Suppression Subtractive Hybridization and Differential Screening Reveals Endodormancy-associated Expression of an *SVP/AGL24*-type MADS-box Gene in Lateral Vegetative Buds of Japanese Apricot

Hisayo Yamane¹, Yukinobu Kashiwa, Tomomi Ooka, Ryutaro Tao, and Keizo Yonemori

Laboratory of Pomology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

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ABSTRACT. To understand the molecular basis of the endodormancy of buds of perennial plants, we searched for the genes that are expressed preferentially in endodormant lateral buds of the deciduous fruit tree Japanese apricot (*Prunus mume* Sieb. et Zucc.) using suppression subtractive hybridization with mirror orientation selection (SSH/MOS). We generated two SSH/MOS libraries containing gene pools that are expressed preferentially in endodormant buds in comparison with paradormant or ecodormant buds to search for the genes that are upregulated by endodormancy induction or down-regulated by endodormancy release, respectively. Differential screening and sequencing indicated that genes involved in gibberellin metabolism, stress resistance, cell wall modification, and signal transduction, such as transcription factors, are upregulated in endodormant buds. After a further expression survey and full-length cDNA cloning, we found that a gene similar to the *SVP/AGL24*-type MADS-box transcription factor showed endodormancy-associated expression. Seasonal expression analysis suggested that the *SVP/AGL24* homolog in Japanese apricot might be involved in endodormancy regulation of its lateral buds.

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

Endodormancy is the result of physiological changes in response to an internal signal that inhibits continuous rapid growth toward budbreak. Certain signals that mediate the induction of endodormancy have been identified and characterized. The role of plant hormones in endodormancy, especially abscisic acid (ABA), is well established. The ABA level is increased in dormant buds during cold and drought stress. Poplars (*Populus tremula* L. var. *sieboldii* Miq. × *P. alba* L.) that overexpress *ABSCISIC ACID INSENSITIVE 3* (*ABI3*) do not form terminal buds, suggesting that ABA affects dormancy acquisition (Rohde and Bhalerao, 2007; Rohde et al., 2002).

Although a complex set of overlapping hormonal signals is responsive to various environmental and physiological conditions related to dormancy (for review, see Horvath et al., 2003; Rohde and Bhalerao, 2007), how the signals are involved in dormancy remains to be elucidated. In contrast to the extensive study of the physiology of dormancy break, the internal factors controlling endodormancy are poorly understood. For stable deciduous fruit production, uniform flowering is important, but has not been achieved recently in protected cultivation in Japan because of irregular lateral bud endodormancy release, putatively caused by global warming (Sugiura et al., 2007). This led us to search for genetic factors controlling endodormancy, with the goal of helping to recover uniform flowering of deciduous fruit tree species in Japan.

Studies using genetic and molecular approaches to understand the mechanisms that regulate bud endodormancy and dormancy release are ongoing for various plant species (for review, see Rohde and Bhalerao, 2007). Some of the quantitative trait loci (QTLs) that regulate dormancy in potato (*Solanum tuberosum* L.) and poplar (*Populus trichocarpa* Torr. & A. Gray) are associated with ABA content or signaling and photoperiod perception. Considerable efforts to identify the genes associated with dormancy have been made using the transcriptome approach in grape (*Vitis vinifera* L.) (Pacey-Miller et al., 2003), raspberry (*Rubus idaeus* L.) (Mazzitelli et al., 2007), poplar (*P. tremula* × *P. alba*) (Rohde et al., 2007; Ruttink et al., 2007), and leafy spurge (*Euphorbia esula* L.) (Jia et al., 2006). Endogenous processes that induce growth cessation and terminal bud set have been partly characterized. Böhlenius et al. (2006) suggested that *CO* (*CONSTANS*) and *FT* (*FLOWERING LOCUS T*), which are flowering regulators in *Arabidopsis thaliana* (L.)

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¹Corresponding author. E-mail: hyamane@kais.kyoto-u.ac.jp.

Heynh.] are involved in short-day-induced seasonal growth cessation of *P. trichocarpa*. In addition, Bielenberg et al. (2008) identified MADS-box genes as candidate genes associated with terminal bud formation of peach [*Prunus persica* (L.) Batsch]. However, thus far, genes that are directly involved in lateral bud endodormancy regulation have not been identified.

We performed suppression subtractive hybridization supplemented with mirror orientation selection (SSH/MOS) and differential screening to identify genes that are expressed preferentially in endodormant buds of Japanese apricot. This species of fruit tree was selected for study because its seasonal endodormancy status has been well characterized (Takamatsu et al., 2004; Yamane et al., 2006). Also, its relatively low chilling requirement for budbreak, and resultant early flowering, often causes unstable fruit yield because a large number of flowers abort due to lack of pollination and fertilization. This occurs because pollinators (bees) tend to be inactive in early spring, or may be attributable to the late spring frost (Miyahara and Tomita, 1982). Thus, delayed flowering time of Japanese apricot is required by means of controlling endodormancy release or controlling flower organ development in the endodormant stage. Accumulating knowledge of endodormancy regulation would enable us to control endodormancy through cultural procedures or breeding.

To search for genetic factors controlling endodormancy, we generated two subtraction libraries. First, we generated a SSH/MOS library containing gene pools that are expressed preferentially in endodormant buds compared with ecodormant buds to search for genes that are down-regulated by endodormancy release. Second, we generated a SSH/MOS library containing gene pools that are expressed preferentially in endodormant buds compared with paradormant buds to search for genes that are upregulated by endodormancy induction. We especially intended to isolate the genes specifically expressed in endodormant buds among paradormant, endodormant, and ecodormant buds. Endodormant buds are different from other dormant buds (paradormant and ecodormant buds) in that they are not able to resume growth under favorable conditions, which suggests that internal factors inhibiting bud burst and growth should be present in endodormant buds and absent in paradormant and ecodormant buds. In addition, the genes expressed specifically in endodormant buds compared with nonendodormant buds may function downstream of the endodormancy-regulation pathway after perception and transduction of the signals that induce endodormancy and endodormancy break. Thus, to search for genes that are highly upregulated in endodormant buds compared with paradormant and ecodormant buds, we conducted additional differential screening. As a result, we obtained a MADS-box gene that shows endodormancy-associated expression. Seasonal expression analysis suggested that the MADS-box gene was upregulated during endodormancy induction and down-regulated during endodormancy release. Full-length cDNA cloning of this gene and phylogenetic analysis revealed that it was similar to *SVP/AGL24* type MADS-box genes of Arabidopsis. We discuss the biological function of the MADS-box gene with special references to its sequence similarity to the *SVP/AGL24*-type MADS-box genes.

Materials and Methods

PLANT MATERIALS. We used field-grown trees (7 years after grafting and 6 years after transplanting, seed-grafted) and 16

pot-grown trees (3 years after grafting, seed-grafted) of the Japanese apricot cultivar Nanko. The trees were grown at the Kyoto University experimental farm, Kyoto, Japan (lat. 34°N, long. 135°E). Although Japanese apricot forms flower buds and vegetative buds as lateral buds, we used only vegetative buds. Both bud types exhibit endodormancy and require chilling exposure for endodormancy release, whereas flower organ development occurs during endodormancy (Takamatsu et al., 2004). We assumed that chilling accumulation promotes endodormancy release, as well as flower organ development, suggesting potential difficulties in distinguishing the genes that are related to endodormancy from those that are related to flower organ development if flower buds were studied. In contrast, vegetative buds do not show visible differentiation during the endodormant stage (Takamatsu et al., 2004), indicating that these organs could be used effectively for the study of endodormancy.

From late Oct. 2005 [0 chilling hours (CH); i.e., below 7.2 °C] to the beginning of Mar. 2006 (>1400 CH), 10 pot-grown 'Nanko' trees were placed in a greenhouse and maintained at 15 °C to 20 °C under natural daylengths to prevent chilling exposure, which promotes endodormancy release. In Mar. 2006, lateral vegetative buds were collected from the pot-grown trees, as well as from field-grown trees. The buds derived from pot-grown trees were used as endodormant buds, whereas those from field-grown trees were used as ecodormant buds because 'Nanko' vegetative buds require more than ≈1000 CH to completely break endodormancy (data not shown). If endodormant and paradormant buds were collected under similar environmental conditions, effects derived from factors unrelated to endodormancy could be excluded; however, none of the artificial treatments prevented paradormant buds from progressing into endodormancy. Thus, lateral buds were collected in June 2006 (2 months after leaf flush) and were used as paradormant buds, whereas lateral buds collected in Oct. 2006 were used as endodormant buds. Bud burst of pot-grown trees was observed within 10 d after being defoliated and decapitated in June, as described below, indicating that the lateral buds in June were not endodormant. For seasonal expression analysis, lateral vegetative buds of field-grown 'Nanko' trees were collected monthly from June to March. The collected buds were immediately frozen in liquid nitrogen and stored at -80 °C until use.

RNA EXTRACTION AND SSH/MOS. Total RNA was isolated from buds using the CTAB method following Yamane et al. (2006). The cDNA was synthesized from RNA extracted from paradormant, endodormant, and ecodormant buds using the SMART PCR cDNA Synthesis Kit (Clontech; TaKaRa Bio, Shiga, Japan) according to the manufacturer's instructions. Two PCR-Select subtractive libraries were constructed using the Clontech PCR-Select cDNA Subtraction Kit (TaKaRa Bio) following the manufacturer's instructions. SSH library 1 contained the genes that were expressed in endodormant buds (collected in Mar. 2006 from the pot-grown trees in the greenhouse) minus the genes that were expressed in ecodormant buds (collected in Mar. 2006 from the field-grown trees). SSH library 2 contained the genes that were expressed in endodormant buds (collected in Oct. 2006 from the field-grown trees) minus the genes that were expressed in paradormant buds (collected in June 2006 from the field-grown trees). The MOS technique was used to eliminate false-positive clones from SSH libraries following the methods of Rebrikov et al. (2000). In

brief, the subtracted cDNA pools were digested by *Xma*I, denatured at 98 °C for 1.5 min, and incubated at 68 °C for 3 h to allow hybridization of each mirror-oriented molecule. The hybridized samples were used for subsequent polymerase chain reaction (PCR). The PCR mixtures and conditions were identical to those used by Rebrikov et al. (2000). The PCR products were cloned using a pGEM-T Easy TA Cloning Kit (Promega, Madison, WI).

DIFFERENTIAL SCREENING BY DOT-BLOT HYBRIDIZATION AND VIRTUAL NORTHERN BLOT HYBRIDIZATION. Differential screening was performed according to the manufacturer's instructions (Clontech). In brief, randomly selected clones from SSH/MOS library 1 were grown overnight on a shaker. A 1-μL aliquot of the bacterial culture was used for PCR using the NP2Rs primer (5'-GGTCGCGGCCGAGGT-3'). The PCR products were spotted on nylon membrane (Biodyne PLUS; Pall Corp., Pensacola, FL). Hybridization was performed using DIG-labeled subtracted cDNA from forward (endodormant minus ecodormant) or reverse (ecodormant minus endodormant) subtractions (*Xma*I-treated secondary PCR products) using the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Mannheim, Germany) with NP2Rs primer. For probe preparation, PCR was performed using initial denaturation at 94 °C for 2 min, 23 cycles at 94 °C for 20 s, 62 °C for 20 s, and 72 °C for 1.5 min; and a final extension at 72 °C for 7 min. After dot-blot hybridization in hybridization buffer (4% skim milk, 5 × SSC, 0.1% sodium lauroylsarcosine, 0.02% SDS, and 20 μM NP2Rs primer) at 72 °C, the membrane was washed four times in 2 × SSC, 0.1% SDS for 20 min at 72 °C and twice in 0.1 × SSC, 0.1% SDS for 20 min at 72 °C. Immunological detection of hybridized nucleic acids was carried out using the anti-DIG-alkaline phosphate conjugate and the chemiluminescent substrate CDP-star (New England Biolabs, Beverly, MA). Chemiluminescence was documented on X-ray film. From SSH/MOS library 1, 1488 SSH/MOS clones were subjected to dot-blot hybridization screening. In total, 432 clones were differentially expressed between the two gene pools. The nucleotide sequences of randomly selected clones were determined using the CEQ8000 and DTCS Quick Start Master Mix (Beckman Coulter, Fullerton, CA). Contig and sequence analysis were carried out using ATGC software (Genetyx, Tokyo). The remaining clones were then subjected to dot-blot screening to eliminate the same clones from the already sequenced clones. As a result, 33 different clones were obtained. Similarly, 132 clones from SSH/MOS library 2 were randomly selected and sequenced. In total, 70 different clones were isolated. Thirty-three and 70 different clones from libraries 1 and 2, respectively, were then subjected to virtual northern blot hybridization to determine the differential expression among the three dormant bud stages: paradormant, endodormant, and ecodormant. Virtual northern blot analysis was performed according to the manufacturer's instructions (Clontech). In brief, 500 ng of cDNA synthesized using the SMART PCR cDNA Synthesis Kit from endodormant (collected in Oct. 2006), ecodormant, and paradormant buds were run in formaldehyde (1%) agarose gel, blotted onto nylon membrane, and probed by the DIG-labeled selected SSH/MOS clones. Hybridization and detection were carried out as described above. The functional annotation of sequences was predicted based on similarities to previously identified sequences obtained by searching two public databases [DNA Data Bank of Japan (DDBJ) and National Center for Biological Information

(NCBI)] using the BLASTX tool for deduced amino acid sequences. E-values >0.01 were considered not significant (i.e., no match). The pfam search results were also considered for annotation.

SEASONAL DORMANCY STATUS OF 'NANKO' VEGETATIVE BUDS. The timing of endodormancy induction and endodormancy release of 'Nanko' vegetative buds was investigated in the middle of each month. In summer, from June to August, two pot-grown trees in the field were defoliated and decapitated monthly. Days to bud burst of at least two buds per tree were counted. In autumn to early spring, from September to March, monthly dormancy status was investigated using cut branches. In brief, three branches with lengths of ≈50 cm were cut from trees and divided into five parts each. The triplicates of three branch parts derived from middle portions of five branch parts were used. A total of nine branch parts, bearing two to three buds each, were placed in water in a growth chamber and were maintained at 25 °C under cool white fluorescent light and a 16-h photoperiod. After 3 weeks in the chamber, buds showing green leaves were considered to have burst. In total, 15 to 18 buds, located in the top portions of the branch parts, were counted.

RNA BLOT ANALYSIS. The seasonal expression pattern of the clone obtained from the SSH/MOS library was investigated by RNA blot analysis. Total RNA was isolated from 'Nanko' vegetative buds, collected monthly, as described above. We ran 5 μg of the total RNA in a formaldehyde (1%) agarose gel, blotted it onto a nylon membrane, and probed it with a partial cDNA sequence of the SSH/MOS clone labeled by DIG-dUTP. The membrane was hybridized at 65 °C overnight and washed, and signals were detected as described above.

CDNA LIBRARY CONSTRUCTION AND SCREENING. Total RNA isolated from endodormant buds of field-grown 'Nanko' collected in October was used to construct a cDNA library following the instruction manual (SMART cDNA library construction kit, Clontech). Poly(A)⁺ RNA was isolated from the total RNA using Oligo-dT cellulose (Wako, Osaka, Japan). Using the first-strand cDNAs synthesized from ≈0.2 μg of poly(A)⁺ RNA, cDNAs were amplified by long-distance PCR with Advantage 2 polymerase, cloned into λTriplEx2 vector (Clontech), and packaged in vitro using MaxPlax Packaging Extract Kit (Epicentre Technologies, Madison, WI). Five thousand plaques were screened by plaque hybridization using the DIG-labeled SSH/MOS clone as a probe. After the low stringency washes (2 × 5 min at room temperature with 5 × SSC and 0.1% SDS followed by 2 × 15 min at 68 °C with 1 × SSC and 0.1% SDS), colorimetric detection with nitroblue tetrazolium (Nacalai Tesque, Kyoto, Japan) and 5-bromo-4-chloro-3-indolyl phosphate (Nacalai Tesque) was conducted as described in Tao et al. (1999). Selected clones were converted to pTriplEx2 plasmids according to the instruction manual (Clontech), and nucleotide sequences were determined as described above.

CONSTRUCTION OF A PHYLOGENETIC TREE. The full-length deduced amino acid sequences of 38 genes belonging to the MIKC-type MADS-box transcription factor gene family in *Arabidopsis* (Pařenicová et al., 2003) and the gene identified in this study were used to construct a phylogenetic tree. After the amino acid sequences were aligned using CLUSTAL X, the phylogenetic tree was constructed by the neighbor-joining method and displayed with the MEGA4 software package (Tamura et al., 2007).

Results and Discussion

SSH/MOS LIBRARY SCREENING, SEQUENCING, AND ANNOTATION. To elucidate the endodormancy regulation in Japanese apricot, we sought to isolate the genes preferentially expressed in endodormant buds compared with the paradormant and ecodormant stages of buds. For this purpose, we constructed two different SSH/MOS libraries containing cDNA clones that were expressed in endodormant buds minus those that were expressed in ecodormant (library 1) or paradormant (library 2) buds. After extensive screening, a total of 7 and 19 clones from SSH/MOS libraries 1 and 2, respectively, exhibited a more than 3-fold increase in intensity in endodormant buds compared with ecodormant or paradormant buds (Fig. 1) when analyzed by virtual northern blot analysis. BLAST search results are shown in Table 1. Of the obtained clones, only one was isolated as a common clone in the two libraries (Table 1); it was similar to the MADS-box transcription factor *Ppdam6* (*Prunus persica dormancy-associated MADS6*; DDBJ/EMBL/GenBank accession number DQ863252; Bielenberg et al., 2008). Clones that were similar to database-registered cytochrome P450 monooxygenase were also included in both libraries; however, the clone from library 1 was considered to differ from that in library 2 because their sequences had very low similarity (data not shown). Seasonal gene expression changes in buds of raspberry (Mazzitelli et al., 2007) and leafy spurge (Jia et al., 2006) were investigated using microarray analysis. Both of these studies indicated that cytochrome P450 monooxygenase was preferentially expressed in endodormant buds, which is in agreement with our results.

Most clones showed preferential expression in paradormant and endodormant buds (Fig. 1B) or in endodormant and ecodormant buds (Fig. 1C), suggesting that distinct genes are involved in endodormancy induction or endodormancy release. The clones down-regulated in ecodormant buds compared with endodormant buds include gibberellin 2

oxidase (GA2ox; Table 1). Several reports have suggested critical roles of gibberellins (GAs), which promote progression from dormancy release, in acting downstream of environmental signals to regulate bud dormancy (Crabbe and Barnola, 1996;

Fig. 1. Virtual northern blot hybridization analysis of suppression subtractive hybridization with mirror orientation selection (SSH/MOS) clones in paradormant, endodormant, and ecodormant lateral vegetative buds of Japanese apricot. We analyzed the expression of selected seven clones from SSH/MOS library 1 (endodormant minus ecodormant) and 19 clones from SSH/MOS library 2 (endodormant minus paradormant). In each panel, left lane: paradormant buds (collected in June); center lane: endodormant buds (collected in October); right lane: ecodormant buds (collected in March). For each clone, the upper panel shows virtual northern blot bands, whereas the lower panel shows ethidium bromide-stained cDNAs as a loading control of the gel. Clone numbers correspond to those in Table 1. The classification of virtual northern blot banding patterns is as follows: (A) preferentially expressed in endodormant buds among three dormancy stages; (B) preferentially expressed in paradormant and endodormant buds among three dormancy stages; (C) preferentially expressed in endodormant and ecodormant buds among three dormancy stages. Asterisks represent clones from SSH/MOS library 1 (see Table 1).

Table 1. List of clones in each suppression subtractive hybridization with mirror orientation selection (SSH/MOS) library. A total of seven and 19 clones were obtained from SSH/MOS library 1 and 2, respectively. A BLAST search of each clone was performed using two public databases [the DNA Data Bank of Japan (DDBJ) and the National Center for Biological Information (NCBI)]. The database-registered clone, which exhibited the strongest similarity to each SSH/MOS clone, is shown in the Hit ID column.

Clones	BLASTX search (DDBJ) results ^z	Hit ID (species)	E-value ^y	BLASTX search (NCBI) results ^z
SSH/MOS library 1 (endodormant minus ecodormant)				
Category A (Virtual northern blot banding pattern A ^v)				
7D	Ppdam6 (dormancy associated MADS 6)	ABJ96360 [<i>Prunus persica</i> (L.) Batsch]	7.00E-96	Ppdam6 (dormancy associated MADS 6)
Category B (Virtual Northern blot banding pattern B ^v)				
2F	ATHB40 (Homeobox protein 40)	O23208 [<i>Arabidopsis thaliana</i> (L.) Heynh.]	2.00E-40	ATHB40 (Homeobox protein 40)
5D	4-Methyl-5 (B-hydroxyethyl)-thiazole monophosphate biosynthesis protein	AY084268 (<i>A. thaliana</i>)	1.00E-55	Putative 4-methyl-5 (b-hydroxyethyl)-thiazol monophosphate biosynthesis enzyme
77	DNAJ protein	AB015601 (<i>Salix gilgiana</i> Seemen)	9.00E-53	DNAJ protein
264	Gibberellin 2 oxidase	AJ132438 (<i>Phaseolus coccineus</i> L.)	2.00E-71	Gibberellin 2 oxidase
266	NADH-ubiquinone oxidoreductase-related-like protein	AP003803 (<i>Oryza sativa</i> L.)	8.00E-19	NADH-ubiquinone oxidoreductase-related like protein
93	Cytochrome P450	AF386512 (<i>Pyrus communis</i> L.)	4.00E-47	Cytochrome P450
SSH/MOS library 2 (endodormant minus paradormant)				
Category A (Virtual northern blot banding pattern A ^v)				
112A (=7D)	Ppdam6 (dormancy associated MADS 6)	ABJ96360 (<i>P. persica</i>)	7.00E-96	Ppdam6 (dormancy-associated MADS 6)
1011	SCPL35 (serine carboxypeptidase-like 35)	AF370244 (<i>A. thaliana</i>)	9.00E-32	SCPL35 (serine carboxypeptidase-like 35)
1028	Putative chain A, crystal structure of phosphopantothienylcysteine synthetase	AP003882 (<i>O. sativa</i>)	4.00E-26	ATCOAB (4-phospho-pantothenylcysteine synthetase): phosphopantothenate-cysteine ligase
1121	Cytochrome P450 monooxygenase	AB218764 (<i>Pisum sativum</i> L.)	1.00E-30	BAS1 (PHYB ACTIVATION TAGGED SUPPRESSOR1)
1004	Putative xyloglucanase inhibitor	AY321357 (<i>Solanum tuberosum</i> L.)	1.00E-33	Putative xyloglucanase inhibitor
1039	Chloroplast omega-3 desaturase	AF517831 (<i>P. persica</i>)	8.00E-54	Chloroplast omega-3 desaturase
103A	Xylogluran endotransglucosylase/hydrolase 3	DQ914796 (<i>Cucumis melo</i> L.)	4.00E-34	Xylogluran endotransglucosylase/hydrolase 3
Category C (Virtual northern blot banding pattern C ^v)				
100E	Alcohol dehydrogenase2	AP008216 (<i>O. sativa</i>)	1.00E-69	Alcohol dehydrogenase
1014	Putative auxin efflux carrier protein	AY553212 (<i>Medicago truncatula</i> Gaertn.)	7.00E-42	Putative auxin efflux carrier protein
1017	Lysyl-tRNA synthetase	CU459220 (<i>Vitis vinifera</i> L.)	1.00E-24	ATP-binding/aminoacyl-tRNA ligase
102B	Putative glycoprotein similar to EP1	AY035116 (<i>A. thaliana</i>)	2.00E-40	Putative glycoprotein EP1
102E	Glucosyltransferase-10	BAB86928 [<i>Vigna angularis</i> (Willd.) Ohwi et Ohashi]	4.00E-31	Glucosyltransferase-10
1038	Pollen ole e 1 allergen	ABD33344 (<i>M. truncatula</i>)	7.00E-26	Pollen ole e 1 allergen
1119	GPI-anchored protein	AK118563 (<i>A. thaliana</i>)	6.00E-38	TMM (TOO MANY MOUTHS); protein binding
111E	Xyloglucan endotransglycosylase 3	ABK30789 (<i>Litchi chinensis</i> Sonn)	1.00E-75	Xyloglucan endotransglycosylase 3
1125	No hit			No hit
112F	UDP-glucose dehydrogenase	AAB58398 [<i>Glycine max</i> (L.) Merrill]	3.00E-31	UDP-glucose 6-dehydrogenase
1134	Putative proline-rich protein APG	AJ276421 (<i>Cicer arietinum</i> L.)	5.00E-38	Putative proline-rich protein APG
113C	Basic helix-loop-helix protein	ABN51065 (<i>Sesamum indicum</i> L.)	1.00E-24	Basic helix-loop-helix protein

^zThe BLASTX searches were performed 1 May 2008.

^yThe E-value from BLASTX, which exhibited the strongest similarity to annotated sequences.

^zThe domain name retrieved by protein profiling database (pfam) search.

^wThe function of the clones were predicted based on the BLASTX results and pfam results.

^vThe classification of virtual northern blot banding patterns of the clones were shown and described in Fig. 1.

Hit ID (species)	E-value ^y	Pfam results ^x	Predicted function ^w	Accession no.
ABJ96360 (<i>P. persica</i>)	7.00E-96	SRF-TF	Transcription factor	AB437345
O23208 (<i>A. thaliana</i>)	1.00E-41	homeobox	DNA/RNA binding	AB437321
AB372266 (<i>Capsicum chinense</i> Jacq.)	9.00E-59	DJ-1/PfpI	Unknown	AB437322
AB015601 (<i>S. gilgiana</i>)	9.00E-53	DNAJ	Stress response	AB437323
AJ132438 (<i>P. coccineus</i>)	2.00E-71	20G-FeII_Oxy	Plant hormone related	AB437324
AP003803 (<i>O. sativa</i>)	7.00E-19	ETC_C1_NDUFA4	Metabolism	AB437325
AF386512 (<i>P. communis</i>)	5.00E-47	p450	Unknown	AB437326
ABJ96360 (<i>P. persica</i>)	7.00E-96	SRF-TF	Transcription factor	AB437345
AF370244 (<i>A. thaliana</i>)	9.00E-32	Peptidase_S10	Protein metabolism	AB437327
NP563904 (<i>A. thaliana</i>)	3.00E-30	DFP	Metabolism	AB437328
NP180239 (<i>A. thaliana</i>)	2.00E-29	p450	Unknown	AB437329
AY321357 (<i>S. tuberosum</i>)	1.00E-33	none	Cell wall modification	AB437330
AF517831 (<i>P. persica</i>)	9.00E-54	FA_desaturase-1	Metabolism	AB437331
DQ914796 (<i>C. melo</i>)	4.00E-34	XET_C	Cell wall modification	AB437332
AY087044 (<i>A. thaliana</i>)	1.00E-68	ADH_N	Metabolism	AB437333
AY553212 (<i>M. truncatula</i>)	7.00E-42	Mem_trans	Plant hormone related	AB437334
NP001047472 (<i>O. sativa</i>)	4.00E-23	tRNA-synt_2	ATP binding	AB437335
AY120736 (<i>A. thaliana</i>)	2.00E-40	B_lectin	Glycoprotein	AB437336
BAB86928 (<i>V. angularis</i>)	4.00E-31	UDPGT	Metabolism	AB437337
ABD33344 (<i>M. truncatula</i>)	7.00E-26	Pollen_Ole_e_I	Defense	AB437338
NM106657 (<i>A. thaliana</i>)	9.00E-50	LRR_1	Signal transduction	AB437339
ABK30789 (<i>L. chinensis</i>)	1.00E-75	XET_C	cell wall modification	AB437340 AB437341
AAB58398 (<i>G. max</i>)	3.00E-31	UDP_MGDP	Metabolism	AB437342
AJ276421 (<i>C. arietinum</i>)	5.00E-38	Lipase_GDSL	Metabolism	AB437343
ABN51065 (<i>S. indicum</i>)	1.00E-24	HLH	Transcription factor	AB437344

Druart et al., 2007; Eriksson and Moritz, 2002; Powell, 1987; Walker and Donoho, 1959; Walser et al., 1981) and seed dormancy (for review, see Finkelstein et al., 2008; Koornneef et al., 2002). This suggests a significant role of GA in the

regulation of dormancy. A recent study of GA metabolism revealed that GA2ox deactivates the bioactive GAs, such as GA1 and GA4, via 2 β -hydroxylation (Thomas et al., 1999). The isolation of GA2ox in our SSH library 1 suggests that bioactive

GAs are down-regulated in endodormant buds by accumulated GA2ox. If this is the case, fewer bioactive GAs may somehow be involved in endodormancy, although seasonal endogenous GA levels appear to be unrelated to dormancy status (Luna et al., 1990). Nevertheless, the upregulation of GA deactivators in endodormant buds does not conflict with previous studies that indicate the promotion of dormancy break by bioactive GA application and the requirement of bioactive GAs for seed germination.

DNAJ protein also showed down-regulation in ecodormant buds compared with endodormant buds. DNAJ protein provides stability to macromolecules like dehydrin, which accumulate in dormant buds (Arora et al., 1992; Artlip et al., 1997; Muthalif and Rowland, 1994; Yakovlev et al., 2008; Yamane et al., 2006). Although this accumulation was thought to be induced by cold acclimation (Muthalif and Rowland, 1994), the differential expression of dehydrin in winter buds between cultivars that have different chilling requirements for budbreak suggests that these molecules play a physiological role in flower bud development and dormancy (Artlip et al., 1997; Yakovlev et al., 2008; Yamane et al., 2006).

Many cell wall modification enzymes such as XTH and xyloglucanase inhibitor and secreted proteins such as glycoprotein and glycosyltransferase were included in SSH library 2 (Table 1). Most of them exhibited upregulation in endodormant buds compared with paradormant buds (Fig. 1). Previous studies have indicated that the lower symplastic cell-to-cell communication through plasmodesmata occurs in dormant buds of birch (Rinne et al., 2001). This suggests that cell wall and/or plasma membrane modification plays an important role in endodormancy regulation. Finally, in addition to a MADS-box gene, several signal transduction-associated factors such as basic helix-loop-helix protein, ATP-binding protein, and nucleotide-binding protein were identified in SSH libraries 1 and 2 (Table 1).

We isolated a common gene from both libraries, suggesting that some genes may be upregulated by endodormancy induction and down-regulated by endodormancy release. The genes that were expressed preferentially in endodormant buds compared with nonendodormant buds might function downstream of the endodormancy-regulation pathway after perception and transduction of the signals that induce endodormancy and endodormancy break. In our SSH libraries, seven clones showed strong preferential expression in endodormant buds compared with nonendodormant buds (Fig. 1A). Among these seven clones, no hybridization bands were detected in non-endodormant buds in clone 7D, suggesting that clone 7D shows endodormancy-associated expression. Clone 7D was found in both libraries and encodes a MADS-box transcription factor (Table 1, Fig. 1A). This clone was subjected to subsequent seasonal expression analysis related to seasonal dormancy status of the lateral buds of Japanese apricot. Furthermore, its phylogenetic analysis was performed based on its full-length cDNA sequence.

AN *SVP/AGL24*-TYPE MADS-BOX GENE SHOWS ENDODORMANCY-ASSOCIATED EXPRESSION. Endodormancy status of the vegetative buds of 'Nanko' was evaluated using pot-grown trees and cut branches. Bud burst was observed in June within 10 d after defoliation and decapitation in both tested pot-grown trees. In July and August, bud burst was observed after 10 and 25 d, respectively, in one of the two pot-grown trees, whereas bud burst was not observed after 28 d in the other tree,

suggesting that the buds started to become endodormant in these months and that the endodormancy proceeded gradually. Bud burst was not observed in any buds in the branches collected from September to November, indicating that the buds are endodormant during these months. Then, 11% of the vegetative buds collected in December and January opened under favorable conditions. Finally, 71% and 94% of the vegetative buds collected in February and March, respectively, opened under favorable conditions. These results suggest that buds were released from endodormancy during December and January, and moved to the ecodormant stage in February.

The seasonal expression changes in clone 7D were monitored by RNA gel blot analysis (Fig. 2). In vegetative buds, no signal was detected in June when buds were not yet endodormant. The faint hybridization signal was detected in July and August when buds started to enter endodormancy. In September, October, and November, when buds were in deep endodormancy, strong hybridization signals were detected. During December and January when buds started to be released from endodormancy, faint hybridization signals were observed. In February and March when buds were in the ecodormant stage, no signal was detected. Taken together, these results clearly indicate that clone 7D was upregulated during the onset of endodormancy and down-regulated during endodormancy release.

The full-length cDNA encoding clone 7D was obtained from the endodormant bud cDNA library. Because the deduced amino acid sequence showed the highest sequence identity (91.7%) with Ppdam6, this clone was named *Pmdam6* (*Prunus mume dormancy-associated MADS6*). The amino acid sequence of Pmdam6 was aligned with 38 known MIKC-type MADS-box genes from Arabidopsis to construct a phylogenetic tree. The resulting phylogenetic tree demonstrated that Pmdam6 was classified in the StMADS11 (*SVP/AGL24*) superclade (Pařenicov et al., 2003; Laseberg et al., 2006), together with *SHORT VEGETATIVE PHASE* (*SVP*) (Hartmann et al., 2000) and *AGAMOUS-LIKE24* (*AGL24*) (Michaels et al., 2003) from Arabidopsis (Fig. 3). In Arabidopsis, *SVP* and *AGL24* were primarily expressed in vegetative tissues and are phylogenetically closely related to each other (Pařenicov et al., 2003). However, functional analysis indicated that they have opposite effects in the regulation of floral transition. Whereas *SVP* acts as a repressor of floral transition (Hartmann et al., 2000), *AGL24* promotes flowering. Recently, woody plant homologs of Arabidopsis flowering-related genes, such as *CO* and *FT* homologs, were found to be involved in endodormancy (Böhlenius et al., 2006). Our finding that a homolog of another flowering-related gene, *SVP/AGL24*, is putatively related to endodormancy in Japanese apricot, in addition to the recent finding that *SVP* homologs in peach are associated with terminal bud formation (Bielenberg et al., 2008), suggests the significant roles of homologs of flowering-related genes of Arabidopsis in bud endodormancy of woody plants.

In peach, MADS-box genes including *Ppdam6* were located in the *EVG* locus. *Evg* peach is a naturally occurring mutant unable to cease terminal meristem growth or form terminal buds (Bielenberg et al., 2004; Rodriguez et al., 1994; Wang et al., 2002). The MADS-box genes were expressed in terminal buds of the wild type, whereas none were expressed in the terminal meristem region of the *evg* mutant, suggesting that they are candidate genes for the regulation of growth cessation and

Fig. 2. RNA blot analysis of suppression subtractive hybridization with mirror orientation selection clone 7D in seasonal lateral vegetative buds of Japanese apricot 'Nanko'. Total RNA (5 µg per lane) extracted from monthly collected vegetative buds was used and hybridized with DIG-labeled 7D probe. The collection months are shown above the lanes. The upper panel shows the hybridization bands, whereas the lower panel shows the ethidium bromide-stained rRNA bands as a loading control for the gel. The endodormancy status of each collection month was investigated and described in the text.

Fig. 3. The phylogenetic tree of Pmdam6 and Arabidopsis MADS-box sequences. After their full-length deduced amino acid sequences were aligned using CLUSTAL X software, the phylogenetic tree was constructed by the neighbor-joining method. Branches with more than 50% bootstrap support are shown.

terminal bud formation in peach (Bielenberg et al., 2004, 2008). However, the endodormancy status of lateral buds of the *evg* mutant or the expression of MADS-box genes in lateral buds in wild types and mutants has yet to be investigated. We focused on lateral bud endodormancy in Japanese apricot and isolated *Pmdam6*, an ortholog of *Ppdam6*, as a strong candidate for endodormancy regulation from among >1500 SSH clones. Its

endodormancy-associated expression in lateral buds suggests that *Pmdam6* might be involved in endodormancy induction and endodormancy release of lateral buds in Japanese apricot. This implies that *Pmdam6* might function as an internal suppressor gene to delay or inhibit bud growth under conditions favorable for bud burst when none of the external inhibitory effects such as apical dominance or inferior environmental conditions exist. Interestingly, gradual down-regulation of the *SVP*-type MADS-box gene was also observed during the dormancy release of raspberry buds (Mazzitelli et al., 2007). This suggests that *SVP*-type MADS-box genes are involved in endodormancy of deciduous fruit tree species. Extensive expression analyses with special references to chilling exposure as well as functional analyses of this gene are under way to address the possible involvement of *Pmdam6* in lateral bud endodormancy induction and endodormancy release in Japanese apricot.

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