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RESEARCH PAPER

Functional analysis reveals the possible role of the C-terminal sequences and PI motif in the function of lily (*Lilium longiflorum*) *PISTILLATA* (*PI*) orthologues

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Abstract

Two lily (*Lilium longiflorum*) *PISTILLATA* (*PI*) genes, *Lily MADS Box Gene 8* and *9* (*LMADS8/9*), were characterized. LMADS9 lacked 29 C-terminal amino acids including the PI motif that was present in LMADS8. Both *LMADS8/9* mRNAs were prevalent in the first and second whorl tepals during all stages of development and were expressed in the stamen only in young flower buds. LMADS8/9 could both form homodimers, but the ability of LMADS8 homodimers to bind to CArG1 was relatively stronger than that of LMADS9 homodimers. *35S:LMADS8* completely, and *35S:LMADS9* only partially, rescued the second whorl petal formation and partially converted the first whorl sepal into a petal-like structure in *Arabidopsis pi-1* mutants. Ectopic expression of *LMADS8-C* (with deletion of the 29 amino acids of the C-terminal sequence) or *LMADS8-PI* (with only the PI motif deleted) only partially rescued petal formation in *pi* mutants, which was similar to what was observed in *35S:LMADS9/pi* plants. In contrast, *35:LMADS9+L8C* (with the addition of the 29 amino acids of the LMADS8 C-terminal sequence) or *35S:LMADS9+L8PI* (with the addition of the LMADS8 PI motif) demonstrated an increased ability to rescue petal formation in *pi* mutants, which was similar to what was observed in *35S:LMADS8/pi* plants. Furthermore, ectopic expression of *LMADS8*-M (with the MADS domain truncated) generated more severe dominant negative phenotypes than those seen in *35S:LMADS9*-M flowers. These results revealed that the 29 amino acids including the PI motif in the C-terminal region of the lily PI orthologue are valuable for its function in regulating perianth organ formation.

Key words: *A. thaliana*, *L. longiflorum*, MADS box genes, PI motif, *PISTILLATA*.

Introduction

The ABCDE model predicts the formation of any flower organ through the interaction of five classes of homeotic genes in plants (Coen and Meyerowitz, 1991; Theissen, 2001; Litt and Kramer, 2010). MADS box genes have a central role in flower development because most ABCDE genes encode MADS box proteins (Coen and Meyerowitz, 1991; Purugganan *et al.*, 1995; Rounsley *et al.*, 1995; Theissen *et al.*, 2000; Theissen and Saedler, 2001; Litt and Kramer, 2010).

B group genes, such as *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), have a major role in specifying petal and stamen development (Jack *et al.*, 1992, 1994; Goto and Meyerowitz, 1994; Krizek and Meyerowitz, 1996; Kramer *et al.*, 1998; Winter *et al.*, 1999; Hernandez-Hernandez *et al.*, 2007; Kanno *et al.*, 2007; Whipple *et al.*, 2007; Irish, 2009). In *Arabidopsis*, mutations in *AP3* or *PI* result in identical phenotypes, in which the second whorl petal is converted into a sepal structure and the third flower whorl stamen is converted into a carpel structure (Bowman *et al.*, 1989; Jack *et al.*, 1992; Goto and Meyerowitz, 1994). Similar conversions for these petals and stamens are observed in the mutants of the *AP3* and *PI* orthologues from a number of core eudicot species (Sommer *et al.*, 1990; Tröbner *et al.*, 1992; Angenent *et al.*, 1993; van der Krol *et al.*, 1993; Yu *et al.*, 1999; Liu *et al.*, 2004; Vandenbussche *et al.*, 2004;

de Martino *et al.*, 2006), basal eudicot species (Drea *et al.*, 2007; Kramer *et al.*, 2007), and monocot species (Ambrose *et al.*, 2000; Nagasawa *et al.*, 2003; Prasad and Vijayraghavan, 2003; Yadav *et al.*, 2007; Yao *et al.*, 2008), indicating that the function of the B class genes *AP3* and *PI* is highly conserved during evolution.

Based on analyses of sequence diversity, B group genes have been proposed to have arisen from an ancestral gene through multiple gene duplication events (Doyle, 1994; Theissen *et al.*, 1996, 2000; Purugganan, 1997; Kramer *et al.*, 1998; Lamb and Irish, 2003; Kim *et al.*, 2004; Stellari *et al.*, 2004; Hernandez-Hernandez *et al.*, 2007). The first duplication is thought to have generated the *PI* and paleo*AP3* lineages. The *PI* lineage is composed of *PI* orthologues that contain a highly conserved PI motif identified in most plant species (Kramer *et al.*, 1998). The paleo*AP3* lineage is composed of *AP3* orthologues identified in lower eudicots, magnolid dicots, and monocots (Kramer *et al.*, 1998). The second duplication from the paleo*AP3* lineage is thought to have generated the eu*AP3* and *TM6* gene lineages, which have been subject to substantial sequence changes in dicots during evolution (Kramer *et al.*, 1998; Kramer and Irish, 1999).

Proteins in the eu*AP3* lineage and in most paleo*AP3* lineages are not able to form homodimers. They have to interact with PI to form heterodimers to regulate petal and stamen development (Schwarz-Sommer *et al.*, 1992; Tröbner *et al.*, 1992; Riechmann *et al.*, 1996; Moon *et al.*, 1999; Vandenbussche *et al.*, 2004; Yao *et al.*, 2008). In contrast, the ability of B class proteins to form homodimers has been reported in the gymnosperms as well as in the paleo*AP3* and *PI* lineages of some non-grass monocots (Winter *et al.*, 1999; Tzeng and Yang, 2001; Hsu and Yang, 2002; Winter *et al.*, 2002; Kanno *et al.*, 2003; Tsai *et al.*, 2004, 2005; Chang *et al.*, 2010). It has been shown that both paleoAP3 and the PI-derived motifs for the paleoAP3 orthologue LMADS1 in lily are essential for the formation of homodimers because the deletion of these two motifs completely abolishes the homodimerization of LMADS1 (Tzeng *et al.*, 2004). Whether the PI motif identified in PI orthologues is also involved in their homodimerization and important for their function still remains unclear and thus requires additional experimental analysis.

Among monocots, research on the B class genes is mostly limited to maize (Ambrose *et al.*, 2000), rice (Nagasawa *et al.*, 2003; Prasad and Vijayraghavan, 2003; Yadav *et al.*, 2007; Yao *et al.*, 2008) and the non-grass monocots tulip (Kanno *et al.*, 2003, 2007) and orchid (Hsu and Yang, 2002; Tsai *et al.*, 2004, 2005; Xu *et al.*, 2006; Guo *et al.*, 2007; Kim *et al.*, 2007; Mondragón-Palomino and Theissen, 2008, 2009; Mondragón-Palomino *et al.*, 2009; Chang *et al.*, 2010), but the functions of these genes still remain under investigation. Due to the economic importance of the monocot lily (*Lilium longiflorum*) in the cut flower market, the A, C, D, and E classes as well as one paleo*AP3* orthologue (*LMADS1*) in the B class of MADS box genes have been analysed for function in this flower (Tzeng and Yang, 2001; Tzeng *et al.*, 2002, 2003, 2004; Benedito *et al.*, 2004; Chen *et al.*, 2008; Hsu *et al.*, 2010). In contrast to most plant species, two *PI*-like genes have been reported in the lily (*L. regale* and *L. formolongi*) genome (Winter *et al.*, 2002; Akita *et al.*, 2008). These two genes may have redundant functions. However, no further functional analyses of these two genes have been reported. In the present study, the isolation and functional analysis of two putative *PI*-like MADS box genes from lily (*L. longiflorum*) are reported. An exploration of the possible function of these two lily *PI* orthologues in the regulation of perianth formation, as well as the role of the conserved PI motif in homodimerization and function, is conducted, and the results are discussed.

Materials and methods

Plant materials and growth conditions

Plants of lily (*L. longiflorum* Thunb. cv. Snow Queen) used in this study were grown in the field in Tein Wei County, Chang Haw, Taiwan. The *Arabidopsis pi-1* mutant line (CS77) in the Landsberg background was obtained from the Arabidopsis Biological Resource Center, Ohio State University, Columbus, OH, USA. Seeds for *Arabidopsis* were germinated and grown as described previously (Chang *et al.*, 2010).

Cloning of LMADS8 *and* LMADS9 *cDNAs from* L. longiflorum

PCR products containing partial sequences that showed high sequence identity to *PI* MADS box genes were identified as described by Tzeng and Yang (2001). Internal gene-specific primers were designed for 3′- and 5′-rapid amplification of cDNA ends (3′- and 5′-RACE) by using the SMART™ RACE cDNA Amplification kit (BD Biosciences Clontech, Palo Alto, CA, USA). The gene-specific primer for 3′-RACE was 5′-AGCGCGGA-GATTGATAGGATCAAGAAGG-3′; and that for 5′-RACE was 5′-CCTCAGAAAATCATTCTGCTTCTCCCGGAC-3′. The RACE identified sequences for two genes, one for *LMADS8* (GenBank accession no. HQ698550) and the other one that contained a sequence distinct from that of *LMADS8* was named *LMADS9* (GenBank accession no. HQ698551).

The cDNA for *LMADS8* were obtained by PCR amplification using the forward primer 5′-GGGGATCCCCATGGGTCGTGG-CAAGATC-3′ and reverse primer 5′-CCGGATCCCTACT-TGTCCTCATGTAAATTAGGCTGG-3′. The cDNA for *LMADS9* was obtained by PCR amplification using the forward primer 5′-GGGGATCCCCATGGGTCGTGGCAAGATC-3′ and reverse primer 5′-CCGGATCCCTATATGCAAGTTC-CATGTCCCTCATA-3′. The specific forward and reverse primers for *LMADS8* and *9* contained the generated *Bam*HI (5′-GGATCC-3′, underlined) recognition site to facilitate the cloning of the cDNA.

The cDNA constructs containing truncated MADS boxes (*LMADS*-M) were obtained by PCR amplification and site-specific mutagenesis. The following 5′ primers with an ATG start codon (in bold) right after the 5′ MADS box domain were used for PCR amplification: 5′-TCTAGA**ATG**TATCAGGTGAACTGCGGC-3′ for *LMADS8*-M, and 5′-GGGGATCC**ATG**TACCAGTT-GAACTCCGGCAAGAAG-3′ for *LMADS9*-M. The individual 3′ primers used for each construct were 5′-CCGGATCCC-TACTTGTCCTCATGTAAATTAGGCTGG-3′ for *LMADS8*-M, and 5′-CCGGATCCCTATATGCAAGTTCCATGTCCCTCATA-3′ for *LMADS9*-M. The specific 5′ primers contained the generated *Xba*I (5′-TCTAGA-3′, underlined) or *Bam*HI (5′-GGATCC-3′, underlined) recognition site to facilitate the cloning of the cDNA.

Construction of LMADS8 *with deletion and* LMAD9 *with addition sequences from* L. longiflorum

The cDNA constructs containing the truncated C-terminal or PI motif of *LMADS8* (*LMADS8-C* or *LMADS8-PI*) were obtained by PCR amplification and site-specific mutagenesis using the forward primer 5′-GGTACCATGGGTCGTGGCAAGATCGA-GATC-3′ and two reverse primers with TAG stop codons (in bold) 5′-GGTACC**CTA**TGCAAATTCCAAGTTCCTCAT-3′ and 5′-GGTACC**TAC**TGGGAACCGAAATCCCCATCT-3′ for *LMADS8-C* and *LMADS8-PI*, respectively. The specific forward and reverse primers for *LMADS8-C* and *LMADS8-PI* contained the generated *Kpn*I (5′-GGTACC-3′, underlined) recognition site to facilitate the cloning of the truncated cDNA.

The cDNA constructs of *LMADS9* containing an additional C-terminal or PI motif from *LMADS8* (*LMADS9+L8C* or *LMADS9+L8PI*) were obtained by primer extension techniques for PCR amplification. For *LMADS9+L8C* construction, the forward primer 5′-GGATCCCCATGGGTCGTGGCAAGATCGA-3′ and five reverse primers 5′-CCATCTTTATGATGATATATGCAAG-TTCCATGTCCCTCATATTCTCC-3′, 5′-CTGGGAACCGAAA-TCCCCATCTTTATGATGATATATGCAAGTTCCATGT-3′, 5′-ACACGAAAGGCCATTGGCATCTGGGAACCGAAATCC CCATCTTTATG-3′, 5′-AATTAGGCTGGATTGGCTGCACA-CGAAAGGCCATTGGCATCTGG-3′, and 5′-GGATCCTACT-TGTCCTCATGTAAATTAGGCTGGATTGGCTGCACACGA-3′ were used sequentially. For *LMADS9+L8PI* construction, the forward primer 5′-GGATCCCCATGGGTCGTGGCAAGATC-GA-3′ and three reverse primers 5′-ACACGAAAGGCCATTGG-CATTATGCAAGTTCCATGTCCCTCATATTCTCC-3′, 5′-AA-TTAGGCTGGATTGGCTGCACACGAAAGGCCATTGGCAT-TATGCA-3′, and 5′-GGATCCTACTTGTCCTCATGTAAAT-TAGGCTGGATTGGCTGCACACGA-3′ were used sequentially. The specific forward and reverse primers for *LMADS9+L8C* and *LMADS9+L8PI* contained the generated *Bam*HI (5′-GGATCC-3′, underlined) recognition site to facilitate the cloning of the cDNA.

Quantitative real-time PCR

For quantitative real-time PCR, the reaction was performed on an MJ Opticon system (MJ Research, Waltham, MA, USA) using SYBER Green Real-time PCR Master Mix (TOYOBO Co., Ltd). The amplification condition was 95 °C for 10 min, followed by 40 cycles of amplification (95 °C for 15 s, 58 °C for 15 s, 72 °C for 30 s, and then plate reading) and melting (50–95 °C with plate readings every 1 °C). *LMADS8* primers 5'-TCCTGTGCCTGCTATTT-TATTTAGAC-3′ and 5′-TTATACTATCAAATTACTACAT-GAACATCAAGAG-3′ were used in lily, and 5′-TCGGTT CCCAGATGCCAATG-3′ and 5′-GTTGCCCGCATAATTAC-GAATATC-3′ were used in *LMADS8* transgenic *Arabidopsis*. *LMADS9* primers 5′-CATGCTTATCGTTACCACTCTACTTG-3′ and 5′-ACTATACTAATAATTACTACATGAACATCTATGC-3′ were used in lily, and 5′-CGAGGAGGCACTAGAGAATGG-3′ and 5′-CGTAACATAAGGGACTGACCAC-3′ were used in *LMADS9* transgenic *Arabidopsis*. The *L. longiflorum ACTIN* gene was used as a normalization control with the primers Liact-F (5′-ATCCCAGCAGCGTCGCACATCC-3′) and Liact-R (5′-GCCA-GATCTTCTCCATGTCATCC-3′) (Wang *et al.*, 2009). The *Arabidopsis* housekeeping gene *UBQ10* was used as normalization control with the following primers: RT-UBQ10-1 (5′-CTCAGGCTCCG-TGGTGGTATG-3′) and RT-UBQ10-2 (5′-GTGATAGTTTTCC-CAGTCAACGTC-3′). The data were analysed using the Gene Expression Macro software (version 1.1, Bio-Rad).

Plant transformation and transgenic plant analysis

The full-length cDNAs of *LMADS8* and *9* or *LMADS8*/*9*-M were cloned into the binary vector pBI121 (Clontech, Palo Alto, CA, USA) under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. These constructs were transformed into *Arabidopsis* plants using the floral dip method as described elsewhere (Clough and Bent, 1998). Transformants were selected in medium containing 50 μg ml^{-1} kanamycin and were further verified by PCR and RT-PCR analyses.

Yeast two-hybrid analysis

The cDNA truncations without the MADS box regions for *LMADS1*, *8*, and *9* were amplified by PCR. The primers for *LMADS1* were 5′-TTCTGCGAATTCTCCACAGAC-3′ and 5′-CCTACAGAATTCGGGTTTCA-3′; for *LMADS8* they were 5′-CATATGTATCAGGTGAACTGCGGCAAGA-3′ and 5′-GAATTCCTACTTGTCCTCATGTAAATTAGGCTG-3′; and for *LMADS9* they were 5′-CATATGTACCAGTTGAAC-TCCGGCAAGA-3′ and 5′-GAATTCCTATATGCAAGTTC-CATGTCCCTC-3′. Specific 5′ and 3′ primers contained the *Eco*RI (5′-GAATTC-3′, underlined) or *Nde*I (5′-CATATG-3′, underlined) recognition sites to facilitate cloning of the cDNAs. PCR fragments were ligated into the plasmid pGBKT7 (binding domain vector) or pGADT7 (activation domain vector) provided by the MATCHMAKER Two-Hybrid System 3 (Clontech). Recombinant plasmids were transformed into yeast using the lithium acetate method (Gietz *et al.*, 1992). The transformants were selected on selection medium according to the manufacturer's instructions. β-Galactosidase activity in the transformants was analysed as described by Tzeng and Yang (2001) and calculated according to Miller (1992).

Electrophoretic mobility shift assay (EMSA)

The cDNA for *LMADS1*, *8*, and *9* used to generate proteins were amplified by PCR. The primers for *LMADS1* were 5′-CATA-TGATGGGGCGGGGAAAGATC-3′ and 5′-GGATCCTCAA-GCCAGTCGGAGATCA-3′; for *LMADS8* they were 5′-GGAATTCCATATGATGGGTCGTGGCAAGATC-3′ and 5′-GAATTCCTACTTGTCCTCATGTAAATTAGGC-3′; and for *LMADS9* they were 5′-GGAATTCCATATGATGGGTCGTGG-CAAGATC-3′ and 5′-CCGGATCCCTATATGCAAGTTC-CATGTCCCTCATA-3′. Specific 5′ and 3′ primers contained the *Nde*I (5′-CATATG-3′, underlined), *Bam*HI (5′-GGATCC-3′, underlined), or *Eco*RI (5′-GAATTC-3′, underlined) recognition sites to facilitate cloning of the cDNAs. The PCR products were cloned into expression vector pET28a (Novagen, Madison, WI, USA) and transformed into expression host BL21(DE3). After induction with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG), the inclusion bodies of LMADS1, LMADS8, and LMADS9 were isolated, solubilized in 6 M urea, and then dialysed with NEB buffer [25 mM HEPES-KOH pH 7.6, 40 mM KCl, 0.1 mM EDTA, 10% glycerol, 5 mM β-mercaptoethanol, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), and 0.5 mg ml^{-1} lysozyme].

DNA probes were prepared from the promoter of *Arabidopsis AP3* by PCR amplification using the reverse primer 5′-GACCA-TATGCGACCTAAAAAGGAAAAGAGTTGC-3′ and forward primer DIG-5′-GCAACTCTTTTCCTTTTTAGGTCGCATAT-GGTC-3′ for the 5′ end digoxigenin (DIG)-labelled probe, or forward primer 5′-GCAACTCTTTTCCTTTTTAGGTCGCA-TATGGTC-3′ for the competitor.

For EMSA, DNA binding reactions were performed in a total volume of 12 μl of binding buffer [10 mM TRIS-HCl, pH 7.5, 1 mM EDTA, 90 μM dithiothreitol (DTT), 2.5 mM NaCl, 4% glycerol, 40 μg ml^{-1} bovine serum albumin (BSA)] containing 40 μg ml^{-1} poly(dI–dC), 80 μM PMSF, 50 μg of probe, and 2 μg of proteins. Binding reaction mixtures were incubated for 20 min at room temperature. To separate the protein–DNA complexes, the reaction mixtures were loaded onto a running non-denaturing 5% polyacrylamide gel, which had been pre-run for 1 h at 4 °C and 100 V in 0.5× TRIS-borate-EDTA buffer. Electrophoresis was further carried out at 4 °C and 100 V for 4 h. After electrophoresis, the gels were blotted onto a nylon membrane (Amersham Biosciences, Bucks,

UK), hybridized by anti-DIG horseradish peroxidase antibody (Roche, Mannheim, Germany), developed by 0.1× CSPD (Roche, Mannheim, Germany), and exposed on X-ray film. For competition experiments, a 10-fold molar excess of the cold competitor DNAs was included in the binding reactions.

Scanning electron microscopy (SEM)

SEM was performed according to the methods of Tzeng and Yang (2001) and Hsu and Yang (2002). Samples were fixed in 2% glutaraldehyde in 25 mM sodium phosphate buffer (pH 6.8) at 4 °C overnight. After dehydration in a graded ethanol series, specimens were critical point dried in liquid CO_2. The dried materials were mounted and coated with gold–palladium in a JBS sputter-coater (model 5150). Specimens were examined in a Topcon scanning electron microscope (model ABT-150S) with an accelerating voltage of 15 kV.

Phylogenetic analyses

The amino acid sequences of the B class MADS box proteins were retrieved via the NCBI server (http://www.ncbi.nlm.nih.gov/). The sequence alignment and phylogenetic tree construction were generated by MEGA 5 software (Kumar *et al.*, 2008; Tamura *et al.*, 2011). Multiple sequence alignment of these protein sequences was performed using the ClustalW program (Thompson *et al.*, 1994) with default parameters. The phylogenetic tree was constructed based on the maximum likelihood method with the following parameters: bootstrap (500 replicates), Jones–Taylor–Thornton (JTT) substitution model (Jones *et al.*, 1992), uniform rates, complete deletion of gaps/missing data, and Nearest Neighbor Interchange (NNI). The bootstrap consensus tree was inferred from 500 replicates (Felsenstein, 1985). The numbers at each node indicate the percentage of bootstrap values from 500 replications, and values <50% were not shown. The MADS box proteins used in this study included AP3 (NM_115294), ApDEF (AB177941), ApGLO (AB079259), Boi1AP3 (U67453), Boi2AP3 (U67455), CMB2 (L40405), CsAP3a (AY948339), CsAP3b (AY948340), CsPIA1 (DQ231247), CsPIB (DQ231249), CsPIC1 (DQ231250), DEF2 (X67508), DEF (X62810), EGM2 (AF029976), FBP1 (M91190), FBP3 (X71417), GDEF1 (AJ009724), GDEF2 (AJ009725), GGLO1 (AJ009726), HPI1 (AF134114), HPI2 (AF134115), LFGLOA (AB359186), LFGLOB (AB359187), LMADS1 (AF503913), LMADS8 (HQ698550), LMADS9 (HQ698551), LRDEF (AB071378), LRGLOA (AB071379), LRGLOB (AB071380), NMH7 (L41727), NTGLO (X67959), OMADS8 (HM140842), OsMADS2 (NM_001051547), OsMADS4 (NM_001062125), OsMADS16 (NM_001065095), PeMADS6 (AY678299), PhaPI9 (AY748818), PhaPI10 (AY771991), PI (NM_122031), PMADS1 (X69946), PMADS2 (X69947), SLM3 (X80490), TGDEFA (AB094965), TGDEFB (AB094966), TGGLO (AB094967), TM6 (DQ539417), ZMM16 (AJ292959), ZMM18 (AJ292960), and ZMM29 (AJ292961).

Results

Isolation of LMADS8 *and* LMADS9 *cDNAs from lily (*L. longiflorum*)*

To isolate the MADS box genes from *L. longiflorum*, a strategy that combined RT-PCR and 5′- and 3′-RACE was used. A DNA fragment was amplified by RT-PCR using total RNA from young floral buds as a template. Sequence comparison led to the identification of partial sequences for several MADS box genes. The full-length cDNA sequences for two *PI*-like B class genes, *LMADS8* and *9*, were isolated.

The *LMADS8* cDNA encoded a 210 amino acid protein that showed high sequence identity to the product of the B class gene *PI* in monocots (Fig. 1A). LMADS8 showed the highest sequence identity to LFGLOA of *L. formolongi* (Akita *et al.*, 2008) and LRGLOA of *L. regale* (Winter *et al.*, 2002) (100% and 99%, respectively) (Fig. 1A). In the MADS box domain, 100% of the amino acids were identical between LMADS8 and LFGLOA/LRGLOA (Fig. 1A). LMADS8 also showed 51% identity and 71% similarity to *Arabidopsis* PI (Fig. 1A). A sequence (KHEHL) that was identical to a highly conserved sequence (KHExL) among the *PI* orthologues was found within the K box domain (Fig. 1A) (Kramer *et al.*, 1998). A consensus PI motif (MPFxFRVQPxQPNLQE), which is unique to B class PI proteins (Kramer *et al.*, 1998; Moon *et al.*, 1999), was identified in the LMADS8 C-terminal region (Fig. 1A).

The cDNA sequence for *LMADS9* encoded a 181 amino acid protein that showed a high sequence identity (100% and 98%) to LFGLOB of *L. formolongi* (Akita *et al.*, 2008) and LRGLOB of *L. regale* (Winter *et al.*, 2002), respectively (Fig. 1A). LMADS9 also showed high sequence identity to LMADS8 (88% total, 95% in the MADS box, and 90% in the K box) (Fig. 1A). Similar to LMADS8, a conserved sequence (KHEHL) was also identified within the K box of LMADS9 (Fig. 1A). Interestingly, LMADS9 seemed to be a truncated form of LMADS8 based on the sequence comparison. Twenty-nine amino acids, including the consensus PI motif, in the C-terminal region of LMADS8 were not present in LMADS9 (Fig. 1A). To confirm this hypothesis further, the cDNA sequences (including the 3'-untranslated region sequence) of *LMADS8* and *LMADS9* were compared. A small deletion of 24 bp of DNA sequence after the stop codon was found in *LMADS9* (Fig. 1B). After this deletion, a sequence of 48 bp that was highly matched to the PI motif of *LMADS8* (only five out of 48 nucleotides are different between *LMADS8* and *LMADS9*) was identified in the *LMADS9* cDNA (Fig. 1B). Thus, this result strongly supported the conclusion that LMADS9 is a truncated form of LMADS8.

The high sequence identity among *LMADS8* and *9* and the *PI* orthologues from various plant species suggests that *LMADS8* and *9* are the putative *PI* orthologues in lily. The amino acid sequences shown in Fig. 1A and sequences of several other MADS box proteins were used to construct a phylogenetic tree of the B functional genes (Fig. 2). On the basis of this analysis, it was concluded that *LMADS8* and *9* are in the *PI* lineage of the monocot Liliales.

Gene expression of LMADS8 *and* LMADS9

To explore the relationships between sequence homology and expression patterns for *LMADS8* and *LMADS9*, *LMADS8/9* expression was detected using quantitative real-time PCR analysis. The *LMADS8* mRNA was absent in the vegetative leaves, root, and stem, but it was detected in the flowers (Fig. 3A). When the expression of *LMADS8* in flower organs of the flower buds during different developmental stages (<5, 5, 15, and 25 mm in length) was analysed

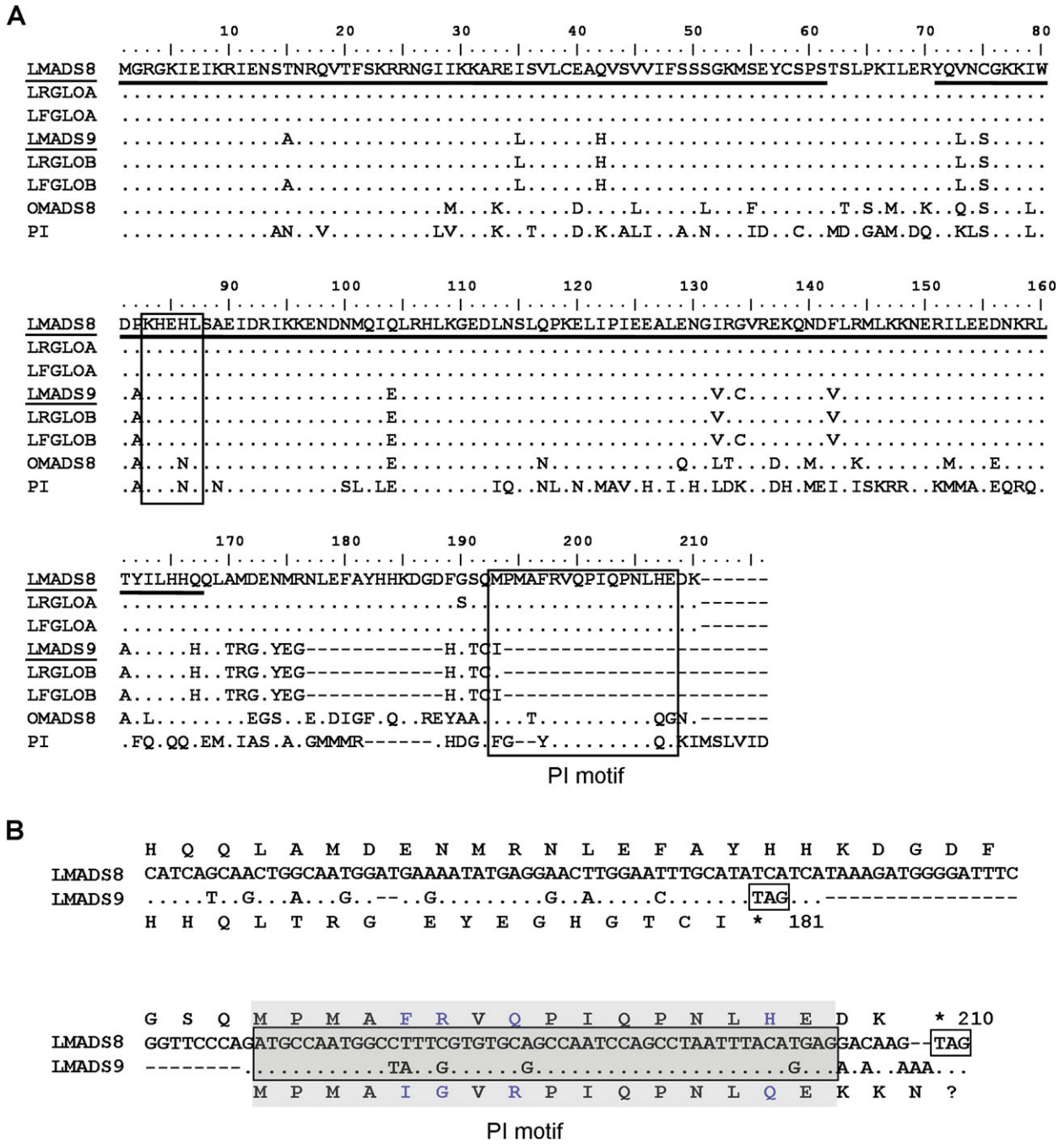

Fig. 1. Sequence information for the LMADS8 and 9 proteins. (A) Sequence comparison of LMADS8 and 9 as well as the related B class MADS domain proteins. The MADS box proteins include LMADS8 and 9 (*Lilium longiflorum*), LRGLOA and B (*Lilium regale*), LFGLOA and B (*Lilium formolongi*), OMADS8 (*Oncidium* Gower Ramsey), and PI (*Arabidopsis thaliana*). The first and second underlined regions represent the MADS domain and K domain, respectively. The box in the K domain represents a sequence (KHExL) that is a highly conserved for PI orthologues. The box in the C-terminal region represents the PI motif (MPFxFRVQPxQPNLQE) that is a highly conserved motif for PI orthologues. Amino acid residues identical to LMADS8 are indicated as dots. To improve the alignment, dashes were introduced into the sequence. The names of the LMADS8 and 9 proteins are underlined. This sequence alignment was generated by the ClustalW Multiple Sequence Alignment Program at the DNA Data Bank of Japan (http://clustalw.ddbj.nig.ac.jp/top-e.html). (B) Sequence comparison of LMADS8 and 9 proteins in the C-terminal region. The PI motif is in grey and boxed. Amino acid residues identical to LMADS8 are indicated as dots. To improve the alignment, dashes were introduced into the sequence.

further, *LMADS8* mRNA was consistently highly expressed in the first and second whorl tepals from young to mature flower buds (Fig. 3A–D). *LMADS8* mRNA was also detected in stamens during early flower development (<5 mm and 5 mm flower buds) (Fig. 3A, B), but its levels were significantly lower in mature flower buds (15 mm and 25 mm flower buds) (Fig. 3C, D). In contrast, *LMADS8* mRNA was not detected in the carpel from young to

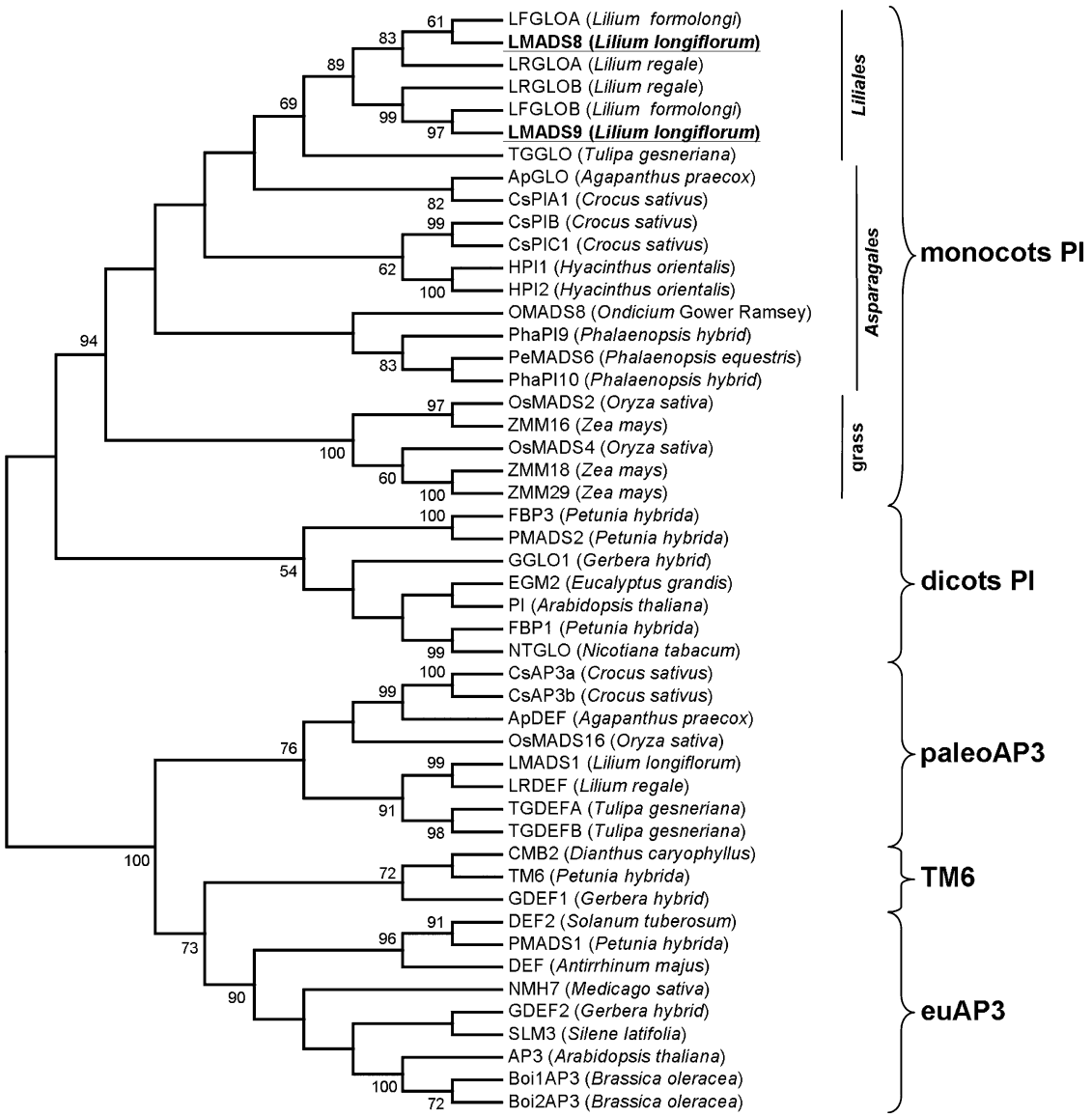

Fig. 2. Phylogenetic analysis of B class MADS domain proteins. Based on the amino acid sequence of the full-length protein, LMADS8 and 9 were closely related to LRGLOA and B (*L. regale*), LFGLOA and B (*L. formolongi*), and TGGLO (*Tulipa gesneriana*) in the PI group of MADS box genes in the monocot Liliales. The names of the LMADS8 and 9 proteins are shown in bold and underlined. The names of the plant species for each MADS box gene are listed after the protein names. The maximum likelihood (ML) phylogenetic tree was generated based on the maximum likelihood method using MEGA 5 software as described in the Materials and methods. The numbers at each node indicate the percentage of bootstrap values from 500 replications, and the bootstrap values <50% were hidden.

mature flower buds (Fig. 3A–D). The expression pattern and the strength of the expression for *LMADS9* were extremely similar to those observed for *LMADS8* (Fig. 3E–H). The *LMADS9* mRNA was also absent in the vegetative leaves, stem, and root (Fig. 3E), and the *LMADS9* mRNA was highly expressed in the first and second whorl tepals during different developmental stages (Fig. 3E–H). In the stamens, *LMADS9* mRNA was also detected only in young flower buds (<5 mm and 5 mm flower buds) (Fig. 3E, F) and was barely detectable in mature flower buds (15 mm and 25 mm flower buds) (Fig. 3G, H). Similar to that of *LMADS8*, *LMADS9* mRNA was also not detected in the carpel from young to mature flower buds (Fig. 3E–H). The expression patterns for *LMADS8* and *LMADS9* were different from those observed for *PI* orthologues in *Arabidopsis* and rice; these orthologues are only expressed in stamens and petals (Goto and Meyerowitz, 1994; Rounsley *et al.*, 1995; Prasad and Vijayraghavan, 2003; Yao *et al.*, 2008). Their expression patterns were also different from those of *PI* orthologues in monocot orchids, which are expressed in all four flower organs (Tsai *et al.*, 2005; Xu *et al.*, 2006; Chang *et al.*, 2010).

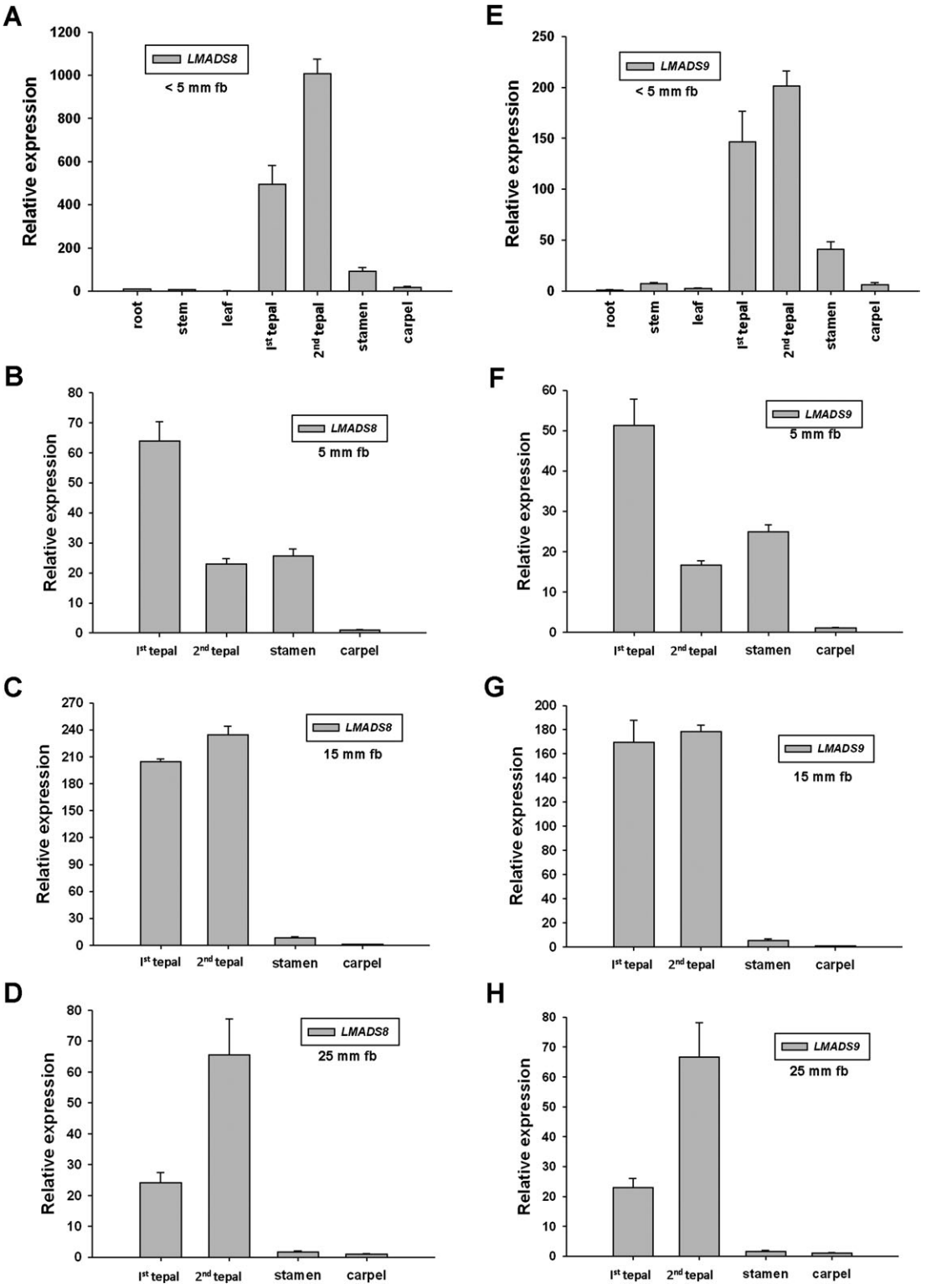

Fig. 3. Detection of expression of *LMADS8* and *LMADS9* in *L. longiflorum*. Total RNA isolated from the root, stem, leaf, and flower organs of the first and second whorl tepals, stamen, and carpel of *L. longiflorum* flower buds at different developmental stages (<5, 5, 15, and 25 mm in length) were used as templates to detect the expression of *LMADS8* (A–D) and *LMADS9* (E–H) by quantitative real-time PCR. The results indicate that *LMADS8* and *LMADS9* have identical expression patterns. *LMADS8* and *LMADS9* mRNAs were absent in the root, stem, and leaf, but were highly expressed in the first and second whorl tepals during all stages of flower development. *LMADS8* and *LMADS9* mRNAs were detected in the stamen only in young flower buds and were almost undetectable in the carpel. In quantitative real-time PCR, the columns represent the relative expression of these genes. The transcript levels were determined using two to three replicates and were normalized using *ACTIN* for lily. The error bars represent standard deviations. Each experiment was repeated three times with similar results.

The ectopic expression of LMADS8 *and* LMADS9 *partially converts sepals into petal-like structures in transgenic* Arabidopsis *plants*

To investigate the function of *LMADS8* and *9* further, cDNAs for these two genes, driven by the CaMV 35S promoter, were transformed into *Arabidopsis* for functional analysis.

Five independent *35S:LMADS8* transgenic *Arabidopsis* plants were obtained. One plant was phenotypically indistinguishable from untransformed wild-type plants, whereas another four plants showed identical altered phenotypes (Fig. 4D). Flowering time, determined by the number of rosette leaves, was not affected in these transgenic plants compared with the wild-type plants. However, flowers produced in the inflorescence of these plants (Fig. 4D) were different from those observed in wild-type plants (Fig. 4A). Unlike in the wild-type flowers (Fig. 4B, C), the partial conversion of the first whorl green sepals into elongated green/white petal-like structures (Fig. 4E, F) was observed in these *35S:LMADS8* flowers. Unlike the wild-type first whorl sepals, which remained tightly associated even after pollination (Fig. 4B, C), these first whorl green/white petal-like structures in *35S:LMADS8* flowers were completely separated immediately after flower opening (Fig. 4E, F). When the epidermal cells of the abaxial side (Fig. 4K) in these first whorl petal-like structures were examined, most of the cells in the converted white portion of the petal-like structures were observed to be morphologically similar to wild-type second whorl petal epidermal cells (Fig. 4M) and distinct from the wild-type first whorl sepal epidermis (Fig. 4J).

Eight independent *35S:LMADS9* transgenic *Arabidopsis* plants were obtained. Three plants were phenotypically indistinguishable from untransformed wild-type plants, whereas another five plants showed altered phenotypes (Fig. 4G) similar to those observed in the *35S:LMADS8* plants. Partial conversion of the first whorl green sepals into green/white petal-like structures (Fig. 4G–I) was also observed in these *35S:LMADS9* flowers. However, the petal-like structure in these *35S:LMADS9* flowers (Fig. 4H) was relatively shorter than that in the *35S:LMADS8* flowers (Fig. 4E). Most of the epidermal cells in the converted white portion of the abaxial side (Fig. 4L) in these first whorl petal-like structures were also morphologically similar to wild-type petal epidermal cells (Fig. 4M).

To explore whether this abnormal phenotype was correlated with *LMADS8/9* expression in the transgenic plants, quantitative real-time PCR analysis was performed. As shown in Fig. 5, higher *LMADS8* (Fig. 5A) and *LMADS9* (Fig. 5B) expression was observed in the transgenic plants with the severe phenotype than in the transgenic plants with wild-type-like phenotypes. This result indicates that the phenotypes generated in the *35S:LMADS8/9* transgenic *Arabidopsis* plants were due to the ectopic expression of the lily *LMADS8/9* gene.

The ectopic expression of LMADS8 *and* LMADS9 *caused different effects in rescuing the* pi *phenotype in* pi-1 *mutants*

To investigate the function of *LMADS8* and *LMADS9* further, the cDNAs of these two genes, driven by the CaMV 35S promoter, were transformed into *pi-1* plants for complementation analysis. The *pi* mutation always causes the conversion of the second whorl petal into a sepal structure and the conversion of the third flower whorl stamen into a carpel structure (Fig. 6A, B) (Bowman *et al.*, 1989; Jack *et al.*, 1992; Goto and Meyerowitz, 1994).

Thirteen independent *35S:LMADS8/pi* transgenic *Arabidopsis* plants were obtained. Seven of these plants showed identical altered phenotypes during reproductive development, whereas the six other plants were phenotypically indistinguishable from untransformed wild-type plants. In these *35S:LMADS8* transgenic *pi-1* plants, the defects of petal formation were fully restored (Fig. 6C, D). In addition, the partial conversion of the first whorl green sepal into a well-expanded green/white petal-like structure (Fig. 6C, D) was observed in these *35S:LMADS8/pi* flowers. In contrast, the defect associated with formation of the third whorl stamen was not restored in these *35S:LMADS8/pi* flowers (Fig. 6C, D).

Seventeen independent *35S:LMADS9/pi* transgenic *Arabidopsis* plants were obtained. Four plants also showed altered phenotypes, in which petal formation was rescued during reproductive development, whereas the other 13 plants were phenotypically indistinguishable from untransformed wild-type plants. However, the restoration of the second whorl petal defects in *35S:LMADS9/pi* (Fig. 6E, F) was weaker than that in *35S:LMADS8/pi* flowers. The second whorl white petals in *35S:LMADS9/pi* flowers (Fig. 6E, F) were clearly shorter than those in *35S:LMADS8/pi* flowers (Fig. 6G). In addition, the partial conversion of the first whorl green sepal into a green/white petal-like structure (Fig. 6E, F) was observed in these *35S:LMADS9/pi* flowers. Similarly, the green/white petal-like structures in these *35S:LMADS9/pi* flowers were much shorter than those in *35S:LMADS8/pi* flowers (Fig. 6H). Similar to what was observed in *35S:LMADS8/pi* flowers, the defect associated with the third whorl stamen formation was not restored in these *35S:LMADS9/pi* flowers (Fig. 6E, F). This finding clearly indicates that *LMADS8* and *LMADS9* were able to rescue the petal, but not the stamen, defect in transgenic *pi-1* mutant plants.

When the epidermal cells in the second whorl petal-like structures in the *35S:LMADS8/pi* (Fig. 6I) and *35S:LMADS9/pi* plants (Fig. 6J) were examined, cells morphologically similar to wild-type petal epidermal cells (Fig. 6L) and distinct from the *pi-1* second whorl sepal-like structure epidermis (Fig. 6K) were observed. When the epidermal cells in the first whorl petal-like structures in the *35S:LMADS8/pi* (Fig. 6M) and *35S:LMADS9/pi* plants (Fig. 6N) were examined, cells morphologically similar to wild-type second whorl petal epidermal cells (Fig. 6L) and distinct from the *pi-1* (Fig. 6O) and wild-type (Fig. 6P) first whorl sepal epidermis were observed.

To explore whether the phenotype was correlated with *LMADS8/9* expression in the transgenic *pi-1* plants,

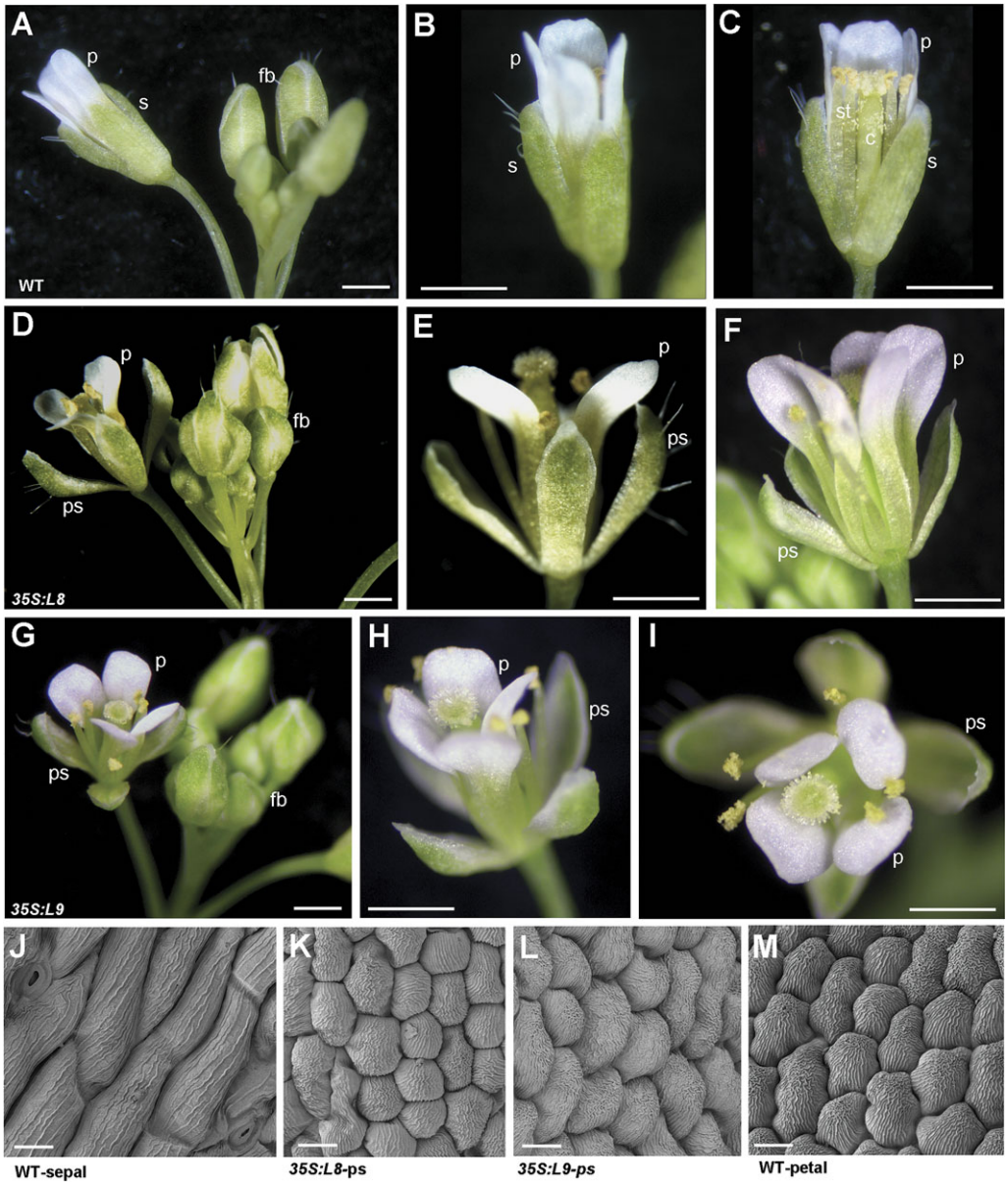

Fig. 4. Phenotypic analysis of transgenic *Arabidopsis* plants ectopically expressing *LMADS8* or *LMADS9*. (A) A wild-type inflorescence contains flower buds (fb) and mature flowers with normal first whorl sepals (s) and second whorl petals (p). (B) A mature wild-type *Arabidopsis* flower consists of four whorls of organs, including four sepals (s), four elongated petals (p), six stamens, and two fused carpels. The stamens and carpels were kept inside by the unopened sepals. (C) A sepal was removed from a mature wild-type *Arabidopsis* flower to reveal the third whorl stamen (st) and fourth whorl carpel (c). s, sepal; p, petal. (D) A *35S:LMADS8* (*35S:L8*) inflorescence contains flower buds (fb) with green/white sepals in the first whorl and fully opened mature flowers with green/white elongated petal-like sepals (ps) in the first whorl and normal petals (p) in the second whorl of the flower. (E) Close-up view of a *35S:LMADS8* transgenic *Arabidopsis* flower. Green/white elongated petal-like sepals (ps) and normal petals (p) were produced in the first and second whorls of the flowers, respectively. (F) Close-up view of a *35S:LMADS8* transgenic *Arabidopsis* flower. Green/white elongated petal-like sepals (ps) and normal petals (p) were produced in the first and second whorls of the flowers, respectively. A petal-like sepal was removed to reveal the inner organs. (G) A *35S:LMADS9* (*35S:L9*) inflorescence contains flower buds (fb) and fully opened mature flowers with green/white petal-like sepals (ps) in the first whorl and normal petals (p) in the second whorl of the flower. (H) Close-up view of a *35S:LMADS9* transgenic *Arabidopsis* flower. Green/white petal-like sepals (ps) and normal petals (p) were produced in the first and second whorls of the flowers, respectively. (I) Close-up view of the top of a *35S:LMADS9* transgenic *Arabidopsis* flower. Green/white petal-like sepals (ps) and normal petals (p) were produced in the first and second whorls of the flowers, respectively. (J) Scanning electron microscopy (SEM) of the surface cells with irregular shapes in the abaxial side of the epidermis of wild-type first whorl sepals. (K) SEM of surface cells of the epidermis in the abaxial side of the first whorl petal-like sepal (ps) of a *35S:LMADS8* (*35S:L8*-ps) flower, which are similar to the mature wild-type second whorl petal epidermis in M. (L) SEM of the surface cells of the epidermis in the abaxial side of the first whorl petal-like sepal (ps) of a *35S:LMADS9* (*35S:L9*-ps) flower, which are similar to the mature wild-type petal epidermis in M. (M) SEM of the surface cells of the epidermis in the abaxial side of a mature wild-type petal. Bar=1 mm in A–I, and 10 μm in J–M.

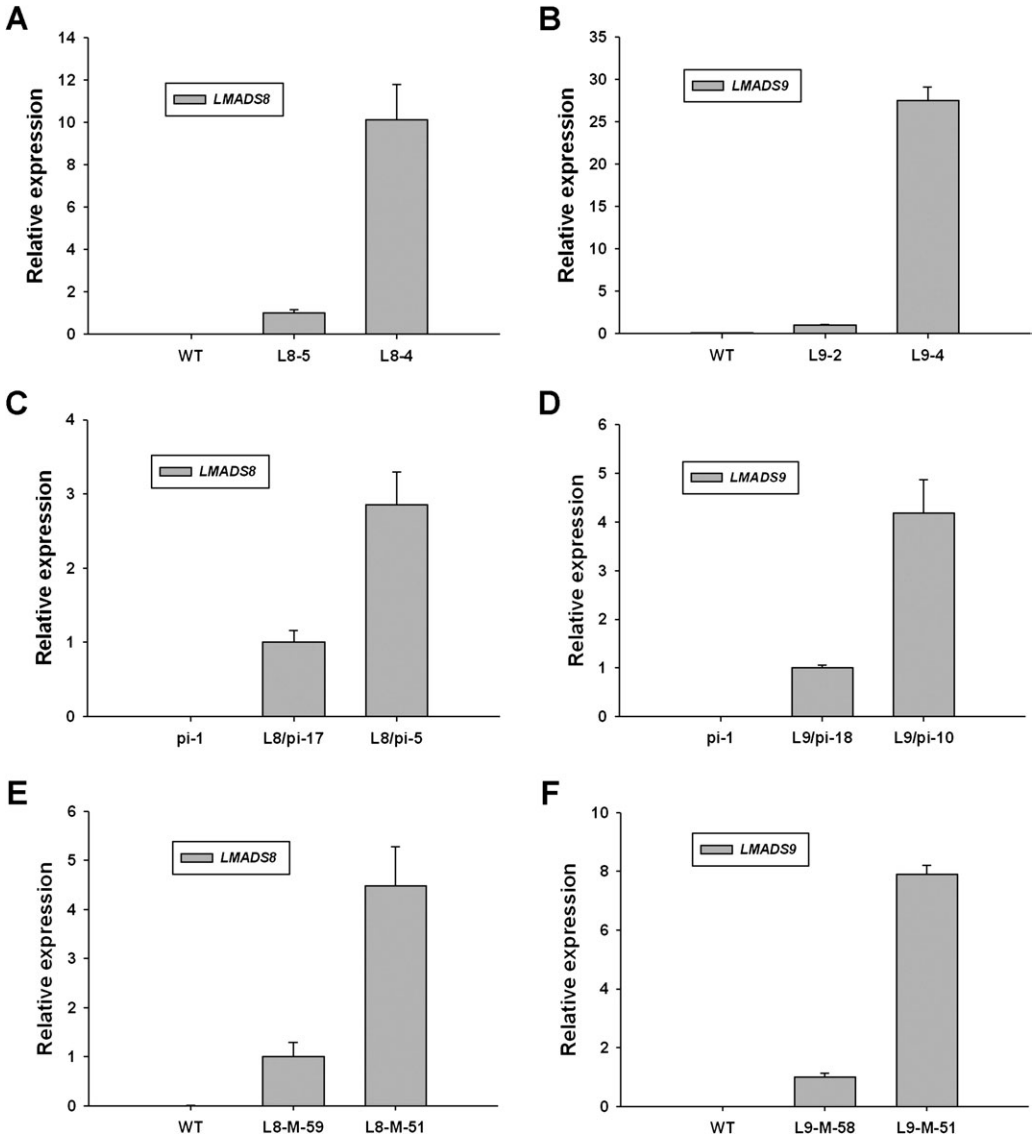

Fig. 5. The detection of gene expression in transgenic Arabidopsis plants. (A) Total RNAs isolated from one severe (L8-4) and one wild-type-like (L8-5) 45-day-old *35S:LMADS8* transgenic *Arabidopsis* and from one untransformed wild-type plant (WT) were used as templates for quantitative real-time PCR. The result indicates that *LMADS8* is expressed at a higher level in the L8-4 than in the L8-5 transgenic plants. *LMADS8* expression was undetectable in untransformed wild-type plants. (B) Total RNAs isolated from one severe (L9-4) and one wild-type-like (L9-2) 45-day-old *35S:LMADS9* transgenic *Arabidopsis* and from one untransformed wild-type plant (WT) were used as templates for quantitative real-time PCR. The result indicates that *LMADS9* is expressed at a higher level in the L9-4 than in the L9-2 transgenic plants. *LMADS9* expression was undetectable in untransformed wild-type plants. (C) Total RNAs isolated from one strongly complementary (L8/pi-5) and one *pi*-like (L8/pi-17) 45-day-old *35S:LMADS8/pi* transgenic *Arabidopsis* and from one untransformed *pi* mutant plant (pi-1) were used as templates for quantitative real-time PCR. The result indicates that *LMADS8* is expressed at a higher level in the L8/pi-5 than in the L8/pi-17 transgenic plants. *LMADS8* expression was undetectable in untransformed *pi* mutant plants. (D) Total RNAs isolated from one strongly complementary (L9/pi-10) and one *pi*-like (L9/pi-18) 45-day-old *35S:LMADS9/pi* transgenic *Arabidopsis* and from one untransformed *pi* mutant plant (pi-1) were used as templates for quantitative real-time PCR. The result indicates that the *LMADS9* is expressed at a higher level in the L9/pi-10 than in the L9/pi-18 transgenic plants. *LMADS9* expression was undetectable in untransformed *pi* mutant plants. (E) Total RNAs isolated from one severe (L8-M-51) and one wild-type-like (L8-M-59) 45-day-old *35S:LMADS8*-M transgenic *Arabidopsis* and from one untransformed wild-type plant (WT) were used as templates for quantitative real-time PCR. The result indicates that *LMADS8*-M is expressed at a higher level in the L8-M-51 than in the L8-M-59 transgenic plants. *LMADS8*-M expression was undetectable in untransformed wild-type plants. (F) Total RNAs isolated from one severe (L9-M-51) and one wild-type-like (L9-M-58) 45-day-old *35S:LMADS8*-M transgenic *Arabidopsis* and from one untransformed wild-type plant (WT) were used as templates for quantitative real-time PCR. The result indicates that *LMADS9*-M is expressed at a higher level in the L9-M-51 than in the L9-M-58 transgenic plants. *LMADS9*-M expression was undetectable in untransformed wild-type plants. In quantitative real-time PCR, the columns represent the relative expression of these genes. Transcript levels of these genes were determined using two to three replicates and were normalized using *UBIQUITIN10*. Error bars represent the standard deviation. Each experiment was repeated three times with similar results.

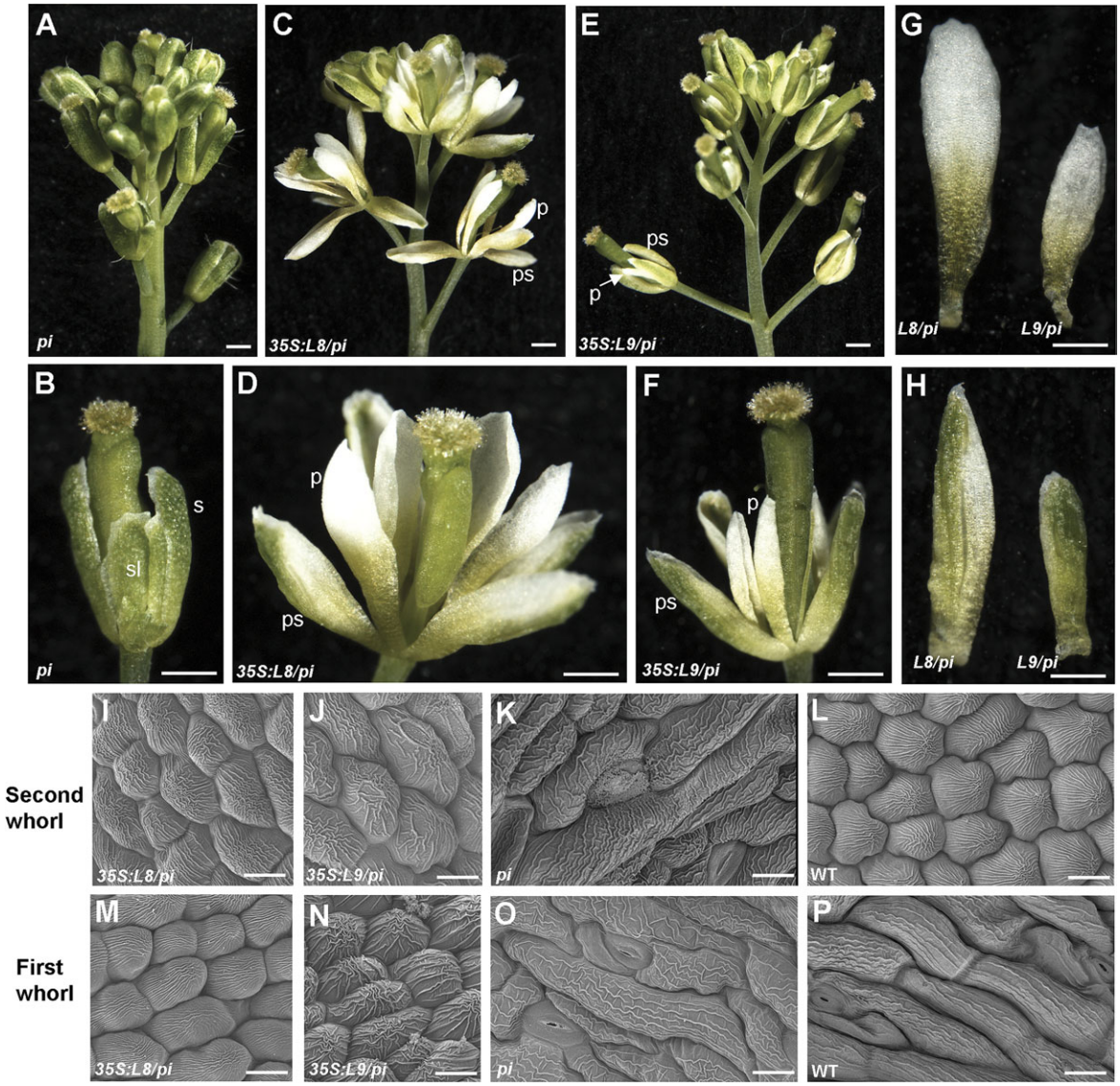

Fig. 6. Phenotypic analysis of *Arabidopsis pi-1* mutants ectopically expressing *LMADS8* or *LMADS9*. (A) Flowers produced in the inflorescence of a *pi-1* mutant plant failed to open during all stages of flower development. (B) Close-up of the unopened *pi-1* flower in A. Green sepal-like structures (sl) were produced in the second whorl of this *pi-1* mutant flower. S, sepal. (C) Flowers produced in the inflorescence of a *35S:LMADS8/pi* (*35S:L8/pi*) plant containing fully elongated second whorl petals (p) and a first whorl petal-like sepal (ps) with white colour during all stages of flower development. (D) Close-up of a *35S:LMADS8/pi* (*35S:L8/pi*) flower in C that contains fully elongated petals (p) and a petal-like sepal (ps) with white colour in the second and first whorls, respectively. (E) Flowers produced in the inflorescence of a *35S:LMADS9/pi* (*35S:L9/pi*) plant containing second whorl short white petals (p) and a first whorl petal-like sepal (ps) during all stages of flower development. (F) Close-up of a *35S:LMADS9/pi* (*35S:L9/pi*) flower in E that contains short petals (p) and a petal-like sepal (ps) with white colour in the second and first whorls, respectively. (G) Close-up of a fully elongated petal from a *35S:LMADS8/pi* (*L8/pi*) flower (left) and a short petal from a *35S:LMADS9/pi* (*L9/pi*) flower (right). (H) Close-up of a fully elongated first whorl petal-like sepal from a *35S:LMADS8/pi* (*L8/pi*) flower (left) and a short first whorl petal-like sepal from a *35S:LMADS9/pi* (*L9/pi*) flower (right). (I–J) SEM of the surface cells of the epidermis of the second whorl organ of a *35S:LMADS8/pi* (*35S:L8/pi*) (I) and *35S:LMADS9/pi* (*35S:L9/pi*) (J) flower, which are similar to the mature wild-type petal epidermis in L. (K) SEM of the surface cells with irregular shapes in the epidermis of the second whorl organ of a *pi-1* (*pi*) mutant flower, which are similar to the mature wild-type first whorl sepal epidermis in P. (L) SEM of the surface cells of the epidermis of a mature wild-type petal. (M and N) SEM of the surface cells of the epidermis of the first whorl organ of a *35S:LMADS8/pi* (*35S:L8/pi*) (M) and *35S:LMADS9/pi* (*35S:L9/pi*) (N) flower that are similar to the mature wild-type petal epidermis in L. (O and P) SEM of the surface cells with irregular shapes in the epidermis of the first whorl sepal of a *pi-1* (*pi*) mutant flower (O), which are similar to the mature wild-type (WT) first whorl sepal epidermis (P). Bar=0.5 mm in A–H, and 10 μm in I–P.

quantitative real-time PCR analysis was performed. As shown in Fig. 5, higher *LMADS8* (Fig. 5C) and *LMADS9* (Fig. 5D) expression levels were observed in the transgenic *pi-1* plants with the mutant phenotype rescued than in the transgenic *pi-1* plants without complementation. This result indicates that the complementation generated in the

35S:LMADS8/9 transgenic *Arabidopsis pi* mutants was due to the ectopic expression of the lily *LMADS8/9* genes.

The ectopic expression of truncated LMADS8 *with the C-terminal sequence or PI motif deleted reduced the ability to rescue the* pi *phenotype in* pi-1 *mutants*

Because *35S:LMADS8* exerted a stronger rescue effect in the *pi-1* mutants than *35S:LMADS9*, it is reasonable to propose that this effect may be due to the additional 29 amino acids in the C-terminal region of LMADS8 (Fig. 7A). To investigate the function of this C-terminal region further, two truncated versions of *LMADS8*, in which either the PI motif (*LMADS8-PI*) or the 29 amino acids of the C-terminal sequence (including the PI motif) (*LMADS8-C*) were deleted, were constructed and transformed into *pi-1* mutants for functional complementation analysis (Fig. 7B). Seven and three independent *35S:LMADS8-C/pi* and *35S:LMADS8-PI/pi* transgenic *Arabidopsis* plants, respectively, that showed identical altered phenotypes for each construct during reproductive development were obtained. Compared with the full-length *LMADS8*, which caused the complete rescue of the *pi-1* mutant in petals (Fig. 7B-1, -2), *LMADS8-PI* rescued the petal formation for *pi-1* mutants in a relatively weaker manner (Fig. 7B-3, -4). The second whorl white petal in the *35S:LMADS8-PI/pi* plants was ~2 mm in length and clearly shorter than that in the *35S:LMADS8/pi* or a wild-type flower, which was ~2.5 mm in length (Fig. 7B-7, -8). Interestingly, *LMADS8-C* showed a partial rescue of the petal formation for *pi-1* mutants (Fig. 7B-5, -6), which was very similar to that observed in *35S:LMADS9/pi* transgenic flowers (Figs 6E, F, 7C-5, -6), and the rescued phenotype was weaker than that in the *LMADS8-PI* and full-length *LMADS8* plants. The length of the second whorl white petal in the *35S:LMADS8-C/pi* plants (1.5 mm) was longer than the second whorl organ in the *pi-1* mutants (1 mm) (Fig. 7B-7, -8) similar to that in the *35S:LMADS9/pi* plants (1.5 mm) (Fig. 7C-7), but shorter than that in the *35S:LMADS8-PI/pi* (2 mm) and *35S:LMADS8/pi* plants (2.5 mm) (Fig. 7B-7, -8). These findings clearly indicated that the different extents of the rescue effect in the *pi-1* mutant phenotype by LMADS8 and LMADS9 were mainly due to the additional sequences in the C-terminal region (including the PI motif) of LMADS8 as had been proposed.

Ectopic expression of LMADS9 *fused to the C-terminal sequence or PI motif of* LMADS8 *enhanced the ability to rescue the* pi *phenotype in* pi-1 *mutants*

Because both *LMADS9* and *LMADS8-C*, which lacked the additional 29 amino acids of the C-terminal sequence, rescued the *pi-1* mutant in a similar matter, it was of interest to determine whether the rescue in the *pi-1* mutants would be modulated by the addition of the 29 amino acids of the LMADS8 C-terminal sequence (L8C) or the PI motif alone (L8PI) to LMADS9. For this purpose, two constructs (*LMADS9+L8C* and *LMADS9+L8PI*) were produced and transformed into *pi-1* mutants for functional complementation analysis (Fig. 7C). Four and seven independent *35S:LMADS9+L8C/pi* and *35S:LMADS9+L8PI/pi* transgenic *Arabidopsis* plants, respectively, that showed identical altered phenotypes for each construct during reproductive development were obtained. The results indicated that *LMADS9 +L8C* almost fully rescued the petal formation in *pi-1* mutants (Fig. 7C-1, -2), similarly to the *LMADS8/pi* transgenic plants (Fig. 7B-1, -2). The *LMADS9+L8PI* plants exhibited a partial rescue of the petal formation in *pi-1* mutants (Fig. 7C-3, -4), which was similar to that observed in the *LMADS8-PI/pi* transgenic plants (Fig. 7B-3, -4). The rescue of the phenotype in the *LMADS9+L8PI* plants (Fig. 7C-3, -4) was stronger than that in plants expressing full-length *LMADS9* (Fig. 7C-5, -6) and weaker than that in plants expressing *LMADS9+L8C* (Fig. 7C-1, -2). The length of the second whorl white petal in *35S:LMADS9+L8PI/pi* plants (2 mm) was clearly shorter than that in *35S:LMADS9+L8C/pi* (2.3 mm) or wild-type plants (2.5 mm), but longer than that in *35S:LMADS9/pi* plants (1.5 mm) or the second whorl organ in the *pi-1* mutants (1 mm) (Fig. 7C-7, -8). These observations further supported the hypothesis that the additional 29 amino acids of the C-terminal sequence, including the PI motif, in LMADS8 play a valuable role in the function of *LMADS8* and *9*.

The ectopic expression of truncated LMADS8 *and* LMADS9 *cDNAs lacking the MADS box region alters petal and stamen formation in* Arabidopsis

MADS box genes have been thought to form dimers and bind to DNA through a DNA-binding domain (the MADS box domain) at the N-terminus (Schwarz-Sommer *et al.*, 1992; Tröbner *et al.*, 1992; Riechmann *et al.*, 1996; Mizukami *et al.*, 1996). Mutant forms of *Arabidopsis AP3* or lily *LMADS1* (*AP3* homologue) lacking the N-terminal MADS box domain region have been reported to generate *ap3* mutations once transformed into wild-type *Arabidopsis* (Krizek *et al.*, 1999; Tzeng and Yang, 2001). It has been reported that the *ap3*-like flowers produced in *35S:LMADS1*-M plants are caused by dominant negative mutations through the formation of non-functional heterodimers between truncated LMADS1 and PI proteins in transgenic *Arabidopsis* (Tzeng and Yang, 2001). To investigate the functions of *LMADS8* and *9* further, truncated cDNAs lacking the MADS box domain (*35S:LMADS8/9*-M) were transformed into *Arabidopsis* to generate dominant negative mutants.

Six out of the 18 independent *35S:LMADS8*-M transgenic *Arabidopsis* plants showed similar abnormal phenotypes. These plants (Fig. 8A) were phenotypically similar to wild-type plants (Fig. 8B) during both vegetative and inflorescence development. However, they produced flowers with altered phenotypes. Unlike mature wild-type flowers, in which the petals and stamens elongate rapidly during late flower development and result in normal flower opening (Fig. 4B), these *35S:LMADS8*-M flowers failed to open during all stages of flower development and remained unsenescent for a long period in the inflorescence (Fig. 8A, C, D). This phenotype was clearly different from that of the

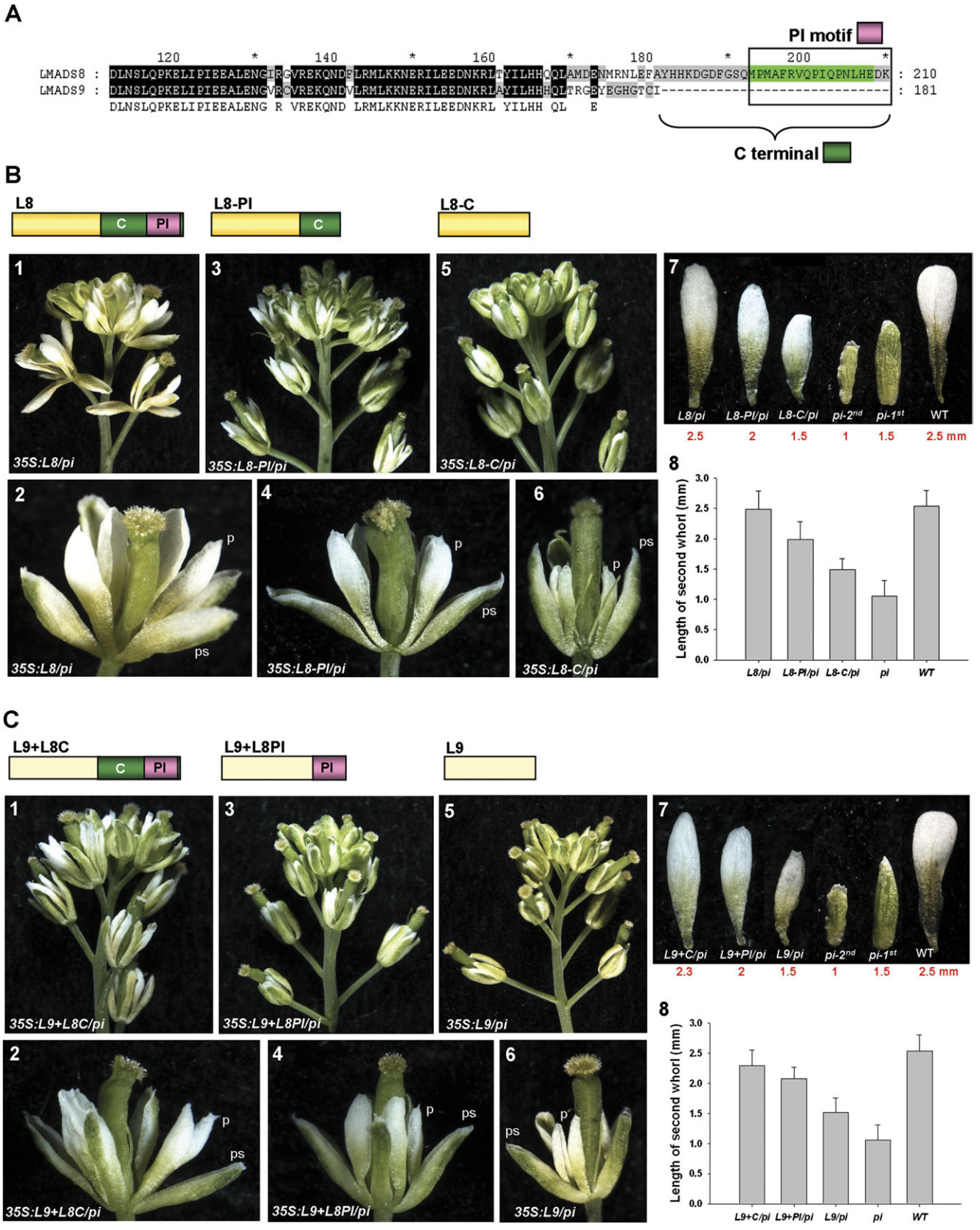

Fig. 7. Phenotypic analysis of *Arabidopsis pi-1* mutants ectopically expressing *LMADS8* with a C-terminal region deletion or *LMADS9* with a C-terminal region addition. (A) The protein structure of LMADS8/9 in the C-terminal region. The LMADS8 protein contains a PI motif (green and boxed region) in the region of the 29 amino acid C-terminus, which was absent in the LMADS9 protein. (B) Flowers produced in the inflorescence of a *35S:LMADS8/pi* (*35S:L8/pi*) plant (B-1, B-2), a *35S:LMADS8-PI/pi* (*35S:L8-PI/pi*) plant (B-3, B-4) plant, and a *35S:LMADS8-C/pi* (*35S:L8-C/pi*) plant (B-5, B-6), containing different degrees of fully elongated second whorl petals (p) and

wild-type inflorescence, which featured senescent flowers and elongated siliques (Fig. 8B). The mature *35S:LMADS8*-M flowers contained all four floral organs when observed upon manual opening (Fig. 8E). However, short and degenerated petal-like structures were observed in the second whorl of the *35S:LMADS8*-M flowers (Fig. 8E) that were different from the white elongated petals in the wild-type flowers (Fig. 4B). In addition, short and degenerated stamen-like structures were observed in the third whorl of the *35S:LMADS8*-M flowers (Fig. 8E), which were phenotypically different from the elongated stamens in the wild-type flowers (Fig. 4B, C). No pollen was produced from the anther-like structures in the *35S:LMADS8*-M flowers (Fig. 8E). Thus, these *35S:LMADS8*-M flowers were sterile, producing no seeds.

As in the *35S:LMADS8*-M plants, similar unopened flowers (Fig. 8F–H) with defective short petals and stamens were observed in the flowers of the six *35S:LMADS9*-M transgenic plants (Fig. 8I). However, the development of the protruding carpel (Fig. 8H, I) was apparently normal in these *35S:LMADS9*-M flowers compared with that in the *35S:LMADS8*-M flowers (Fig. 8D, E). Occasionally, flowers with longer petals were also produced in these *35S:LMADS9*-M transgenic plants (Fig. 8G, J). However, these flowers were still sterile due to the production of short and defective stamens (Fig. 8K).

As shown in Fig. 5, higher *LMADS8*-M (Fig. 5E) and *LMADS9*-M (Fig. 5F) expression levels were observed in the transgenic plants with the severe phenotype than in the transgenic plant that was indistinguishable from the wild-type plants. This discrepancy indicates that the phenotypes generated in the *35S:LMADS8/9*-M transgenic *Arabidopsis* plants were due to the ectopic expression of the lily *LMADS8/9*-M gene.

Homodimer and heterodimer formation of the LMADS1, 8, and 9 proteins

LMADS1, the PaleoAP3 homologue from the monocot lily, has been reported to be able to form homodimers (Tzeng and Yang, 2001; Tzeng *et al.*, 2004). Because *LMADS8* and *9* showed high sequence homology to monocot *PI* orthologues, it was interesting to study the possible interaction between LMADS1 and LMADS8/9. Thus, a yeast two-hybrid analysis was performed. As shown in Fig. 9, LMADS8 and 9 were able to form heterodimers with LMADS1. Similar to LMADS1 (Tzeng and Yang, 2001), LMADS8 and 9 were able to form homodimers (Fig. 9B). When the interaction between LMADS8 and LMADS9 was analysed, β-galactosidase activity was also detected (Fig. 9B), indicating that LMADS8 and 9 are able to form not only homodimers, but also heterodimers with each other.

To confirm further the results obtained from the yeast two-hybrid analysis, an EMSA, a technique established for the investigation of dimerization and DNA binding of MIKC-type MADS domain proteins (Schwarz-Sommer *et al.*, 1992; Riechmann *et al.*, 1996; Winter *et al.*, 2002; Tzeng *et al.*, 2004), was employed. As shown in Fig. 10, similar to LMADS1, LMADS8 and 9 proteins bound efficiently to the CArG1 probe, and protein–DNA complexes were observed. The ability of LMADS8 homodimers to bind CArG1 was clearly similar to that of LMADS1 and relatively stronger than that of LMADS9 (Fig. 10).

Discussion

To investigate the role of B class MADS box genes in the regulation of flower development in the lily (*L. longiflorum*), two genes, *LMADS8* and *LMADS9*, were identified and characterized in this study. Based on sequence alignment, the conserved motifs in the C-terminal regions of the encoded proteins, and phylogenetic tree analysis, *LMADS8* and *9* were classified as *PI*-like genes and were found to be closely related to monocot *PI* orthologues (Figs 1, 2). The deletion of 29 amino acids, including the consensus PI motif in the C-terminal region, in LMADS9 suggested the possibility that LMADS9 might be a truncated form of LMADS8 in lily.

The presence of two *PI* orthologues in lily is not surprising because duplicated B class genes are often seen in basal eudicots (Di Stilio *et al.*, 2005) and non-grass monocots, such as Liliales (Liliaceae) and Asparagales (Kramer *et al.*, 2003; Kanno *et al.*, 2007). However, the

a first whorl petal-like sepal (ps) with white colour during all stages of flower development. (B-7) Length comparison (from left to right) of elongated second whorl organs from a *35S:LMADS8/pi* (*L8/pi*) flower (2.5 mm), a *35S:LMADS8-Pl/pi* (*L8-Pl/pi*) flower (2 mm), a *35S:LMADS8-C/pi* (*L8-C/pi*) flower (1.5 mm), second (*pi*-2nd) (1 mm) and first (*pi*-1st) (1.5 mm) whorl organs of a *pi*-1 flower, and the elongated second whorl petal of a wild-type (WT) flower (2.5 mm). (B-8) The variation and statistical analysis of the length for the second whorl organs from *35S:LMADS8/pi* (*L8/pi*), *35S:LMADS8-Pl/pi* (*L8-Pl/pi*), *35S:LMADS8-C/pi* (*L8-C/pi*), *pi-1* (*pi*) mutant and wild-type (WT) plants. The flower organ length was measured for five plants, with two measurements for each plant. Standard deviations are shown at the top of the columns. (C) Flowers produced in the inflorescence of a *35S:LMADS9+L8C/pi* (*35S:L9+L8C/pi*) plant (C-1, C-2), a *35S:LMADS9+L8Pl/pi* (*35S:L9+L8Pl/pi*) plant (C-3, C-4) plant, and a *35S:LMADS9/pi* (*35S:L9/pi*) plant (C-5, C-6), containing different degrees of fully elongated second whorl petals (p) and first whorl petal-like sepals (ps) with white colour during all stages of flower development. (C-7) Length comparison (from left to right) of elongated second whorl organs from a *35S:LMADS9+L8C/pi* (*L9+C/pi*) flower (2.3 mm), a *35S:LMADS9+L8Pl/pi* (*L9+Pl/pi*) flower (2 mm), a *35S:LMADS9/pi* (*L9/pi*) flower (1.5 mm), second (*pi*-2nd) (1 mm) and first (*pi*-1st) (1.5 mm) whorl organs of a *pi-1* flower, and the elongated second whorl petal of a wild-type (WT) flower (2.5 mm). (C-8) The variation and statistical analysis of the length for the second whorl organs from *35S:LMADS9+L8C/pi* (*L9+C/pi*), *35S:LMADS9+L8Pl/pi* (*L9+Pl/pi*), *35S:LMADS9/pi* (*L9/pi*), *pi-1* (*pi*) mutant, and wild-type (WT) plants. The flower organ length was measured for five plants, with two measurements for each plant. Standard deviations are shown at the top of the columns.

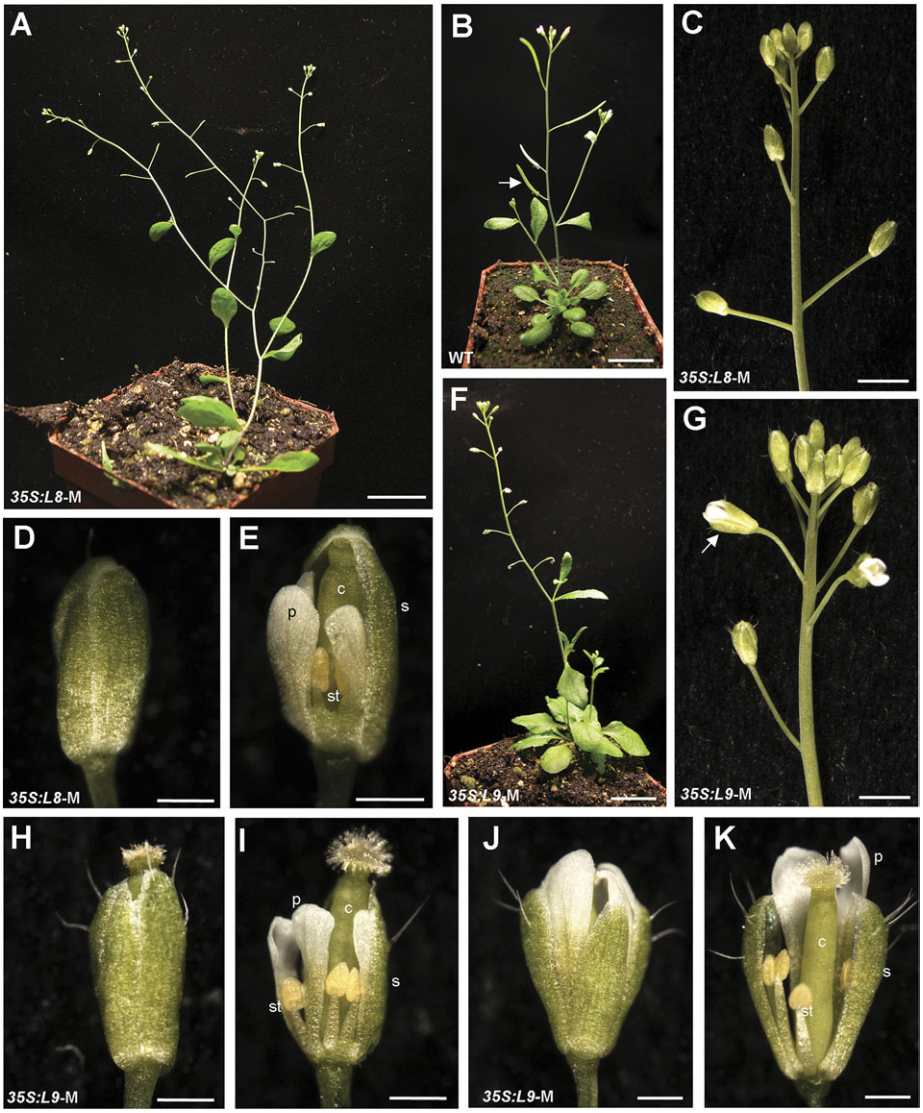

Fig. 8. Phenotypic analysis of transgenic *Arabidopsis* plants ectopically expressing *LMADS8*-M or *LMADS9*-M. (A) A 35-day-old *35S:LMADS8*-M (*35S:L8*-M) transgenic *Arabidopsis* plant produced unopened flowers. (B) A 35-day-old wild-type (WT) plant produced normal flowers and elongated silique (arrowed).(C) Close-up of the unopened flowers in the inflorescence of a *35S:LMADS8*-M (*35S:L8*-M) plant in A. (D) Close-up of an unopened flower in C. (E) When opened manually, short petal-like structures (p), short stamen-like structures (st), and an unelongated carpel (c) were observed in the inner three whorls of a mature *35S:LMADS8*-M (*35S:L8*-M) flower from D. Two sepals (s) were removed to reveal the inner organs. (F) A 35-day-old *35S:LMADS9*-M (*35S:L9*-M) transgenic Arabidopsis plant produced unopened flowers. (G) Close-up of the flowers in the inflorescence of a *35S:LMADS9*-M (*35S:L9*-M) plant in F. Most flowers were unopened during all stages of flower development, whereas slightly opened flowers with relatively long petals were occasionally observed (arrowed). (H) Close-up of an unopened flower in G. (I) When opened manually, short petal-like structures (p), short stamen-like structures (st), and a normal carpel (c) were observed in the inner three whorls of a mature *35S:LMADS9*-M flower from H. Two sepals (s) were removed to reveal the inner organs. (J) Close-up of a slightly opened flower in G. (K) Close-up of the flower opened manually from J. Short stamen-like structures (st) along with normally elongated petals (p) and carpel (c) were observed in the inner three whorls. Two sepals (s) were removed to reveal the inner organs. Bar=1 cm in A, B, and F; 0.5 cm in C, G; and 0.1 cm in D, E, and H–K.

appearance of the duplicated paleo*AP3* or *PI* genes occurred differently in plant species (Kramer *et al.*, 2003). For example, plants such as tulips and orchids tend to have duplicated paleo*AP3* genes rather than *PI* genes. Two (*TGDEFA* and *TGDEFB*), four (*PeMADS2*, *PeMADS3*, *PeMADS4*, and *PeMADS5*), and three (*OMADS3*, *OMADS5*, and *OMADS9*) paleo*AP3* orthologues have been identified in tulips (*Tulipa gesneriana*, Liliaceae) and orchids (*Phalaenopsis equestris* and *Oncidium* Gower Ramsey) (Kanno *et al.*, 2003; Tsai *et al.*, 2004; Chang *et al.*, 2010). Only one *PI*-like gene is found in these plants. In contrast, relatively few species contain duplicated *PI*-like genes as found in *L. longiflorum*. For example, two (*LRGLOA* and *LRGLOB*), two (*LFGLOA* and *LFGLOB*), and three (*MaGLOA1*, *MaGLOA2*, and *MaGLOB*) *PI*-like genes have been reported in *L. regale*, *L. formolongi*, and

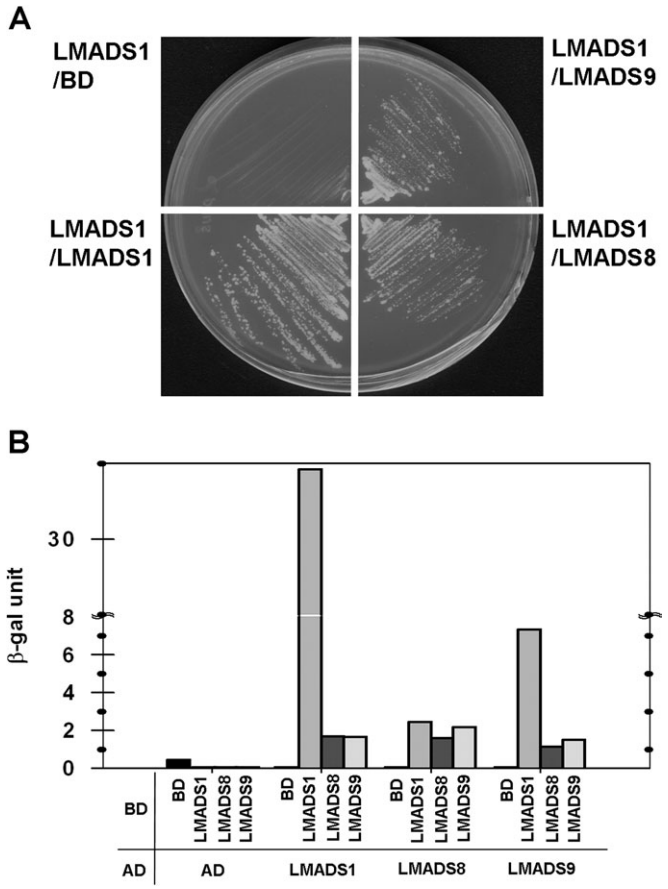

Fig. 9. Protein interactions among B class proteins of *L. longiflorum* in a yeast two-hybrid assay. (A) Interaction between LMADS1 and LMADS8/9 proteins in a yeast two-hybrid assay. The yeast strain AH109 co-transformed with truncated LMADS1 in an activation domain plasmid (AD, Prey) and LMADS1, 8, and 9 in a binding domain plasmid (BD, Bait) was streaked on a minimal synthetic dropout (SD) medium without adenine, histidine, leucine, and tryptophan. Yeast cells transformed with LMADS1 in the activation domain plasmid (AD) and the binding domain plasmid (BD) alone were used as controls for background activity.
(B) β-Galactosidase activity in yeast cells transformed with combinations of truncated LMADS1, LMADS8, or LMADS9 in either the binding domain plasmid (BD, Bait) or the activation domain plasmid (AD, Prey) was calculated according to Miller (1992). Yeast cells transformed with LMADS1, 8, and 9 in the binding domain plasmid (BD) or the activation domain plasmid (AD) alone were used as controls for background activity. Yeast transformed with vectors (AD+BD) only was used as a negative control.

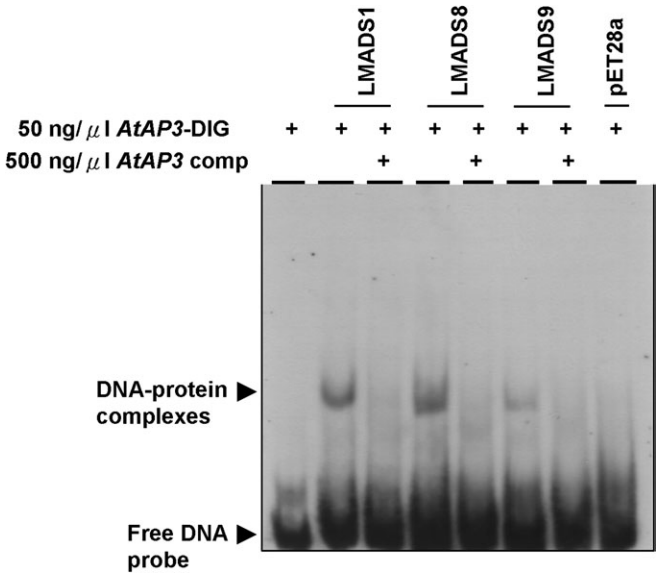

Fig. 10. Homodimerization of LMADS1, 8, and 9 in electrophoretic mobility shift assays (EMSAs). Equal amounts of LMADS1, LMADS8, and LMADS9 were incubated with the DIG-labelled DNA probe CArG1, and the protein–DNA complexes were subjected to polyacrylamide gel electrophoresis. For the competition assay, a 10-fold molar excess of the cold competitor DNA was included in the binding reactions. The results indicate that the LMADS1, LMADS8, and LMADS9 proteins were able to bind to CArG1 and form protein–DNA complexes. These protein–DNA complexes disappeared and only free probes were seen once the cold competitor DNA was added.

Muscari armeniacum, respectively (Winter *et al.*, 2002; Kanno *et al.*, 2007; Akita *et al.*, 2008). Interestingly, as in *L. longiflorum*, only one paleo*AP3* gene is found in these three species, indicating that the duplication events for B class genes occurred more frequently for paleo*AP3*-like genes than for *PI* genes in monocots during evolution.

The expression pattern for these two lily *PI* orthologues, *LMADS8* and *9*, was interesting. First, their expression patterns were almost identical (Fig. 3). In addition to their close phylogenetic relationship (Fig. 2), this result strongly supported the idea that these orthologues were raised through a gene duplication event and retained similar transcriptional regulation and function in lily. Because ~88% identity was observed between the LMADS8 and 9 proteins (Fig. 1A), it appeared that there were only few sequence diversifications after duplication. Although high sequence identity was observed, the deletion of 29 amino acids, including the consensus PI motif, from the C-terminal region of LMADS9 was observed when compared with LMADS8. This result revealed that LMADS9 might be a truncated form of LMADS8 in lily, in which the C-terminal region of LMADS9 was lost after gene duplication. This assumption was further supported by the identification of a sequence of 48 bp that was highly matched to the PI motif of *LMADS8* (only five out of 48 nucleotides and four out of 16 translated amino acids are different between LMADS8 and LMADS9) in the *LMADS9* cDNA after the stop codon (Fig. 1B). This finding strongly indicated that after gene duplication, *LMADS9* experienced a 24 bp DNA deletion before the PI motif as well as a point mutation that produced a premature stop codon. This caused the 29 amino acid truncation of LMADS9 compared with LMADS8. Interestingly, similar phenomena were observed for *PI* orthologues in *L. regale* and *L. formolongi* in which two *PI*-like genes, *LRGLOA* and *LRGLOB* in the former and *LFGLOA* and *LFGLOB* in the latter, were identified, and the sequence analysis indicated that *LRGLOB*/*LFGLOB* was a truncated

form of *LRGLOA*/*LFGLOA* (Winter *et al.*, 2002; Akita *et al.*, 2008) (Fig. 1A). These results indicate the vast similarity of the genome contents and organization for these three species of lilies. Thus, the duplication, point mutation, and deletion events of the two *PI*-like genes should have occurred before the divergence of these three species of lilies during evolution. Furthermore, truncated forms have also been described for AP3 orthologues (ThtAP3-2a and ThdAP3-2a) in *Thalictrum dioicum* (Ranunculaceae) (Kramer *et al.*, 1998; Di Stilio *et al.*, 2005), indicating that a similar duplication and mutation occurred in both *PI*-like and *AP3*-like genes during evolution.

The second interesting characteristic of *LMADS8* and *9* is their spatial and temporal expression pattern. The expression patterns for *LMADS8* and *9* were different from the expression patterns of *PI* orthologues in dicots, which are normally expressed in petals and stamens (Goto and Meyerowitz, 1994; Rounsley *et al.*, 1995; Prasad and Vijayraghavan, 2003; Yao *et al.*, 2008). In contrast, the mRNAs for *LMADS8* and *9* were strongly detected in the first and second whorls tepals during all stages of flower development and in the stamens only during early flower development (Fig. 3). This expression pattern was also different from that of some *PI* orthologues in non-grass monocots, which are expressed in all four flower organs, such as *TGGLO* in tulip (*T. gesneriana*), *TriaGLO* in *Tricyrtis affinis*, *CsPIc* in *Crocus sativus*, *PeMADS6* in *P. equestris*, *DcOPI* in *Dendrobium crumenatum*, and *OMADS8* in *Oncidium* orchid, as well as *MaGLOA1*, *MaGLOA2*, and *MaGLOB* in *M. armeniacum* (Tsai *et al.*, 2005; Tsaftaris *et al.*, 2006; Xu *et al.*, 2006; Kanno *et al.*, 2007; Chang *et al.*, 2010). *LMADS8* and *9* also showed a slightly different expression pattern from that of *LFGLOB*/*LFGLOA*, which is expressed strongly in whorls 1–3, but very weakly in whorl 4 (Akita *et al.*, 2008). The expression of *LMADS8* and *9* in the first whorl organ is explainable because lily plants produce almost identical first and second whorl tepals. The genes controlling second whorl organ formation in these plants are very probably also expressed in the first whorl organ (Tzeng and Yang, 2001; Kanno *et al.*, 2007). These results reveal that *LMADS8* and *9* may have an important role in regulating all processes involved in tepal formation, while only participating in early stamen formation.

The function of *LMADS8* and *9* in flower formation was further revealed by functional complementation analysis. Because an efficient genetic transformation system is still not available for lily (*L. longiflorum*), the functional analysis for *LMADS8*/*9* was performed in transgenic *Arabidopsis* in this study. When *LMADS8* and *9* were ectopically expressed in *Arabidopsis pi* mutants, the restoration of the mutant phenotype through the production of normal petal organs in the second whorl along with the green/white petal-like sepals in the first whorl in *pi-1* mutants was observed in transgenic plants (Fig. 6). This result provided further evidence to support the conclusion that *LMADS8* and *9* simply function as B genes in the *PI* lineage. This concept was further supported by the production of dominant negative mutations with degenerated short petal-like structures in the second whorl and short stamen-like structures in the third whorl of flowers ectopically expressing *LMADS8* or *LMADS9* truncated cDNAs lacking the MADS box domain (*35S:LMADS8*/*9*-M) (Fig. 8). These results indicate that *LMADS8* and *LMADS9* are able to participate in the *Arabidopsis* MADS functional protein complex in the sepals/petals/stamens to perform their functions.

One interesting finding of this study is the different phenotypes generated by the ectopic expression of *LMADS8* and *9* or *LMADS8*-M and *9*-M in transgenic *Arabidopsis*. *35S:LMADS8* completely rescued the second whorl petal formation and partially converted the first whorl sepal into an expanded green/white petal-like structure for *Arabidopsis pi* mutants, whereas *35S:LMADS9* only partially rescued the petal formation for *pi* mutants (Fig. 6). In addition, *35S:LMADS8*-M generated more severe dominant negative phenotypes than those in *35S:LMADS9*-M flowers (Fig. 8), suggesting the *LMADS8*-M could exert a greater effect in the *Arabidopsis* MADS functional protein complex than *LMADS9*-M. Because 29 amino acids, including the consensus PI motif, are missing from the C-terminal region of LMADS9 when compared with LMADS8, LMADS9 is a truncated form of LMADS8 in lily as described above. The different degrees of the phenotypes observed for *35S:LMADS8*/*pi*, *35S:LMADS9*/*pi*, and *35S:LMADS8*-M/*LMADS9*-M suggested a valuable role for these 29 amino acids, including the PI motif, in the C-terminal region in the function of the lily *PI* orthologues. The absence of this C-terminal region apparently weakened, but did not abolish, the ability of LMADS9 to participate in the *Arabidopsis* MADS functional protein complex when compared with LMADS8, which contained the entire C-terminal region.

This assumption is further supported by the deletion experiment for LMADS8 and the addition experiment for LMADS9. The results (Fig. 7) indicated that *LMADS8-C* (with the 29 amino acids of the C-terminal sequence deleted) only partially rescued the petal formation for *pi-1* mutants, which was similar to the rescue observed in *35S:LMADS9*/*pi* transgenic plants. The *LMADS8-PI* (with only the PI motif deleted) also partially rescued the petal formation for *pi-1* mutants, and the rescued phenotype was stronger than that in the *LMADS8-C* plants and weaker than in plants expressing full-length LMADS8. Similarly, *LMADS9+L8C* (with the addition of the 29 amino acids of the LMADS8 C-terminal sequence) almost fully rescued the petal formation in *pi-1* mutants similar to the rescue observed in *LMADS8*/*pi* transgenic plants. The *LMADS9+L8PI* (with the addition of only the PI motif of LMADS8) showed a partial rescue of the petal formation in *pi* mutants similar to that observed in *LMADS8-PI*/*pi* transgenic plants. These results clearly indicated that the more sequence that is deleted from this C-terminal region, the less the degree of the rescue in the *pi-1* mutant by the LMADS8 proteins is. In contrast, the more sequences added from this C-terminal region, the greater ability of the LMADS9 proteins to rescue the *pi-1* mutation. When considering these data together, the additional 29 amino acids of the C-terminal sequence, including the PI motif, in LMADS8 most probably play a valuable role in the function of *LMADS8* and *9* as proposed.

Through yeast two-hybrid analysis (Fig. 9), LMADS8 and LMADS9 were observed to form heterodimers with each other and with the lily paleoAP3 orthologue LMADS1. LMADS8 and LMADS9 were also found to be capable of forming homodimers. Interestingly, the ability of LMADS8 homodimers to bind to CArG1 was relatively stronger than that of LMADS9 homodimers, as indicated by EMSA (Fig. 10), which suggested that the absence of the PI motif in the C-terminal region of lily PI orthologues will affect, to some extent, the ability of these homodimers to bind to the CArG1 sequence and perform their function. Interestingly, this result was similar to that for the lily paleoAP3 orthologue LMADS1. It has been reported that the ability of LMADS1 to form homodimers decreases when the C-terminal paleoAP3 motif is deleted (Tzeng *et al.*, 2004). Thus, both paleoAP3 and the PI motifs of the paleoAP3 orthologue LMADS1 and the PI orthologue LMADS8 are needed, but not essential, for their homodimers to bind to the CArG1 sequence and function. The finding of similar functional roles for the PI motif of PI orthologues (LMADS8/9) and the paleoAP3 motifs of the paleoAP3 orthologue (LMADS1) provides useful information for the study of homo- and heterodimerization and the function of B class genes during evolution. This postulation was, however, different from the reports that the C-terminal motif of the *Arabidopsis* PI is dispensable in its floral organ identity function (Piwarzyk *et al.*, 2007) and that the function of PI orthologues in pea (*Pisum sativum*) and legume (*Medicago truncatula*) is not affected in orthologues lacking the C-terminal PI motif (Berbel *et al.*, 2005; Benlloch *et al.*, 2009). The present findings suggested that the conserved motifs in the C-terminus of the *AP3*/*PI* subfamily of MADS box proteins may play functional roles that diverged between monocots and dicots during evolution.

In summary, two *PI* orthologues of the B class MADS box genes, *LMADS8* and *LMADS9*, that specify flower development in the lily *L. longiflorum* were characterized. The mRNAs for *LMADS8* and *LMADS9* were flower specific and expressed in all stages of first and second whorl tepal development and in the early stage of stamen formation. Different effects on floral organ complementation or conversion observed in *35S:LMADS8*/*LMADS8-PI*/*LMADS8-C-pi* and *35S:LMADS9*/*LMADS9+L8PI*/*LMADS9+L8C-pi*, *35S:LMADS8*-M, and *35S:LMADS9*-M transgenic *Arabidopsis* plants and the differential ability of LMADS8 and LMADS9 homodimers to bind to the CArG1 sequence suggest a valuable role for the 29 amino acids, including the PI motif, in the C-terminal region in the function of LMADS8 and 9. In addition to *LMADS1*, the characterization of the *LMADS8* and *9* genes provides useful information for understanding the relationships between the lily B class MADS box genes as well as their roles in regulating perianth formation. Because most of the ABCDE (Tzeng and Yang, 2001; Tzeng *et al.*, 2002, 2003, 2004; Chen *et al.*, 2008; Hsu *et al.*, 2010) genes of *L. longiflorum* have been isolated and characterized in the authors' laboratory, further investigation of the role of ACDE genes in interactions with the B genes characterized in this study should lead to a deeper understanding of flower organ formation in non-grass monocots.

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