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Expression of *DORMANCY-ASSOCIATED MADS-BOX* (*DAM*)-like genes in apple

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Abstract

Apple (*Malus* × *domestica* Borkh.) is a perennial woody plant that undergoes a period of dormancy (in cv. Jonathan between late September and mid-December) to survive freezing temperatures of winter. *DORMANCY-ASSOCIATED MADS-BOX* (*DAM*) genes play important roles in the regulation of growth cessation and terminal bud formation in peach. To understand the role of *DAM* orthologs in apple, we isolated and characterized four *DAM*-like genes (designated as *MdDAMa*, *MdDAMb*, *MdDAMc*, and *MdDAMd*) and monitored their expression in apical buds throughout the season by real-time quantitative polymerase chain reaction analyses. The transcription of *MdDAMa* peaked in October and that of *MdDAMc* was elevated from August to October, whereas *MdDAMb* and *MdDAMd* were practically undetectable. The tandemly arranged genes *MdDAMa*/*MdDAMb* and *MdDAMc*/*MdDAMd* were localized to chromosomes 16 and 8, respectively. Based on these observations, we infer that *MdDAMa* and *MdDAMc* acted in a dominant fashion on each locus and were correlated with the period of endodormancy.

Additional key words: gene expression, *Malus* × *domestica*, RT-qPCR.

Introduction

Apple (*Malus* × *domestica* Borkh.) is adapted to mid-latitude climates with annual growth cycles consisting of dormant and active growth phases. Low winter temperatures are required to align the growth rhythm with the annual cycle. There are three categories of dormancy in boreal and temperate zone woody plants: para-dormancy, endodormancy, and ecodormancy (Lang *et al.* 1985, Welling and Palva 2006, Horvath 2009). The paradormancy, also known as summer dormancy, is synonymous with apical dominance. The endodormancy, also termed as physiological dormancy, begins in early to mid-autumn, maintains during winter, and then gradually releases (Kuroda and Sagisaka 2001, this paper). The ecodormancy prevents the premature breaking of buds that are released from the physiological dormancy and have achieved full growth competence, preventing a frost damage from late winter to early spring.

The vernalization pathway promotes flowering in annual plants such as the *Brassica* species that are exposed to prolonged winter temperatures. Although the MCM1-ARG80-AGAMOUS-DEFICIENS-SRF (MADS)-BOX transcription factor encoded by the *FLOWERING LOCUS C* (*FLC*) down-regulates the *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* [*SOC1* (*AGL20*), *MADS-BOX* gene] and the *FLOWERING LOCUS T* [*FT*, coding a phosphatidyl-ethanolamine binding protein

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Abbreviations: *CBF* - *C-REPEAT BINDING FACTOR*; *CENL1* - *CENTRORADIALIS-LIKE1*; *FLC* - *FLOWERING LOCUS C*; GDR - genome database for *Rosaceae*; *FT* - *FLOWERING LOCUS T*; *Md* - *Malus* × *domestica*; PEBP - phosphatidyl-ethanolamine binding protein; PlnTFDB - plant transcription factor database; QTL - quantitative trait locus; RT-qPCR - real time quantitative polymerase chain reaction; *SVP* - *SHOOT VEGETATIVE PHASE*.
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(PEBP)] to integrate the effect of vernalization on a flowering initiation in *Arabidopsis* (Lee *et al.* 2007, Li *et al.* 2008), *FLC* ortholog genes in *Rosaceae* have not been reported. Apple and peach endodormancy appear to respond to a cold temperature, that of peach is controlled by two cues, *i.e.*, a cold temperature and a short day length (Wisniewski *et al*. 2011, Artlip *et al*. 2013).

The mutant *evergrowing* (*evg*) of peach fails to enter dormancy and terminates growth under decreasing temperatures in the cold season (Wang *et al.* 2002). The *EVG* locus has been identified and contains six *MADS-BOX* genes of the *SHOOT VEGETATIVE PHASE* (*SVP*)/*AGL24* gene family that are arranged in tandem and show a high degree of sequence similarity to each other (similarities in the amino acid sequence 74 - 86 %) and thus appear to have arisen by gene duplication (Bielenberg *et al.* 2004, 2008). These genes are known as *DORMANCY-ASSOCIATED MADS-BOX1-6* (*DAM1-6*) (Bielenberg *et al.* 2004, 2008, Jiménez *et al.* 2009, Li *et al.* 2009). Poplar overexpressing constitutively *PmDAM6* of *Prunus mume* exhibits growth cessation, terminal bud set, and lateral bud endodormancy under a long (14-h) day and a temperature of 22 °C, suggesting that *PmDAM6* is involved in growth regulation and dormancy induction (Sasaki *et al.* 2011).

Inactive *DAM6* chromatin is induced by histone H3 lysine 27 trimethylation and down-regulates its gene expression, and then releases dormancy, as similarly observed in the gene regulation by histone modifications of *FLC* and other regulatory genes of vernalization in several grass plants (Horvath *et al.* 2010, Leida *et al.* 2012).

The peach *DAM* genes were first characterized, and dormancy mechanisms were subsequently studied in other perennial plants, such as *Rubus idaeus*, *Euphorbia esula*, *Prunus mume*, *Pyrus pyrifolia*, *Poncirus trifoliata*, and *Actinidia* spp. (Mazzitelli *et al.* 2007, Horvath *et al.* 2008, Yamane *et al.* 2008, Li *et al.* 2010, Ubi *et al.* 2010, Wu *et al.* 2012, Saito *et al.* 2013). An accumulating evidence for tree species suggests that the mechanisms of dormancy resemble those underlying vernalization at the molecular level.

The aim of this study was to identify the orthologs of *DAM* genes in apple and to analyze their expression patterns along the growth-dormancy cycle.

Materials and methods

Plants and sprouting tests: Samples from fruit-bearing terminal buds on 2-year-old branches of apple (*Malus* × *domestica* Borkh.) cultivars Jonathan and Fuji were collected from an experimental field at the NARO Institute of Fruit Tree Science in Morioka, Japan (latitude 39.7 ° N, longitude 141.1 ° N).

For a sprouting test, seven fruit-bearing shoots (length > 30 cm) of cv. Jonathan were collected in years 2011 - 2012 and 2012 - 2013 and placed in test tubes containing a distilled water with 0.3 % (m/v) sodium hypochlorite. These were then maintained at a temperature of 25 °C, a 16-h photoperiod, a low irradiance of 0.2 μmol m^{-2} s^{-1}, and a relative humidity of 60 %. The distilled water in the vials was changed at 1-week intervals. The dormancy status was defined as the stage at which green leaves emerged after 30 d.

Database searching and comparison of *DAM* homolog genes: Sequence database searching was performed using *BLAST2* from the National Center for Biotechnology Information (*NCBI*, http://www.ncbi.nlm.nih.gov/), *Genome Database for Rosaceae* (*GDR*, Velasco *et al.* 2010, http://www.rosaceae.org/), and *Plant Transcription Factor Database v. 2.0* (*PlnTFDB*, Zhang *et al.* 2011, Center for Bioinformatics, the Peking University, China, http://planttfdb.cbi.pku.edu.cn/). Amino acid sequences were analyzed using the *ClustalX* multiple sequence alignment program *v. 1.83* (Jeanmougin *et al.* 1998). The settlement of multiple parameters was based on the following settings: gap opening, 10.0; gap extension, 0.2; delay divergent sequence, 30; protein weight matrix, *Gonnet 250*; output by clustering neighbor-joining as random number generator seed, 111; and number of bootstrap trials, 1 000. A sequence alignment was generated using *GeneDoc* (Nicholas *et al.* 1997), and a phylogenetic tree was displayed using the *Njplot* program (Perrière and Gouy 1996).

Screening and cloning cDNAs: To isolate full-length cDNAs of *DAM* homologs, we screened an apple cv. Fuji cDNA library that was constructed using *Lambda FIX® II/Xho I Partial Fill-In Vector* kit (*Stratagene*, La Jolla, CA, USA). mRNAs were extracted from fruit-bearing buds collected during July, September, and November. Approximately 1.5 × 10^5 plaques were screened with *MdDAMa* PCR fragment as a probe. Nucleotide sequences were determined using *DTCS Quick Start* kit for dye-terminator cycle sequencing (*Beckman Coulter*, Fullerton, CA, USA) and an automated DNA sequencer *CEQ 8000* (*Beckman Coulter*). To obtain the *DAM* homolog genes, a PCR amplification was performed with Fuji first-strand cDNAs. The PCR-products were cloned into the *pT7Blue T-Vector* (*Novagen*, Darmstadt, Germany), and then sequenced.

Expression analyses by real-time quantitative PCR: For expression analyses, total RNA samples were extracted from over 10 fruit-bearing buds from three

different trees (cv. Jonathan) that were collected during a period from June or July to next April in 2006 - 2007 and in 2009 - 2010. First-strand cDNAs were synthesized from 2 μg of total RNA in 0.040 cm^3 of a reaction mixture using *ReverTra Ace* kit (*Toyobo*, Tokyo, Japan). For the 2006 - 2007 samples, real-time quantitative polymerase chain reaction (RT-qPCR) analyses were performed with *Applied Biosystems 7500 Real-Time PCR System* (*Applied Biosystems*, Foster City, CA, USA). RT-qPCR reactions were performed with 0.15 μg of the first-strand cDNA and *GeneAce SYBR® qPCR Mix α Low ROX* (*Nippon gene*, Tokyo, Japan) in a total volume of 0.010 cm^3. Thermal cycler programs were as follows: 95 °C for 10 min followed by 40 cycles of 98 °C for 15 s and 62 °C for 34 s. The software analysis of absolute quantitation was performed with the sequence detection software *v. 2.0* (*Applied Biosystems*). For the 2009 - 2010 samples, RT-qPCR analyses were also performed with the *Applied Biosystems 7300 Real-Time PCR System* (*Applied Biosystems*). RT-qPCR reactions were performed with 0.15 μg of the first-strand cDNA mixture as a template and *power SYBR Green Master mix* (*Applied Biosystems*) in a total volume of 0.012 cm^3. Thermal cycler programs were as follows: 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The software analysis of absolute quantitation was performed with the sequence detection software *v. 1.3.1* (*Applied Biosystems*). Primer sets for RT-qPCR analyses are shown in Table 2. Standards were determined with a diluted plasmid containing each cloned target sequence, respectively. The quantitative value of the target transcripts was calculated as the coefficient of variation, and normalized to the transcript level of *SAND* (acc. No. in *GDR*: MDP0000088431, Chao *et al.* 2012, Saito *et al.* 2013, Imai *et al.* 2014), *UBIQUITIN* (U74358, Takos *et al.* 2006, Imai *et al.* 2014, Tanaka *et al.* 2014), *HISTONE H3* (AY347801, Kotoda *et al.* 2010, Imai *et al*. 2014), or the average of these three genes, respectively. The average gene-specific variation for the three genes, *SAND*, *UBIQUITIN*, and *HISTONE H3*, was calculated using an equation described by Vandesompele *et al.* (2002).

Table 1. Summary of apple *DAM*-like genes. Sequence database searches were performed in *GDR* and *PlnTFDB* (* indicates the region located on each chromosome by mega base pair).

Clone name	No. of obtained cDNAs (phage/PCR)	EST clones	Contig No.	Chromosome No.
MdDAMa	5/-	Mdo003921	MDC020688.360	16 (17.74 M*)
MdDAMb	0/8	ADL36743, Mdo014533	MDC012498.125	16 (17.71 M)
MdDAMc	1/4	-	MDC008471.150	8 (23.86 M)
MdDAMd	0/0	-	MDC020948.189	8 (23.93 M)
MdSVPa	0/2	DQ402055, HM122599, Mdo003914	MDC016474.222	11 (18.05 M)
MdSVPb	0	-	MDC017035.448	4 (0.414 M)

Table 2. PCR primer sequences for gene expression analyses.

Primers	Oligonucleotide sequence
MdDAMacF (common)	5′-ATCGACTACTTGCCGGCAAGGC-3′
MdDAMaR	5′-GGTTGATCCGATTTTTCCCCACCGA-3′
MdDAMcR	5′-CAAGCATCGATTGATTCGATTTTTCCCCAG-3′
SAND	5′-CCCAGGACTTTGAGCTTTATGC-3′ (forward)
(MDP0000088431)	5′-TATCACCATGAAAAGGGGCTTG-3′ (reverse)
UBIQUITIN	5′-CTCCGTGGTGGTTTTTAAGT-3′ (forward)
(U74358)	5′-GGAGGCAGAAACAGTACCAT-3′ (reverse)
HISTONE H3	5′-GTCAAGAAGCCCCACAGATAC-3′ (forward)
(AY347801)	5′-CTGGAAACGCAGATCAGTCTTG-3′ (reverse)

Results and discussion

For apple, a perennial woody plant in temperate zones, dormancy is important to prevent winter frost damage and to maintain the annual growth rhythm. Here we identify that the period for a deep endodormancy of cv. Jonathan is between late September and mid-December (Fig. 1). To elucidate the relationship of apple *DAM*-like genes and dormancy, we identified the *DAM*-like sequences and analyzed their expressions throughout the season. These sequences were designated as *MdDAMa*, *MdDAMb*, *MdDAMc*, *MdDAMd*, *MdSVPa*, and *MdSVPb*.

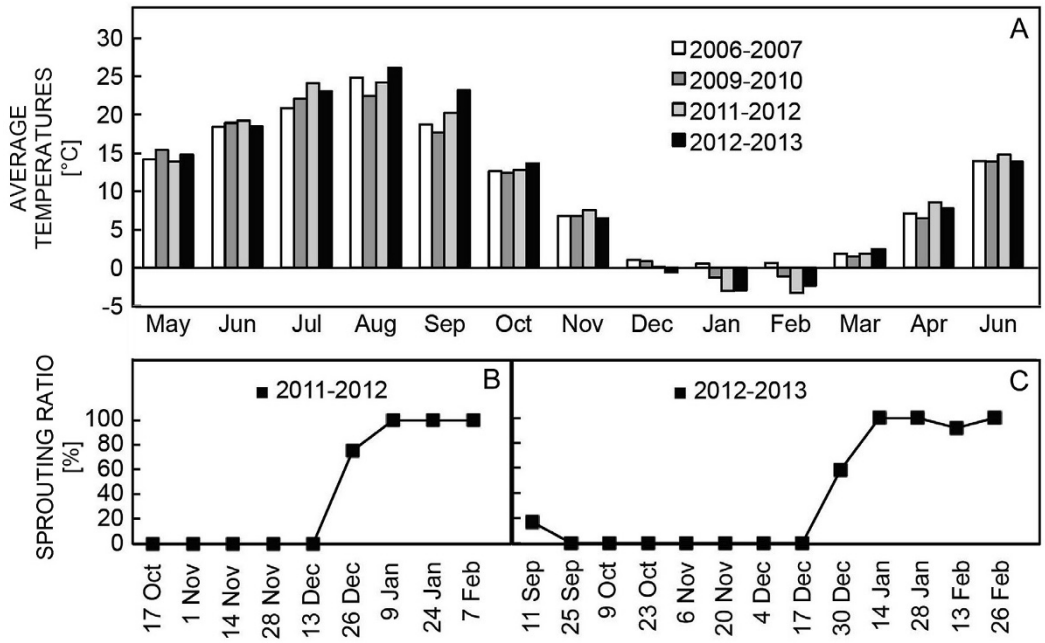

Fig. 1. Average temperatures in Morioka, Japan (*A*), and the changes in a sprouting ratio in apple cv. Jonathan (*B*,*C*).

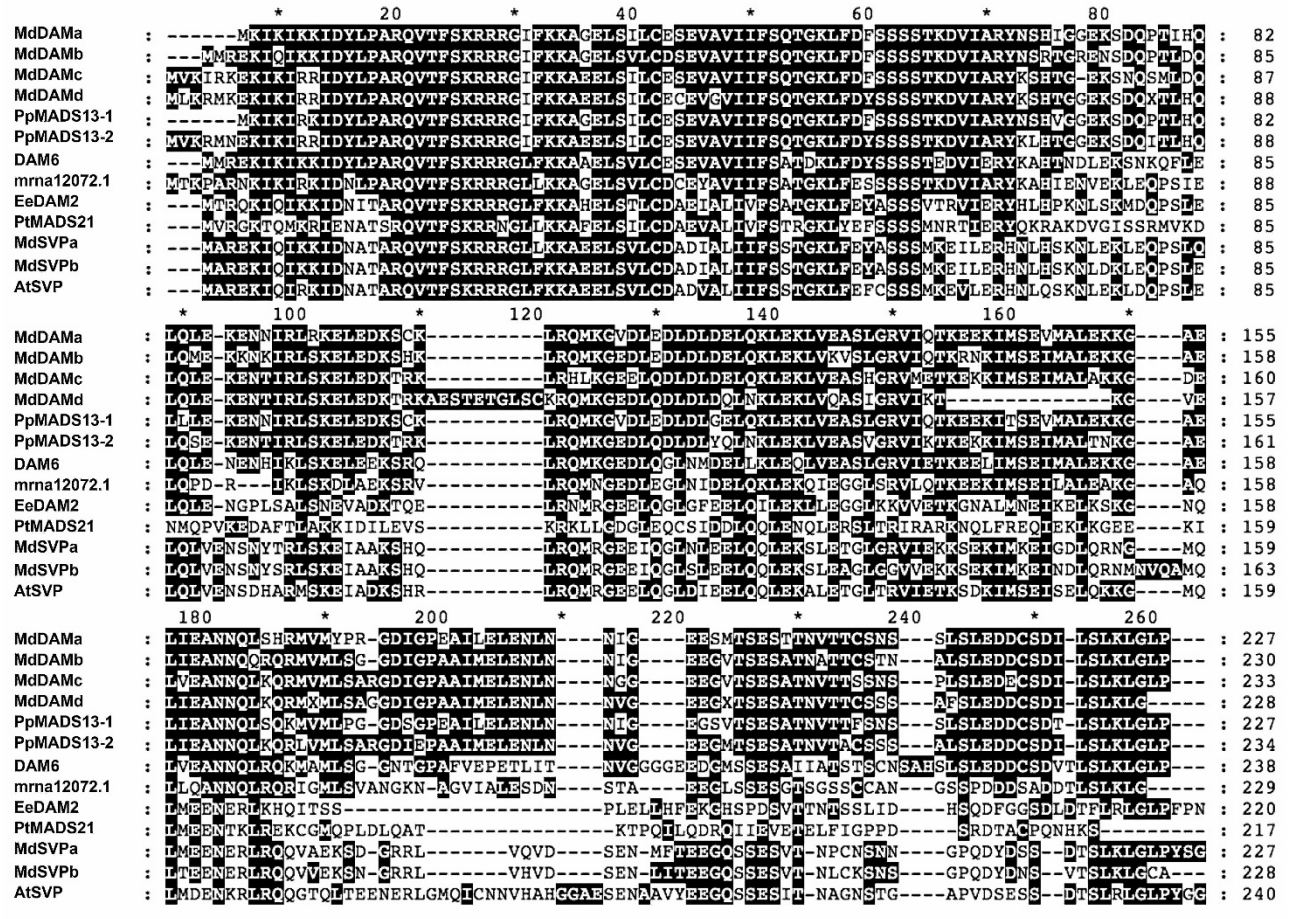

Fig. 2. The comparison of the deduced amino acid sequences of *DORMANCY-ASSOCIATED MADS-BOX* (*DAM*)-like genes. The alignment of *SVP*/*AGL24*-type MADS-BOX proteins. Acc. Nos. of *Malus* × *domestica* genes are shown in Table 1 and those of others in Fig. 3.

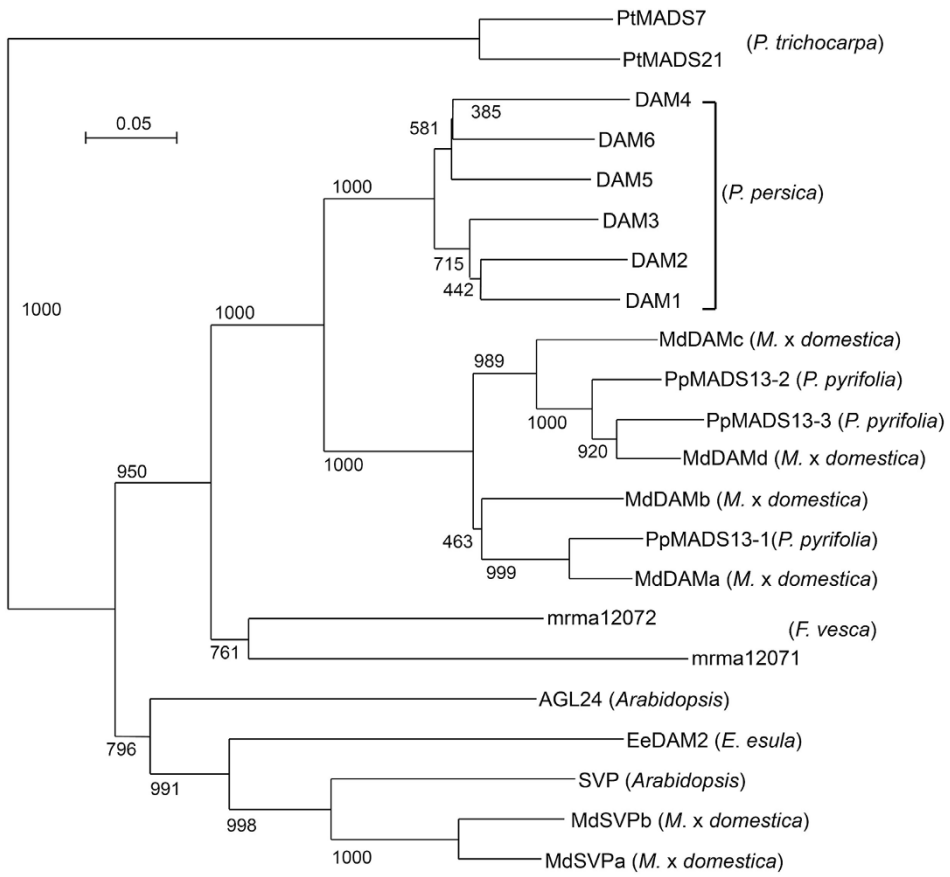

Fig. 3. Phylogenetic relationships of *SVP*/*AGL24*-type MADS-BOX proteins. Bootstrap values are shown at the nodes. The *scale bar* on top indicates 0.05 substitutions/site. Acc. Nos: *Arabidopsis thaliana* (BAD43004, NP_194185), *Euphorbia esula* (EU339320), *Fragaria vesca* (mrma12071, mrma12072), *Populus tomentosa* (EEE79434, ABF51526), *Prunus persica* (DQ863253, DQ863255, DQ863256, DQ863250, DQ863251, and DQ863252), *Pyrus pyrifolia* (AB504716, AB504717, and AB774474). Acc. Nos. of *Malus* × *domestica* genes are shown in Table 1.

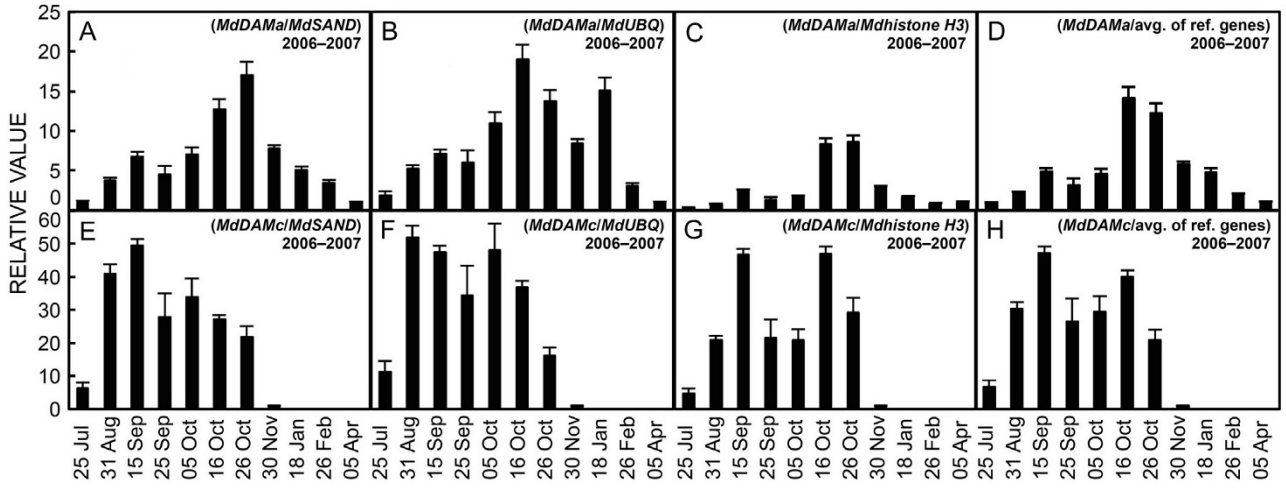

Fig. 4. Expression patterns of *DAM*-like genes in apple from July to April in 2006 - 2007. The expression patterns of *MdDAMa* (*A*,*B*, *C*,*D*) and *MdDAMc* (*E*,*F*,*G*,*H*), respectively, in the apical buds of fruit-bearing shoots (cv. Jonathan). Transcriptions were normalized against *SAND*, *UBIQUITIN*, *HISTONE H3*, and the average of these three genes. The transcript accumulations of *MdDAMa* and *MdDAMc* are expressed relative to the sample of 5th April and 30th November (base value 1), respectively. Means ± SD from three replicate wells of a RT-qPCR plate.

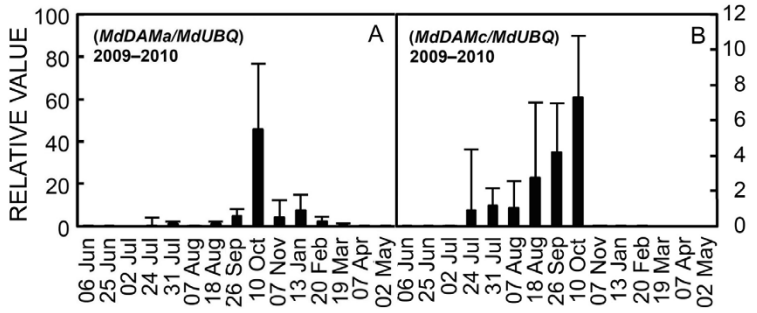

Fig. 5. Seasonal expression patterns of *DAM*-like genes in apple. The expression patterns of *MdDAMa* (*A*) and *MdDAMc* (*B*) from June to May in 2009 - 2010, respectively, in the apical buds of fruit-bearing shoots (cv. Jonathan; samples collected during May were flowers). Transcriptions were normalized against *UBIQUITIN*. The transcript accumulations of *MdDAMa* and *MdDAMc* are expressed relative to the sample of 18th and 7th August (base value 1), respectively. Means ± SD from three replicate wells of a RT-qPCR plate.

The results of our phylogenetic analysis suggest that *Rosaceae DAM*-like genes, including *MdDAMa*, *MdDAMb*, *MdDAMc*, and *MdDAMd*, constituted a gene cluster that is unique to *Rosaceae* (Figs. 2 and 3). On the other hand, *MdSVPa* and *MdSVPb* shared a high sequence homology with *A. thaliana SVP* (*AtSVP*; Figs. 2 and 3) indicating that their functions might be different from other *DAM*-like genes.

We monitored the expressions of the *DAM*-like genes during a year-cycle of apical buds (fruit-bearing shoots) throughout 2006 - 2007 or 2009 - 2010 by RT-qPCR. The *MdDAMa* transcription peaked in October and subsequently decreased (Figs. 4 and 5). The *MdDAMc* transcription increased from late July to early October and decreased from late October until it was practically undetectable from November to May next year (Figs. 4 and 5). On the other hand, the expressions of *MdDAMb* and *MdDAMd* were undetectable (data not shown). Interestingly, *MdDAMa*/*MdDAMb* and *MdDAMc*/*MdDAMd* were tandemly localized on chromosomes 16 and 8, respectively, and constituted a clade (Table 1, Fig. 3). Based on these observations, we infer that *MdDAMa* and *MdDAMc* underwent a positive selection and acted in a dominant fashion on each locus.

Similarly, the expression profiles of apple dormancy process-related genes were defined in the southern hemisphere region of Brazil (Falavigna *et al*. 2014). One of the 17 dormancy-associated candidate genes, *ABD14* (*MdDAMa*), is upregulated in cv. Royal Gala during late autumn and in cv. Fuji in mid-winter. After a maximum, the expression gradually declines in these cultivars. However, *ABD14* rapidly declines after an expression peak in cv. Castel Gala (a bud mutant of cv. Royal Gala) which have a low chilling requirement (15 °C) in relation to original cv. Royal Gala (5 °C) for dormancy release (Hawerroth *et al*. 2013, Falavigna *et al*. 2014), indicating an association of *ABD14* with dormancy maintenance. In Japanese pear cv. Kosui, the expression profiles of *MdDAM*-like genes, *PpMADS13-1* and *PpMADS13-2*, are highest from November to December in Tsukuba, Japan (latitude: 36.3 ° N, longitude: 140.5 ° E) suggesting that these genes may be correlated with the establishment of endodormancy (Ubi *et al*. 2010, Saito *et al*. 2013). On the other hand, a *PpMADS13-3* expression is highest from September to December, which occurred before the upregulation of the other *DAM*-like genes, and is similar to that of *MdDAMc* (Saito *et al*. 2013, Figs. 4 and 5). A down-regulation of all *DAM*-like gene expressions in pear and apple was observed after late autumn. Because endodormancy in apple starts in late September (Fig. 1) in Morioka, Japan, the expression patterns of *MdDAMa* and *MdDAMc* were consistent with some functions during the endodormancy period.

The expression of a peach *C-REPEAT BINDING FACTOR* (*PpCBF*) transcriptional activator gene is induced by a cold stress (Wisniewski *et al.* 2011). Transgenic apple plants that constitutively express *PpCBF* show an increased cold tolerance, growth cessation, and leaf senescence in response to the short day. The promoter regions of the *DAM* genes contain a conserved element that is highly homologous to the consensus *CBF*-binding sites (Wisniewski *et al.* 2011). *MdDAMa* had also the CRT/DREB (A/GCCGAC) response elements within the first -663 and -956 bp upstream of the coding region (data not shown). In the near future, investigations on whether *MdDAMa* directly interacts with MdCBFs in apple are necessary.

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