populus tomentosa* Carr.; Pt (MADS9), *Populus trichocarpa* Torr. & Gray.

than that of *PpMADS13-1* in 'Kosui' (Figures 2b and 3b). Results of the expression analysis of *MADS13* in the biological replicates of 'Kosui' and 'Hengshanli' during endodormancy using *HistoneH3a* as a reference gene are also shown in Supplementary Figures S1 and S2 available as Supplementary Data at *Tree Physiology* Online.

Expression of PpMADS13-1, PpMADS13-2 and PpMADS13-3 in response to HC and heat treatments

About 41% of the lateral leaf buds sprouted after HC treatment in contrast to only 4% sprouting in the control at 21 days after treatment (Figure 4a, inset), indicating that HC treatment enhanced bud release in 'Kosui'. In the control, expression of *PpMADS13-1*, *PpMADS13-2* and *PpMADS13-3* declined (Figure 4a–c), whereas a further significant reduction in the expression of these genes were recorded by HC treatment (Figure 4a–c). Thus, the increase in sprouting ratio by HC was inversely related to the reduction in *PpMADS13* expression. On the other hand, since Takemura et al. (2011) showed that lack of chilling inhibited the induction of bud dormancy in Japanese pear, we investigated the effect of heat treatment on *PpMADS13* expression. The expression of *PpMADS13-1*, *PpMADS13-2* and *PpMADS13-3* was normally induced upon endodormancy establishment (14 December) and decreased during endodormancy release (26 January) in the lateral leaf buds from field-grown control trees, while the expression of *PpMADS13-1*, *PpMADS13-2* and *PpMADS13-3* from buds treated with heat remained at almost constant levels without notable induction, albeit some significant differences were observed at some stages (Figure 5a–c). The results of the expression analysis of the biological replicate for *PpMADS13-1, PpMADS13-2* and *PpMADS13-3* in response to HC and heat treatments using *HistoneH3a* as a

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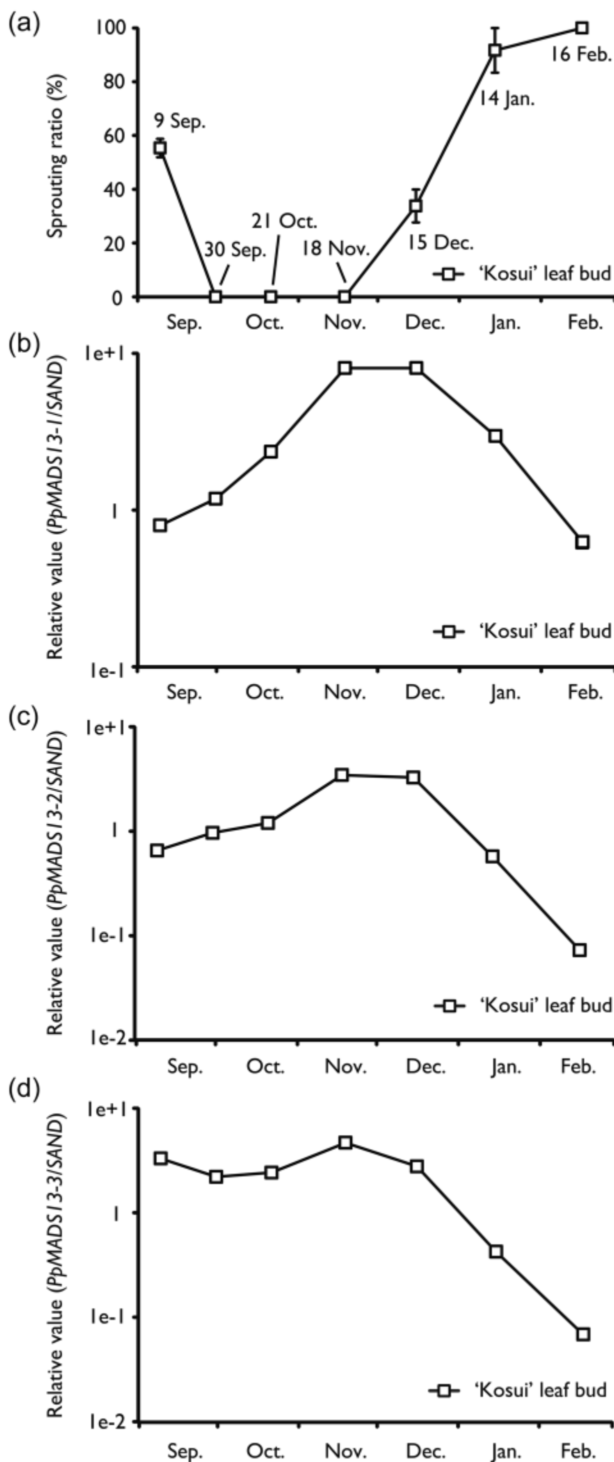

Figure 2. Changes in sprouting ratio (a) and relative expression levels of *PpMADS13-1* (b), *PpMADS13-2* (c) and *PpMADS13-3* (d) in the lateral leaf buds of 'Kosui' during endodormancy. Error bars are not shown because SE lies within the symbols. Data for the biological replicates are shown in Supplementary Figure S1 available as Supplementary Data at *Tree Physiology* Online.

reference gene are also shown in Supplementary Figures S3 and S4 available as Supplementary Data at *Tree Physiology* Online.

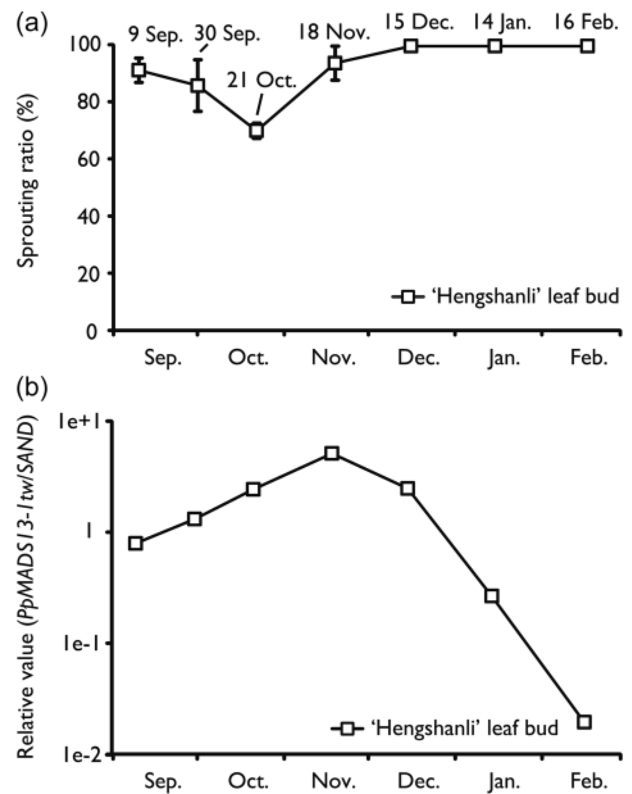

Figure 3. Changes in sprouting ratio (a) and relative expression levels of *PpMADS13-1tw* (b) in the lateral leaf buds of pear cultivar 'Hengshanli' (less chilling requirement) during endodormancy. Error bars are not shown because SE lies within the symbols. Data for the biological replicates are shown in Supplementary Figure S2 available as Supplementary Data at *Tree Physiology* Online.

Identification of putative cis-acting regulatory elements and methylation status in the 5′ upstream regions of MADS13-1 genes from 'Kosui' and 'Hengshanli'

A 2516 bp nucleotide position on the 'Kosui' *PpMADS13-1* upstream region of the translation start site was cloned and sequenced. The ~1500 bp upstream region of the translation start site was well conserved between 'Kosui' *PpMADS13-1* and the putative apple *DAM* gene. Based on the results of several sequences of 5′ rapid amplification of cDNA ends products, two putative TATA boxes were identified ~500 bp upstream of the translation start site in the *MADS13-1* promoter (Figure 6a). In silico analysis of putative transcription factor binding sites identified several cold gene expression-related *cis*-acting regulatory elements and a C-repeat/dehydration-responsive element (CRT/DRE) in the 'Kosui' *PpMADS13-1* putative promoter region (Figure 6a). We also isolated the 5′ upstream region of 'Hengshanli' *PpMADS13-1tw* that was 97% similar to that of 'Kosui'. There were no large differences in the *cis*-acting regulatory elements, including CRT/DRE found in the *PpMADS13-1* and *PpMADS13-1tw* genes (Figure 6a).

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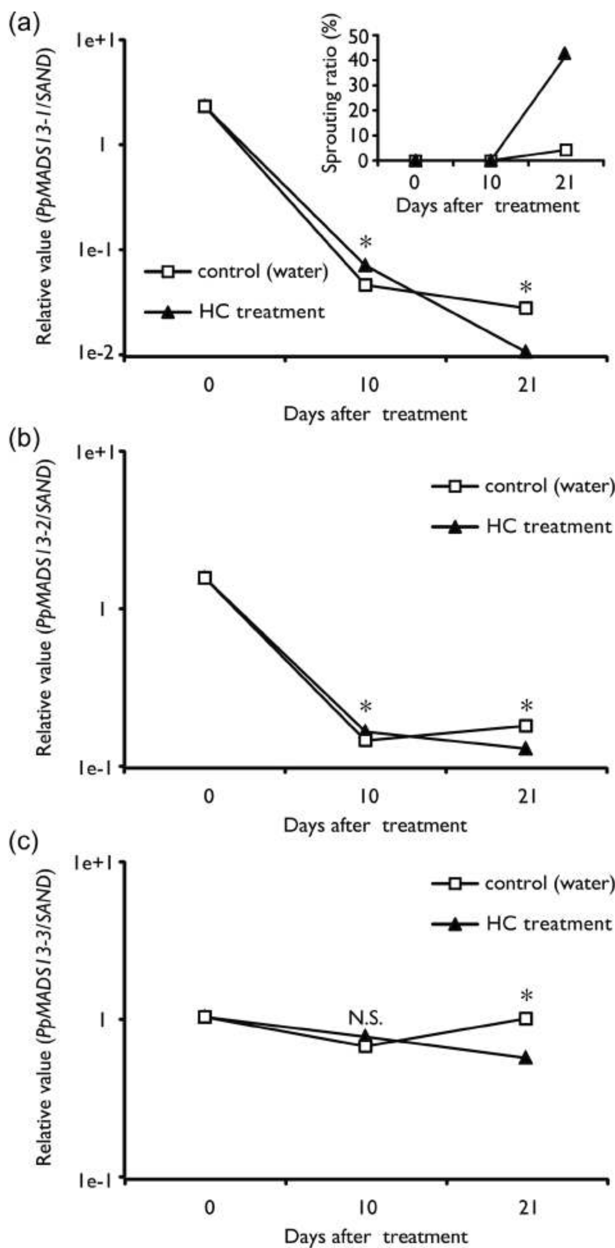

Figure 4. Relative expression levels of *PpMADS13-1* (a; the change in sprouting ratio is shown in the inset), *PpMADS13-2* (b) and *PpMADS13-3* (c) in lateral leaf buds after hydrogen cyanamide (HC) treatment of 'Kosui.' Succulent shoots of 'Kosui' were treated with HC on 19 December 2011. Distilled water was sprayed as the control. After treatment, all shoots were incubated at 25 °C, and gene expression was analyzed 0, 10 and 21 days after incubation. Asterisks indicate a significant difference at $P < 0.05$ versus the control at each date by the *t*-test. Error bars are not shown because SE lies within the symbols. Data for the biological replicates are shown in Supplementary Figure S3 available as Supplementary Data at *Tree Physiology* Online.

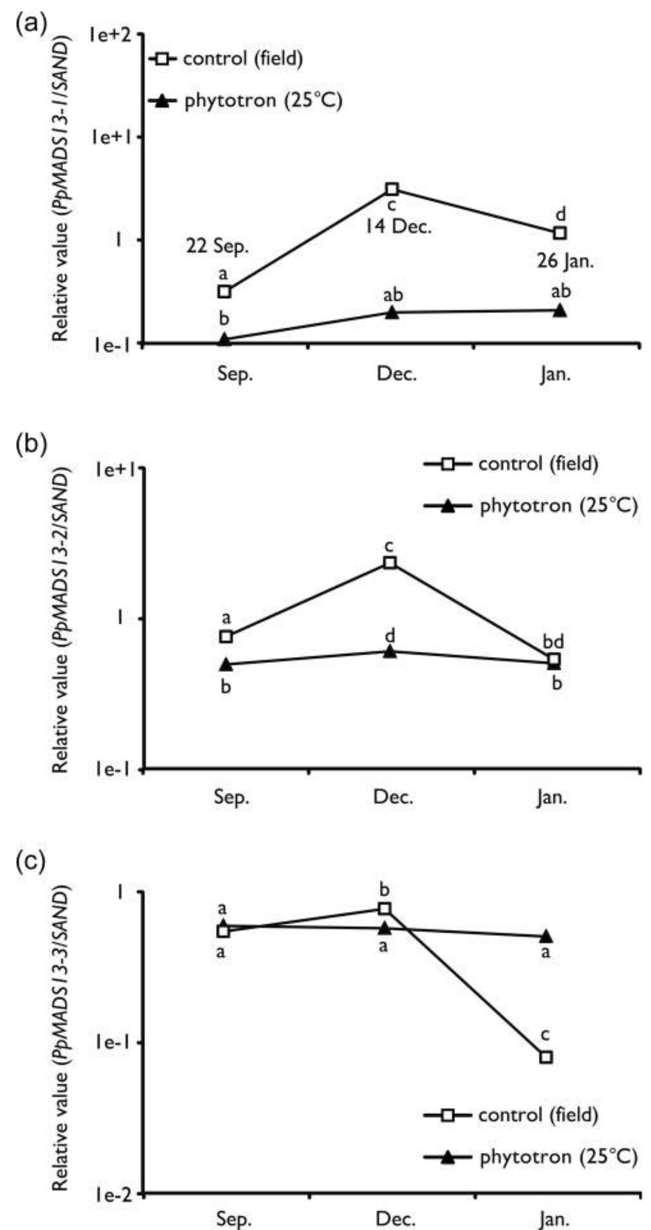

Figure 5. Relative expression levels of *PpMADS13-1* (a), *PpMADS13-2* (b) and *MADS13-3* (c) after prolonged heat treatment; 'Kosui' potted trees were transferred to the phytotron (25 °C) on 20 September 2011 and kept for 128 days under a natural photoperiod. Trees in the orchard were used as the control. Sampling was conducted 85 (14 December) and 128 (26 January) days after incubation. Identical letters are not significantly different at $P < 0.05$ by Tukey's test. Error bars are not shown because SE lies within the symbols. Data for the biological replicates are shown in Supplementary Figure S4 available as Supplementary Data at *Tree Physiology* Online.

Methylation levels in the 5′ upstream region of *PpMADS13-1* and *PpMADS13-1tw* varied from 0 to 41% and showed low levels around the putative TATA box region in both cultivars (Figure 6b). Contrastingly, the positive control gene, *Ppcrt1*, showed 78–96% methylation levels (Figure 6b), indicating that the relatively low methylation levels of both *PpMADS13-1* and *PpMADS13-1tw* were not due to the experimental method. Thus, the lower expression of 'Hengshanli' *PpMADS13-1tw* might not be due to repression of promoter activity by the methylation status of the promoter region of this gene.

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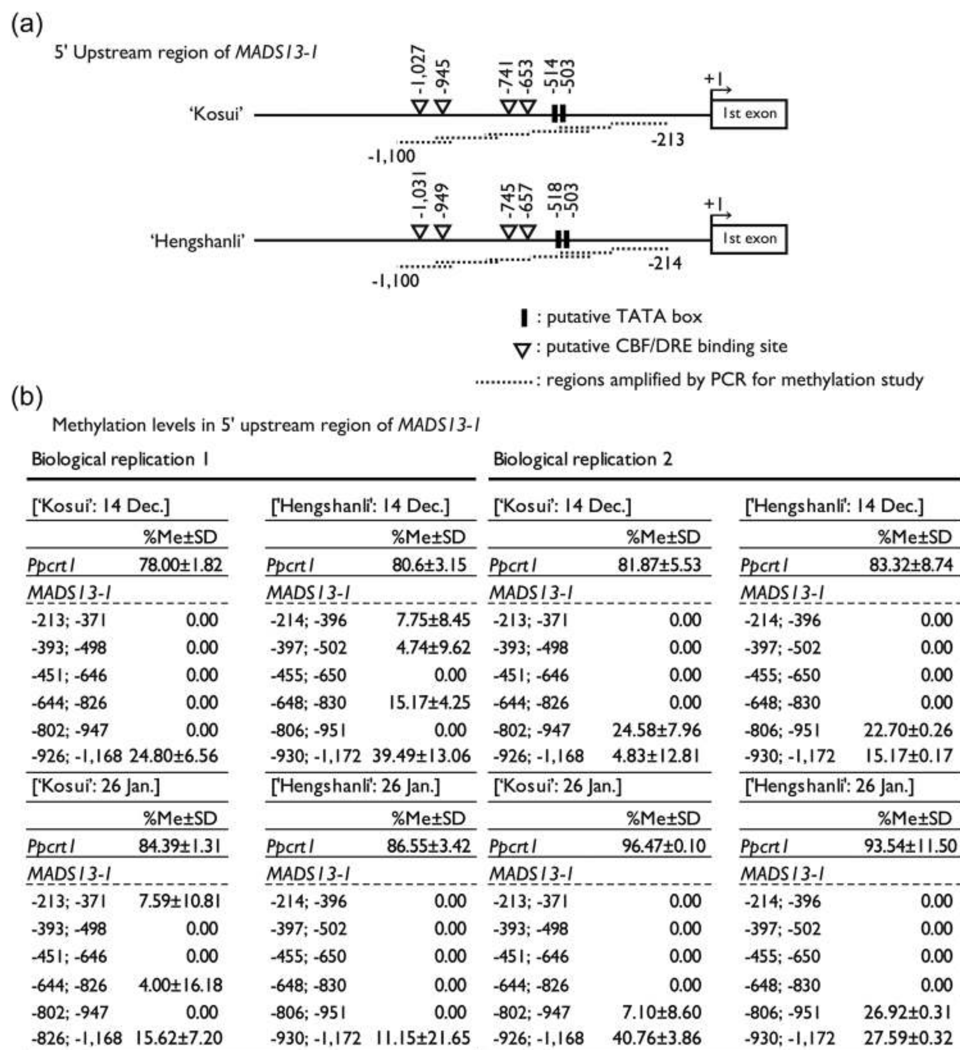

Biological replication 1

['Kosui': 14 Dec.]

	%Me±SD
Ppcrt1	78.00±1.82
MADS13-1	
-213; -371	0.00
-393; -498	0.00
-451; -646	0.00
-644; -826	0.00
-802; -947	0.00
-926; -1,168	24.80±6.56

['Hengshanli': 14 Dec.]

	%Me±SD
Ppcrt1	80.6±3.15
MADS13-1	
-214; -396	7.75±8.45
-397; -502	4.74±9.62
-455; -650	0.00
-648; -830	15.17±4.25
-806; -951	0.00
-930; -1,172	39.49±13.06

['Kosui': 26 Jan.]

	%Me±SD
Ppcrt1	84.39±1.31
MADS13-1	
-213; -371	7.59±10.81
-393; -498	0.00
-451; -646	0.00
-644; -826	4.00±16.18
-802; -947	0.00
-826; -1,168	15.62±7.20

['Hengshanli': 26 Jan.]

	%Me±SD
Ppcrt1	86.55±3.42
MADS13-1	
-214; -396	0.00
-397; -502	0.00
-455; -650	0.00
-648; -830	0.00
-806; -951	0.00
-930; -1,172	11.15±21.65

Biological replication 2

['Kosui': 14 Dec.]

	%Me±SD
Ppcrt1	81.87±5.53
MADS13-1	
-213; -371	0.00
-393; -498	0.00
-451; -646	0.00
-644; -826	0.00
-802; -947	24.58±7.96
-926; -1,168	4.83±12.81

['Hengshanli': 14 Dec.]

	%Me±SD
Ppcrt1	83.32±8.74
MADS13-1	
-214; -396	0.00
-397; -502	0.00
-455; -650	0.00
-648; -830	0.00
-806; -951	22.70±0.26
-930; -1,172	15.17±0.17

['Kosui': 26 Jan.]

	%Me±SD
Ppcrt1	96.47±0.10
MADS13-1	
-213; -371	0.00
-393; -498	0.00
-451; -646	0.00
-644; -826	0.00
-802; -947	7.10±8.60
-926; -1,168	40.76±3.86

['Hengshanli': 26 Jan.]

	%Me±SD
Ppcrt1	93.54±11.50
MADS13-1	
-214; -396	0.00
-397; -502	0.00
-455; -650	0.00
-648; -830	0.00
-806; -951	26.92±0.31
-930; -1,172	27.59±0.32

Figure 6. (a) Schematic representation of the locations of C-repeat/dehydration-responsive element (CRT/DRE) motifs in the putative promoter of *MADS13-1*. A line and white box indicate the 5′ upstream region and the first exon, respectively. The CRT/DRE motifs were identified using the PLACE database and are shown above the promoter sequence relative to the nucleotide position from the ATG start codon. The ATG start codon is designated as a black arrow. Black boxes indicate the putative TATA box. Dotted lines below the promoter sequence indicate the regions amplified by PCR for the methylation study. (b) Methylation status of the *MADS13-1* promoter. Two independent experiments were performed as biological replicates (right and left panels). The methylation levels of retrotransposon *Ppcrt1* (AB550651) were evaluated as a positive control.

Genomic structure of MADS13-1 from 'Kosui' and 'Hengshanli'

The genomic structure of *MADS13-1* from 'Kosui' and 'Hengshanli' consisted of eight exons and seven introns flanked with start and stop codons, which included EcoRI and XbaI cutting sites. The modular domains comprising MIKC-type proteins were reflected in each exon structure; a MADS domain was encoded by the first exon, an I region by the second and third exons, the K domain by the fourth—sixth exons and the C region by the seventh—eighth exons. When the corresponding genomic clones from 'Kosui' and 'Hengshanli' were compared, single nucleotide polymorphisms and differences in the number of repeated sequences were observed (data not shown). Notably, an additional sequence of 2317 bp that was absent in 'Kosui' *PpMADS13-1* was found in the first intron of 'Hengshanli' *PpMADS13-1tw*. To confirm if this insertion was related to the lower chilling requirement for endodormancy release in 'Hengshanli', the presence of the insertion in the first intron of *MADS13-1* was investigated among the 10 pear genotypes using a primer set amplified over the flanking region of 2317 bp. The CU for leaf bud endodormancy release in each genotype has been reported previously (Tamura et al. 2001, Takemura et al. 2012). Unexpectedly, the designed primer amplified both the 1076 and 3218 bp fragments in 'Kosui' (Figure 7b). The same amplification pattern observed in 'Kosui' was obtained in 'Niitaka' and 'Okusankichi', while the other six genotypes showed one band, which possibly corresponds to the band from 'Hengshanli' *PpMADS13-1tw* type with 3218 bp (Figure 7c).

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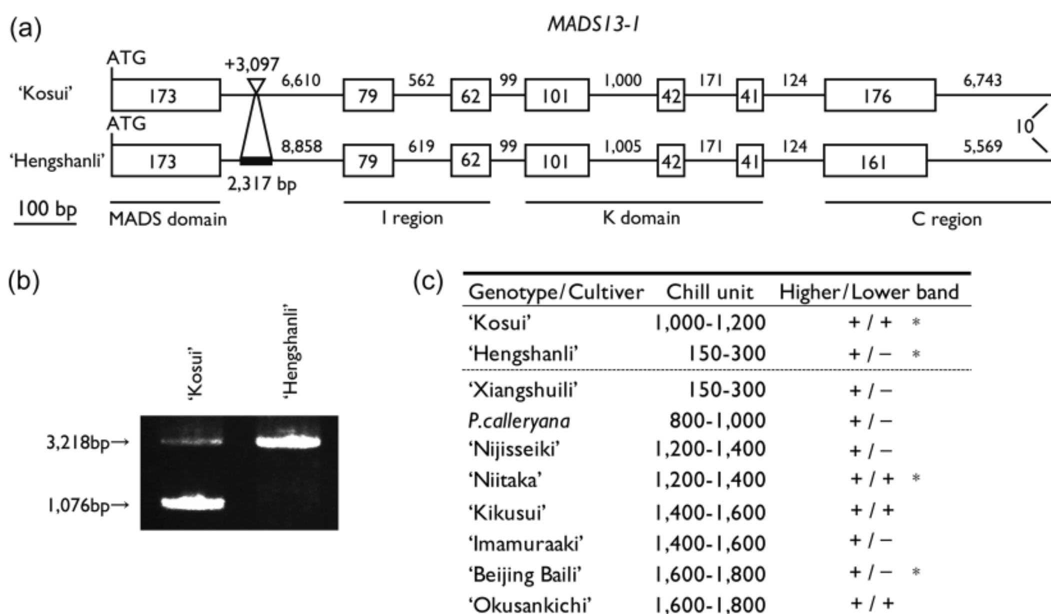

Genotype/Cultiver	Chill unit	Higher/Lower band
'Kosui'	1,000-1,200	+ / + *
'Hengshanli'	150-300	+ / − *
'Xiangshuili'	150-300	+ / −
P.calleryana	800-1,000	+ / −
'Nijisseiki'	1,200-1,400	+ / −
'Niitaka'	1,200-1,400	+ / + *
'Kikusui'	1,400-1,600	+ / +
'Imamuraaki'	1,400-1,600	+ / −
'Beijing Baili'	1,600-1,800	+ / − *
'Okusankichi'	1,600-1,800	+ / +

Figure 7. (a) Schematic overview of *MADS13-1* introns and exons in 'Kosui' and 'Hengshanli'. Exons and introns are designated as boxes and lines, respectively. The MADS domain, I region, K domain and C region are indicated below the schematic drawing. (b) Polymerase chain reaction amplification of the insertion in the first intron of *MADS13-1* in 'Kosui' and 'Hengshanli'. (c) Summary of the insertion in the first intron among pear genotypes. The sequences of bands with asterisks were confirmed by sequencing. Chill units (CUs) were retrieved from Tamura et al. (2001) except for 'Hengshanli' and 'Xiangshuili'. The CUs of 'Hengshanli' and 'Xiangshuili' were from Takemura et al. (2012) and Sato et al. (unpublished data), respectively.

Discussion

Characterization of MADS13 expression during endodormancy phase transition

In this study, we have shown that the expression of two *DAM* genes, namely *PpMADS13-1* and *PpMADS13-2*, was up-regulated towards endodormancy establishment and down-regulated towards endodormancy release in Japanese pear 'Kosui' (Figure 2a–c). In contrast, an obvious induction of *PpMADS13-3* expression was not observed upon endodormancy establishment; *PpMADS13-3* expression was maintained at a constant level from autumn to the onset of endodormancy release, although down-regulation of *PpMADS13-3* expression was also observed similar to *PpMADS13-1* and *PpMADS13-2* (Figure 2d). The function of *PpMADS13-3* may be different from the other two 'Kosui' *MADS13* genes, although the precise function of *PpMADS13-3* is not yet known. Peach *DAM1*, *DAM3* and *DAM4*, which are closely related with growth cessation, were up-regulated and their expression levels peaked in September (Li et al. 2009). Indeed, the induction of *PpMADS13-3* expression in another year was recorded at the end of July (see Supplementary Figure S5 available as Supplementary Data at *Tree Physiology* Online). Thus, *PpMADS13-3* expression resembled those of peach *DAM1*, *DAM3* and *DAM4*.

When comparing the *MADS13-1* expressions in 'Kosui' and 'Hengshanli', similar expression patterns were observed in both cultivars without large differences in *MADS13-1* expression levels until endodormancy establishment (Figures 2b, 3b). In contrast, the expression level of *PpMADS13-1tw* in 'Hengshanli' declined earlier than that of *PpMADS13-1* in 'Kosui' towards endodormancy release (Figures 2b and 3b), which may be ascribed to the difference in chilling requirement between 'Kosui' and 'Hengshanli' as proposed in peach and Japanese apricot, in which the threshold of expression levels for an inhibitory effect of growth resumption was dependent on the cultivars with different chilling requirements for bud release (Yamane et al. 2011*b*). On the other hand, the trend of *MADS13-1* expression nearly, but not completely, coincided with the changes in sprouting ratio, which may indicate the involvement of other factors besides *MADS13-1* in the regulation of endodormancy phase transitions. Recent work on apricot (*Prunus armeniaca* L.) demonstrated that a QTL for chilling requirement was associated with the allelic composition of *ParSOC1*, a homologue of *Arabidopsis AGL20* (Trainin et al. 2013). Thus, although the presence of other regulators may be assumed in pear, *MADS13* must be one of the key regulators for endodormancy phase transitions as suggested in other plants (Horvath et al. 2010, Jiménez et al. 2010, Yamane et al. 2011*a*).

Characterization of DAM expression after HC and heat treatments

A lower expression of *MADS13* was observed in the lateral leaf buds treated with HC than that with water (control) concomitant with the acceleration of endodormancy release in 'Kosui' (Figure 4a–c). Enhanced bud release after HC treatment and the

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simultaneous down-regulation of *DAM* gene expression was also shown in peach (Yamane et al. 2011*a*). Thus, the close relationship between the expression of pear *DAM* genes and the transition of endodormancy was again confirmed in pear. On the other hand, peach *DAM5* and *DAM6* were shown to be induced by a short photoperiod and be down-regulated when exposed to chilling temperatures (Li et al. 2009, Jiménez et al. 2010). When the potted 'Kosui' trees were maintained at 25 °C in the phytotron under a natural photoperiod from autumn to winter, induction of *PpMADS13-1*, *PpMADS13-2* and *PpMADS13-3* expression was not observed on 14 December even under short-day length, one of the crucial environmental factors for endodormancy establishment, which implies that expression of 'Kosui' *MADS13* was mainly regulated by low temperature, not the photoperiod (Figure 5a–c). Indeed, growth cessation, dormancy induction and release are known to be regulated by low temperature, but not photoperiod in pear (Heide and Prestrud 2005, Takemura et al. 2011). Recently, Yamane et al. (2011*a*) found that ambient cool temperatures in early autumn (September) stimulate peach *DAM5* and *DAM6* expression. Peach *DAM* could be responsive to both short photoperiods and cool temperatures for dormancy establishment, and the different responses of *DAM* genes to photoperiod observed between pear and peach may be ascribed to the divergent evolution of the tribes Pyreae and Amygdaloideae. However, we cannot rule out the possibility of the involvement of photoperiod in growth cessation and endodormancy establishment because pear transcriptome analysis during endodormancy transition of leaf buds using a 10 K cDNA microarray identified several light- and photoperiod-related genes such as NAC domain transcription factor and pseudo-response regulator (Nishitani et al. 2012).

Comparison of 'Kosui' PpMADS13-1 and 'Hengshanli' PpMADS13-1tw 5′ upstream regions and methylation status

To identify potential transcriptional regulators involved in *MADS13-1* expression, we identified putative transcription factor binding sites. Analysis of the ~1000 bp 5′ upstream region of the *MADS13-1* genes revealed the presence of up to four CRT/DRE motifs (Figure 6a), among which CBF/DRE binding sites at 741 and 945 bp possess the CCGAC sequence. The presence of CRT/DRE motifs in promoters enhances or modulates target gene expression by DNA-binding proteins belonging to the AP2/ERF family, such as CBF or DREB, both of which are defined as major regulators of the cold tolerance pathway in *Arabidopsis* (Cook et al. 2004). The presence of these transcription binding sites was also reported in the putative promoter regions of leafy spurge and peach *DAM* genes (Horvath et al. 2010, Yamane et al. 2011*a*). Interestingly, the induction of *CBF*-like genes during endodormancy coincided with the induction of leafy spurge *EeDAM1* (Horvath et al. 2008). Thus, the possibility that cold-responsive pear *MADS13-1* expression was controlled by CBF protein was also investigated; however, there were no large differences in the number or positions of CRT/DRE motifs in the putative promoter regions of the *MADS13-1* genes (Figure 6a) despite their different expression levels observed during December to February in 'Kosui' and 'Hengshanli' (Figures 2b and 3b).

As another possible modulator of *MADS13-1* expression, the methylation status in the putative promoter region of the *MADS13-1* genes was investigated by qAMP (Figure 6b). In chestnut (*Castanea sativa* Mill.), global methylation levels of genomic DNA declined during the transition from dormancy to bud burst (Santamaría et al. 2009). Methylation of genomic DNA prevents movement of RNA polymerases along DNA strands and is associated with repression of transcription (Zilberman et al. 2007). Ubi et al. (2010) already reported the methylation status of the genome sequences of *PpMADS13-1* and *PpMADS13-2* including their coding regions, but changes in methylation status were not coincident with changes in the expression of *PpMADS13-1* and *PpMADS13-2*. On the other hand, DNA methylation in the putative promoter region is known to interfere with transcription factor access and is associated with gene silencing or a reduction in transcription (Zeisel 2012). However, relatively low methylation levels were detected in the putative promoter regions of 'Kosui' *PpMADS13-1* and 'Hengshanli' *PpMADS13-1tw* towards ecodormancy (Figure 6b) without consistently correlating with different expression levels at this stage (Figures 2b and 3b). It was reported that the expression of a *MADS-box FLOWERING LOCUS C* was regulated via chromatin remodelling at low temperature in *Arabidopsis* (Heo and Sung 2011). Chromatin remodelling of *DAM* has already been reported in leafy spurge and peach (Horvath et al. 2010, Leida et al. 2012). Interestingly, histone acetyl transferase, one of the key enzymes for chromatin remodelling, was known to be recruited by CBF protein in *Arabidopsis* (Amasino 2004). The CBF binding site in the *DAM* promoter was found in leafy spurge, peach and Japanese pear. Thus, the interaction of the *DAM* gene and the CBF protein including chromatin remodelling may modulate *DAM* gene expression (Horvath 2009).

Genomic structure of pear MADS13-1 from 'Kosui' and 'Hengshanli'

The screening of putative apple *DAM* genes in GDR indicated that three *MADS13*-like genes were annotated in chromosomes 8 and 16 (Figure 1a). Considering the similarity of the genomic structure between apple and Japanese pear, a similar genomic structure was expected. Both *MADS13-2* and *MADS13-3* were annotated to the same putative apple *DAM* gene MDP000052790 in chromosome 8, while *MADS13-1* was annotated to MDP0000322567 in chromosome 16 (Figure 1c). In addition, MDP0000259294 of chromosome 8 also showed similarity to *MADS13-2* and *MADS13-3* (Figure 1c). These results suggested the possibility of the

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presence of other *DAM* genes showing high similarity to apple MDP0000259294 and/or of an allelic relationship between *MADS13-2* and *MADS13-3*. Indeed, Southern hybridization revealed 3–5 strong signals, suggesting the presence of other *MADS13* genes in the pear genome (Figure 1b), although it is impossible to determine the true copy number of *DAM* genes by Southern hybridization alone. Recently the draft genome of the Chinese pear (*Pyrus bretschneideri* Rehd.) was reported (Wu et al. 2013), which would help clarify the detailed genomic structure of *MADS13* genes in the tribe Pyreae.

Japanese pear 'Kosui' requires 1000–1200 CU for endodormancy release (Tamura et al. 2001), whereas fewer than 200 CU is sufficient for 'Hengshanli' (Takemura et al. 2012). We examined whether these differences could be ascribed to differences in the genomic structure of 'Kosui' *PpMADS13-1* and 'Hengshanli' *PpMADS13-1tw*. Sequence analysis revealed a 2317 bp insertion in the first intron of 'Hengshanli' *PpMADS13-1tw* (Figure 7a). Similarly, the presence of a relatively long insertion in the first intron of peach *DAM5* (598 bp) and *DAM6* (2604 bp) was found and assumed to affect the expression levels of the *DAM* genes in the low-chill peach cultivar 'Okinawa' (Yamane et al. 2011*b*). Therefore, to elucidate the relationship between insertions in the first intron of *MASDS13-1* and dormancy characteristics, the presence of the insertion was investigated in several pear genotypes by PCR using a primer set amplified over the flanking region of 2317 bp. Unexpectedly, the designed primer amplified both 1076and 3218 bp fragments in 'Kosui', while 'Hengshanli' possessed only the 3218 bp fragment. Therefore, the two bands amplified in 'Kosui' would be allelic, in which 'Kosui' possesses the allelic forms of *PpMADS13-1* type and *PpMADS13-1tw* type, and 'Hengshanli' has two sets of *PpMADS13-1tw* type (Figure 7b). The high-chill cultivars, 'Imamuraaki' (1400–1600 CU) and 'Beijing Baili' (1600–1800 CU), showed similar allelic structure to the low-chill 'Hengshanli', demonstrating that an insertion in the first intron might not be related to dormancy characteristics but simply show the genetic variations of pear genotypes (Figure 7c). The observation that the moderate-chill cultivars, 'Kosui' (1000–1200 CU) and 'Niitaka' (1200–1400 CU), also possessed the higher band corresponding to 3218 bp of 'Hengshanli' lends further support to this conclusion (Figure 7c). Since we only used a limited number of pear genotypes, confirming this conclusion will necessitate further studies using more pear genotypes that differ in their chilling requirement along with their expression of *MADS13-1*.

Conclusion

We have shown an inverse relationship between the expression patterns of pear *MADS13* genes and endodormancy phase transition. A pear cultivar with a lower chilling requirement, 'Hengshanli', showed earlier reduction of *PpMADS13-1* expression level compared with 'Kosui' towards endodormancy release. To determine the factors responsible for the difference in *MADS13-1* expression level observed between 'Kosui' and 'Hengshanli', we investigated *cis*-acting regulatory elements, the methylation status of the putative promoter region of *MADS13-1* genes and an insertion in the first intron of *MADS13-1*, but found no large differences. However, we cannot rule out the possibility that these factors are involved in regulated expression of other *MADS13* genes including *MADS13-2* and *MADS13-3*.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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