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Cloning and characterisation of *APETALA3*-like and *PISTILLATA*-like B class MADS-box genes from sweet cherry

Kenji Beppu*, Hidemi Sumida and Ikuo Kataoka

*Faculty of Agriculture, Kagawa University, Miki, Kagawa 761-0795, Japan. *E-mail: beppuk@ag.kagawa-u.ac.jp*

Abstract

We isolated *APETALA3* (*AP3*)-like and *PISTILLATA* (*PI*)-like cDNA clones called *PaTM6* and *PaPI* from sweet cherry (*Prunus avium*). *PaTM6* showed very high similarity to the *TM6* lineage of *AP3* of other Rosaceae species. *PaTM6* contained three amino acid residues (F, T, M) within the MADS box and the (H/Q)YExM sequence near the K box, both of which are characteristic of the *AP3* subfamily. A paleo AP3 motif was present at the C-terminal end of *PaTM6*. *PaPI* showed very high similarity to *PI* of other Rosaceae species. *PaPI* had the serine residue and the KHExL sequence within the MADS box and near the K box, respectively, both of which are characteristic of the *PI* subfamily. A PI motif was present at the C-terminal end of *PaPI*. Both *PaTM6* and *PaPI* genes were expressed specifically in petals and stamens, the same expression patterns as those of class B MADS-box genes. These results indicated that *PaTM6* and *PaPI* are homologues of *AP3* and *PI*, respectively.

Key words: *AP3*, class B gene, double pistils, *PaPI*, *PaTM6*, *PI*, *Prunus avium*

Introduction

Recently, attempts have been made to produce sweet cherry in southwestern Japan to harvest fruits earlier than in the northern major production areas and to supply local markets. In this region, however, the occurrence of double fruits is a major problem (Beppu and Kataoka, 2011). This malformation is due to abnormal differentiation of pistil primordia in the previous growing season (Philp, 1933; Tucker, 1934).

We demonstrated, under controlled conditions that the occurrence of double pistils in 'Satohnishiki' markedly increased when the trees were exposed to temperatures above 30 °C (Beppu and Kataoka, 1999). Furthermore, extremely high temperature induced the formation of not only of double pistils but also of pistil-like appendages that replaced anthers (Beppu and Kataoka, 1999; Ryugo, 1988). Thus, the B and C classes of homeotic genes, which control stamen and pistil formation, may be involved in these phenomena. Generally, the class B and C genes together induce the formation of stamens, and the class C gene is required for the formation of pistils (Weigel and Meyerowitz, 1994). To evaluate the transcriptional levels of class B and C MADS-box genes of sweet cherry grown at different temperatures, these genes must first be isolated. In the Rosaceae, the family to which sweet cherry belongs, the class B and C genes of rose and apple have been cloned (Kitahara and Matsumoto, 2000; Kitahara *et al*., 2001, 2004; Yao *et al*., 2001; Linden *et al*., 2002). Based on their sequences, we previously isolated the *AGAMOUS*–like C class genes of sweet cherry (Beppu *et al*., 2015).

In this study, we isolated the *AP3*-like and *PI*-like B class MADS-box genes of sweet cherry and evaluated their expression in each organ of the flower.

Materials and methods

Isolation of class B MADS box gene: RNA isolation: Flower buds of 'Satohnishiki' sweet cherry were collected on 29 July, when the buds were the most sensitive to high temperature inducing double pistil formation (Beppu *et al*., 2001). They were stored at -80 °C until use. Total RNA was isolated from 0.3 g plant material by the modified cetyltrimethylammonium bromide (CTAB) method, as described by Kotoda *et al*. (2000). One microgram of the total RNA was used for first strand cDNA synthesis by SuperScript II RT (Life Technologies, MD) with an adapter-dT primer (5'CGA CGT TGT AAA ACG ACG GCC AGT TTT TTT TTT TTT TTT -3') consisted of M13-20 sequence primer and oligo (dT)16.

Cloning of homologues by PCR: We used a combination of reverse transcription (RT)-PCR and rapid amplification of cDNA ends (RACE) techniques to isolate *AP3* and *PI* homologues. First, partial sequences of *AP3* and *PI* homologues were isolated by reverse RT-PCR with AP3-F1-ra (5'-CCA GAC SAA CAG GCA GGT GAC CTA-3')/ AP3-R2-ra (5'-AGA TGA GTR ATG GAG GAG-3') and PI-F1-ra (5'-CTC AAG YAA CAG GCA GGT GAC CTA-3')/ PI-R2-ra (5'-TGG AGA TTT GGC TGA WTA GGC-3'). These primers were designed from conserved regions of *AP3* and *PI* in Rosaceae [rose (Kitahara and Matsumoto, 2000; Kitahara *et al*., 2001) and apple (Yao *et al*., 2001; Linden *et al*., 2002; Kitahara *et al*., 2004)], respectively.

PCR was performed using a programme of 30 cycles at 94 °C for 30 sec, 52 °C for 30 sec, and 72 °C for 1 min with an initial denaturing of 94 °C for 3 min and a final extension of 72 °C for 7 min. The PCR reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM each of dNTPs, 400 nM each of primers, 0.1 mg of template cDNA, and 1 unit of TaKaRa Ex Taq polymerase (Takara Shuzo Co., Shiga, Japan) in a 50 mL reaction volume. PCR products were subcloned into the TA cloning vector (pGEM-T Easy Vector System; Promega, WI, USA). Nucleotide sequences of several clones were determined with the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan).

3'- and 5'-RACE to obtain full length clones were performed. For 3' RACE of *AP3* and *PI* homologues, Pa-AP3-F5 (5'-AAG GTG AAG AAC TTG GAG GAA AGA-3') and Pa-PI-F7 (5'-TAT GAG CTG CAC AAA CAG GAG ATG A-3') primers were used, respectively, with M13-20 primer as the adapter primer. These primers were designed from the sequences obtained by RT-PCR described above. PCR condition for 3' RACE and TA cloning was same as RT-PCR described above. 5' RACE was carried out using a 5'-Full RACE Core Set (Takara Shuzo). First, the reaction mixtures were prepared according to the manufacture instructions. These mixtures were for first strand cDNA synthesis by RT using the 5'-phosphorylated RT-primer Pa-AP3-P (5'-CAA CCG CAG ATT CAT-3') and Pa-PI-P (5'-GGC AAA CGG TAT CTG-3'). The conditions of the RT reaction were 10 min at 30 °C, 60 min at 50 °C, 2 min at 80 °C, followed by cooling 4 °C. The cDNA first strand was self-ligated by T4 RNA ligase according to the manufacture instructions. For the first and second PCRs of the self-ligated cDNA first strand, two sets of primers were designed. For the first PCR the primers were Pa-AP3-S1 (5'-GGA AGT ACC ACG TGA TCA AA-3') and Pa-AP3-A1 (5'-TCA TGA CCC AAC CTC TGC CT-3'), and Pa-PI-S1 (5'-ACA AGC AGT CCA AGT TCG TC-3') and Pa-PI-A1 (5'-ATT CAA CCA TCT TTC CAG AG-3'). For the second PCR the primers were Pa-AP3-S2 (5'-GAA CTT GGA GGA AAG AAG AG-3') and Pa-AP3-A2 (5'-GTA GGG CTA ATA TAC TCG TG-3'), and Pa-PI-S2 (5'-GAG CAT AAG CGC CTC ACT TA-3') and Pa-PI-A2 (5'-CTT AAT GAT CCC ATT CCT CC-3'). The reaction mixtures of the first and second PCRs were prepared according to the manufacturer's instructions. The first PCR was performed using a programme of 25 cycles at 94 °C for 30 sec, 54 °C for 30 sec, and 72 °C for 1 min with an initial denaturing of 94 °C for 3 min and a final extension of 72 °C for 7 min. The second PCR was performed similarly except the cycle number (30 cycles). The second PCR products were subcloned into the TA cloning vector and sequenced as described above.

Comparison and phylogenetic analysis: The deduced amino acid sequences of *AP3* homologue genes of apple (*MdTM6,* Accession number AB081093), Barberton daisy (*GDEF1,* AJ009724), birthwort (*AeAP3-1,* AF230697), grape (*VvTM6,* DQ979341; *VvAP3,* EF418603), hydrangea (*Hydrangea TM6,* AF230703), mandarin (*CitMADS8;* AB218614), petunia (*Petunia TM6,* AF230704; *pMADS1,* X69946), poplar (*LtAP3,* AF052878), potato (*STDEF,* X67511), rapeseed (*BAP3,* AF124814), rose (*MASAKO B3*, AB055966; *MASAKO euB3,* AB099875), sweet cherry (*PaTM6,* AB763909, this study), tobacco (*NTDEF,* X96428), tomato (*Lycopersicon TM6*, X60759; *LeAP3,* AF052868) and wintersweet (*CfAP3-1,* AF230699) were aligned using the program Clustal W. Those of *PI* homologue genes of apple (*MdPI,* AJ291490), Barberton daisy (*GGLO1,* AJ009726), cucumber (*CUM26,* AF043255), grape (*VvPI,* DQ988043), peach (*PpMADS10,* EU005663), petunia (*pMADS2,* X69947), rose (*MASAKO BP,* AB038462), sweet cherry (*PaPI,* AB763910, this study) and thale cress (*PI,* D30807) were also aligned.

Expression analysis of class B MADS box gene: Floral organs, sepals, petals, stamens and pistils and young leaves were collected at anthesis. They were stored at -80°C until use. Total RNA was isolated as described previously (Beppu *et al*., 2002) and first strand cDNA synthesis was done as described above.

Based on the sequences of *AP3*-like and *PI*-like genes obtained above, specific primer sets for each gene were made. RT-PCR was performed with the primers, AP3-RT-F (5'-GTA AAA TGC ACG AGT ATA TT-3') and AP3-RT-R (5'-GTG GAT CCT CAG AGG CTA AT-3') for *AP3* like gene, PI-RT-F (5'-GAA AGA TGG TTG AAT ACT GC-3') and PI-RT-R (5'-TCA TCT CCT GTT TGT GCA GC-3') for *PI* like gene. *Actin* was used as the RT-PCR control (Yamane *et al*., 2003). PCR was performed as described above. The PCR products were run in 1% (w/v) agarose gels for 0.5 h at 100 V using the Mupid 2 electrophoresis system (Advance Co. Ltd., Tokyo, Japan).

Results and discussion

Isolation of class B MADS-box gene: The deduced amino acid sequences of *Prunus avium TM6* (*PaTM6*) (DDBJ/EMBL/GenBank Accession Nos. AB763909) are shown in Fig. 1 with those of other Rosaceae species. *PaTM6* was 708 bp in length with an open reading frame corresponding to 235 amino acid residues. The deduced amino acid sequence of *PaTM6* contained a MADS box and a K box. The MADS-box domain of *PaTM6* spanned amino acids 1 to 60 and was a highly conserved region, whereas the K-box domain spanned amino acids 89 to 154 and was a moderately conserved domain. Three of four characteristic amino acid residues: phenylalanine, threonine and methionine, within the MADS box of the *DEF* (= *AP3*) subfamily (Theißen *et al*., 1996) were represented by amino acid residues 29, 36 and 47. A highly conserved sequence of *AP3* homologues (H/Q)YExM (Kramer *et al*., 1998) was present in the corresponding region with a slight divergence (amino acid residues 83–87). These results suggest that *PaTM6* is a sweet cherry homologue of *AP3*.

AP3 homologue genes in higher eudicots have been divided into two groups, the *TM6* and *euAP3* lineages (Kramer *et al*., 1998). The *PaTM6* protein showed high similarity to proteins known to correspond to *TM6* lineage members, in particular *MdTM6* of apple (*Malus domestica*) (85%) and *MASAKO B3* of rose (*Rosa rugosa*) (76%), both of which, like sweet cherry, are Rosaceae species (Fig. 2). *PaTM6* had the classical TM6 motif: the paleo AP3 motif (YGxHDLRLA) (Kramer *et al*., 1998) in the C-terminal region (amino acid residues 227–235). These results indicated that *PaTM6* belongs to the *TM6* lineage.

The deduced amino acid sequences of *P. avium PISTILLATA* (*PaPI*) (DDBJ/EMBL/GenBank Accession Nos. AB763910) are shown in Fig. 3 with those of other Rosaceae species. *PaPI* was 633 bp in length with an open reading frame corresponding to 210 amino acid residues. The deduced amino acid sequence of *PaPI* also contained a well-conserved MADS domain from amino acids 1–59 and a conserved K box from amino acids 89–150. A characteristic amino acid residue, serine, within the MADS box of the *GLO* (=*PI*)-subfamily (Theißen *et al*., 1996) was identified (amino acid residue 14). A highly conserved sequence of *PI* homologues, KHExL (Kramer *et al*., 1998), was present in the corresponding region (amino acid residues 83–87). *PaPI* carried the PI motif (MPFxFRVQPxQPNLQE) in the C-terminal region between amino acid residues 193 and 208. This motif is present in almost all described *PI* type proteins (Kramer *et al*., 1998). *PaPI* protein had high similarity to proteins known to correspond to *PI* subfamily members (Table 1). In particular, *PaPI* showed the highest similarity to *PpMADS10* of peach (*P.*

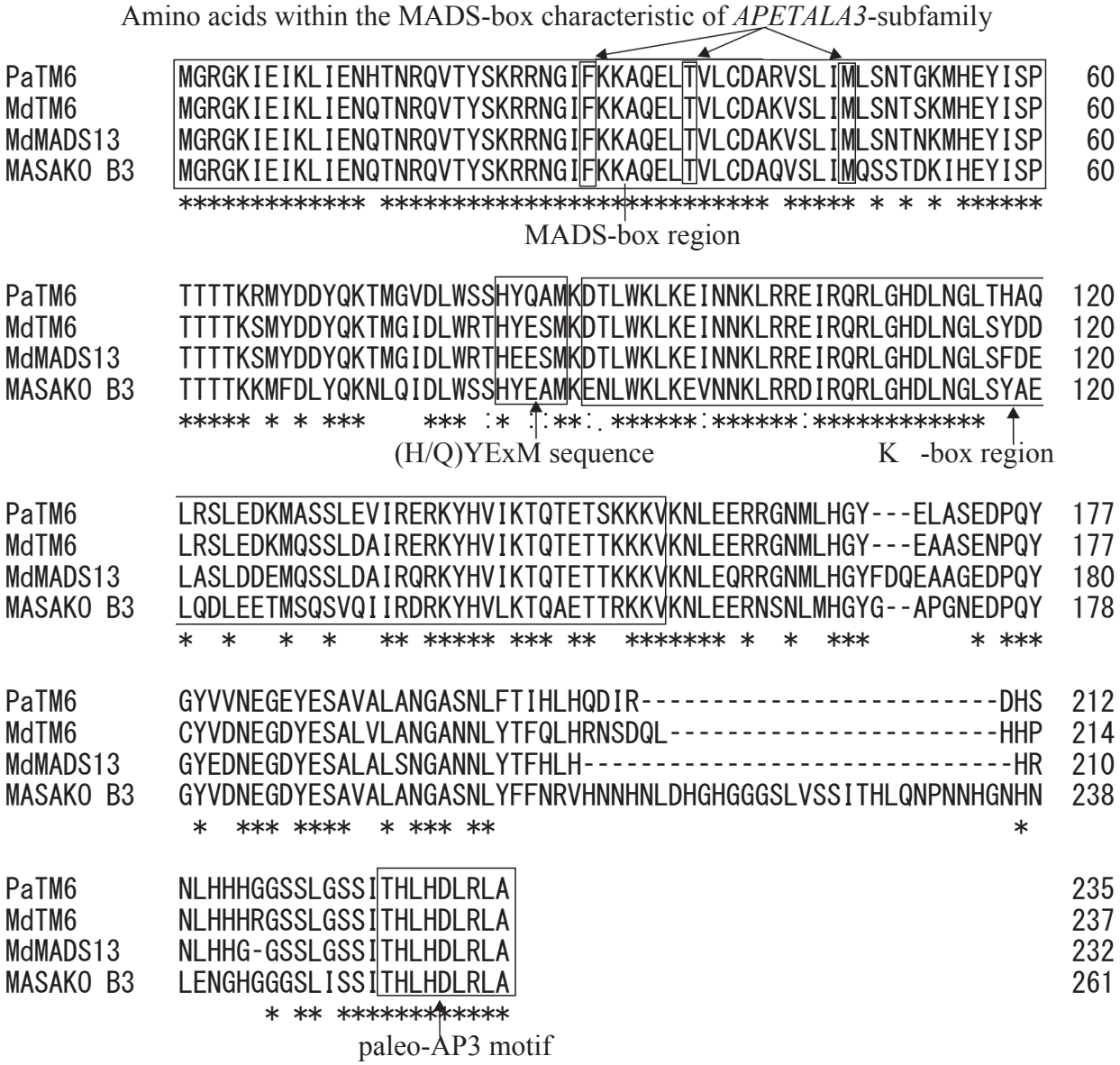

Fig. 1. Deduced amino acid sequences of AP3 homologue genes in Rosaceae species, sweet cherry [*PaTM6* (this study)], apple [*MdTM6* (Kitahara *et al*., 2004), *MdMADS13* (van der Linden *et al*., 2002)] and rose [*MASAKO B3* (Kitahara and Matsumoto, 2000)].

persica) (97%), in the same genus as sweet cherry, followed by *MdPI* of apple (88%). These results suggest that *PaPI* is a sweet cherry homologue of *PI*.

Expression analysis of class B MADS-box gene: In *Arabidopsis*, expressions of *AP3* and *PI* in mature flowers are restricted to petals and stamens (Jack *et al*., 1992; Goto and Meyerowitz, 1994). The same expression patterns have been reported in Rosaceae species such as rose and apple (Kitahara *et al*., 2001; Linden *et al*., 2002). In the present study, the expression patterns of *PaTM6* and *PaPI* were characterised by RT-PCR analysis. Fragments of the expected size (392 bp) were amplified by RT-PCR from petals and stamens with AP3-RT-F and AP3-RT-R primers (Fig. 4). RT-PCR with PI-RT-F and PI-RT-R primers also amplified a fragment of the expected size (404 bp) from petals and stamens. No RT-PCR fragment was observed in leaves, sepals and pistils with these primer sets. These results suggested that both *PaTM6* and *PaPI* genes were expressed specifically in petals and stamens, thus displaying the same expression patterns as *AP3* and *PI*, respectively (Jack *et al*., 1992; Goto and Meyerowitz, 1994). The detection of *PaTM6* and *PaPI* transcripts in petals and stamens supports the hypothesis that *PaTM6* and *PaPI* are homologues of *AP3* and *PI*, respectively.

Based on sequence similarity and tissue-specific expression patterns, *PaTM6* and *PaPI* were inferred to be homologues of *APETALLA3* and *PISTILLATA*, respectively. Next we plan to analyse transcription levels of the class B genes *PaTM6* and *PaPI* and the class C genes *PaAG* and *PaSHP* (Beppu *et al*., 2015) in the flower buds of sweet cherry grown at different temperatures, with the aim of clarify their involvement in pistil doubling.

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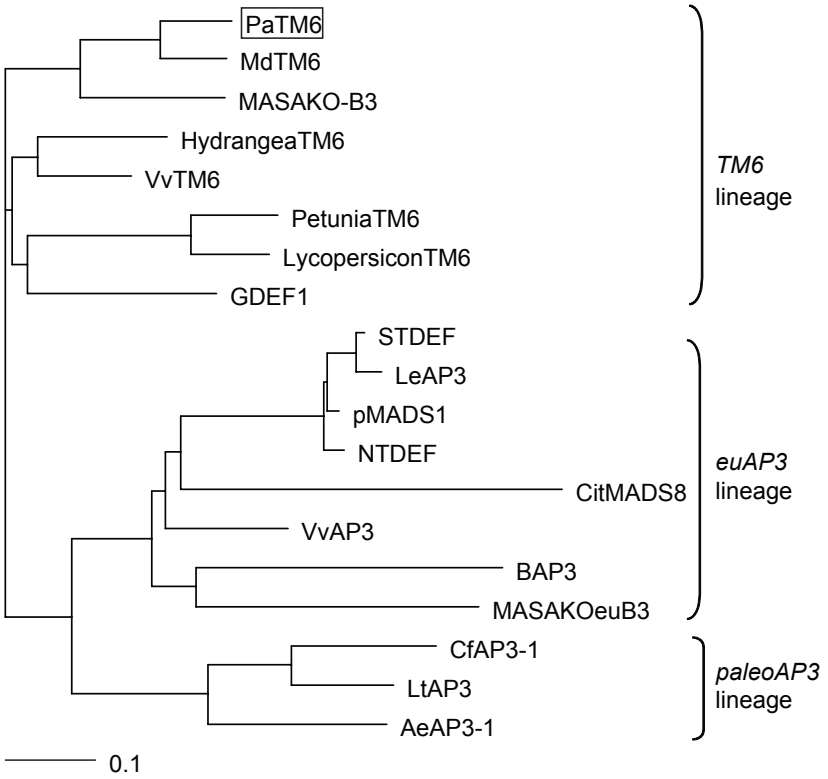

Fig. 2. Phylogenic trees of the deduced amino acid sequences of *PaTM6* (box) and other *AP3* homologues (see Materials and methods).

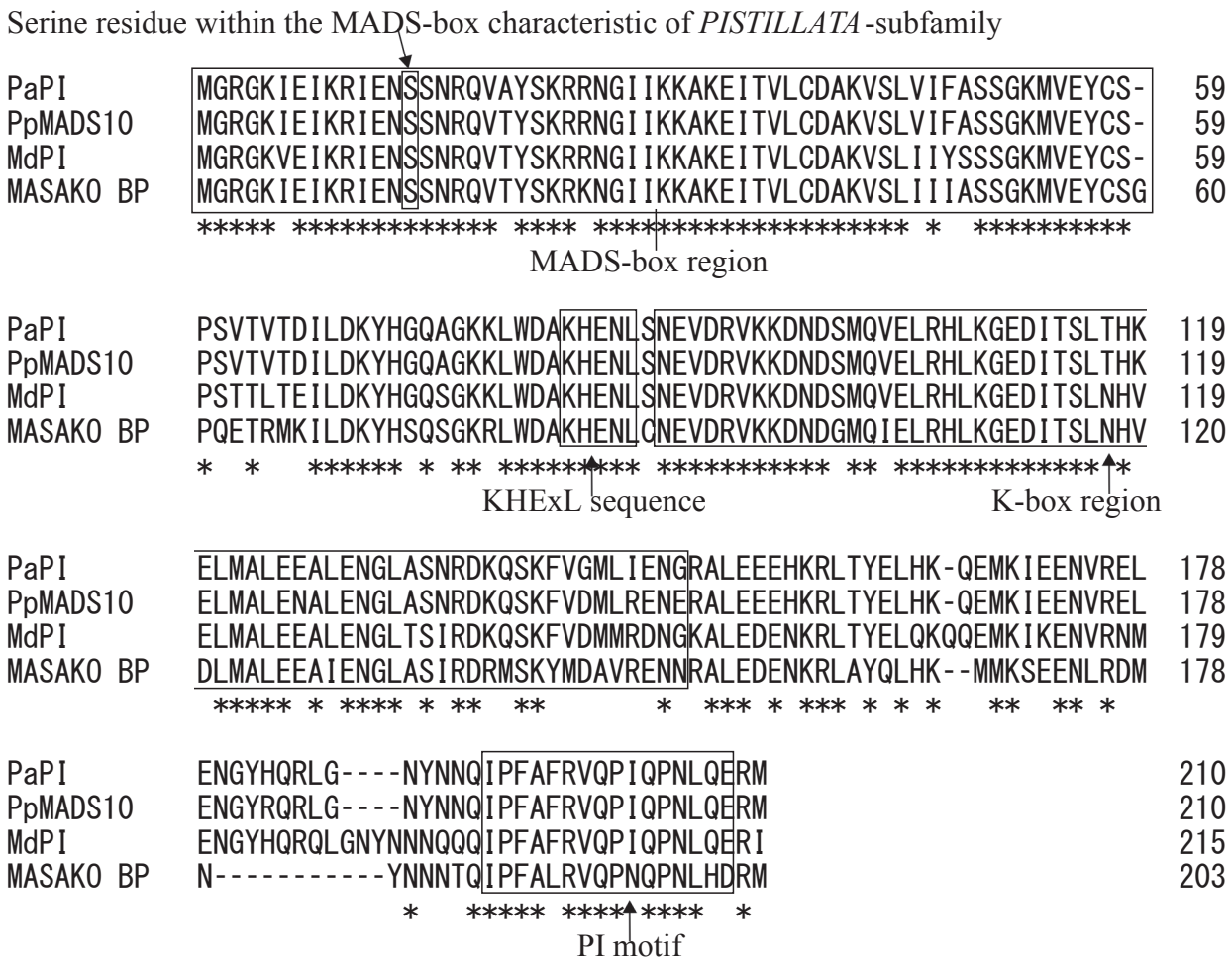

Fig. 3. Deduced amino acid sequences of *PI* homologue genes in Rosaceae species, sweet cherry [*PaPI* (this study)], peach [*PpMADS10* (Zhang *et al*., 2008)], apple [*MdPI* (Yao *et al*., 2001)] and rose [*MASAKO BP* (Kitahara and Matsumoto, 2000)].

Table 1. Identities of deduced amino acid sequences of *PaPI* and other *PI* homologues (see Materials and methods) (%)

	PpMADS10	*MdPI*	*MASAKO-BP*	*VvPI*	*CUM26*	*pMADS2*	*GGLO1*	*PI*
PaPI	97	88	76	76	75	70	63	59
PpMADS10		88	77	76	76	70	65	60
MdPI			78	72	74	68	67	62
MASAKO-BP				66	69	66	62	54
VvPI					77	70	69	59
CUM26						72	72	60
pMADS2							71	59
GGLO1								59

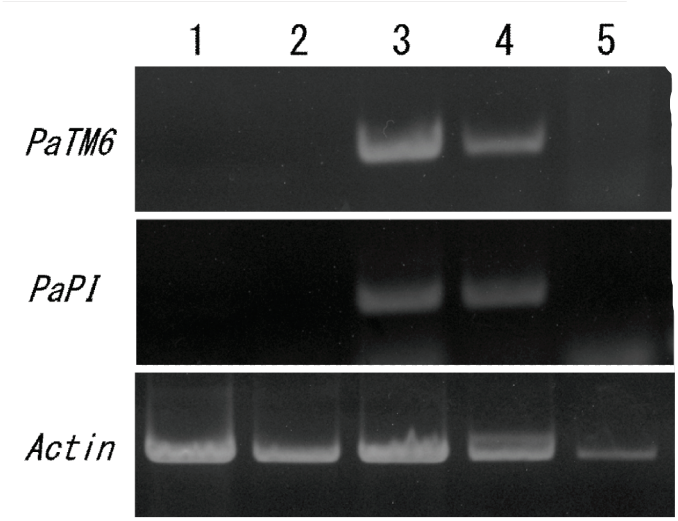

Fig. 4. RT-PCR analyses of *PaTM6* and *PaPI* genes in different tissues. Lane 1, leaves; 2, sepals; 3, petals; 4, stamens; 5, pistils.

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