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Genes from the *APETALA3* and *PISTILLATA* Lineages are Expressed in Developing Vascular Bundles of the Tuberous Rhizome, Flowering Stem and Flower Primordia of *Eranthis hyemalis*

MARTIN SKIPPER*

Botanical Institute, University of Copenhagen, Gothersgade 140, DK-1123, Copenhagen K, Denmark

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In *Arabidopsis thaliana* expression of the B-class MADS-box genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) is confined to petals and stamens but in other plant species these genes are also transcribed in non-flower tissues; in *Solanum tuberosum* they are transcribed specifically in vascular bundles leading to petals and stamens. Transcription analysis of B-class genes in *Eranthis hyemalis* using reverse transcribed *in situ* PCR revealed that both *AP3* and *PI* are expressed in developing vascular bundles in the tuberous rhizome, flowering stem and floral primordia. In addition, *AP3* and *PI* transcripts are also found in stems and leaves. These results suggest a more complex role of B-class genes in *Eranthis* and possible involvement in the development of vascular tissue.

Key words: *Eranthis*, MADS-box genes, *AP3*, *PI*, transcription analysis, vascular bundles, RT-ISPCR.

INTRODUCTION

The *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) genes comprise the B-class floral identity genes in higher dicots, e.g. *Arabidopsis thaliana* Schur and *Antirrhinum majus* L. (Coen *et al*., 1991; Meyerowitz *et al*., 1991; Kramer *et al*., 1998). Both belong to the MADS-box gene family that encodes DNA-binding proteins. The proteins of *AP3* and *PI* genes form heterodimers in *Arabidopsis* and *Antirrhinum* and both are essential for petal and stamen identity (Jack *et al*., 1992; Schwarz-Sommer *et al*., 1992; Tröbner *et al*., 1992; Goto and Meyerowitz, 1994). Like other MIKC-type MADS-box genes, so far only found in plants (Alvarez-Buylla *et al*., 2000), AP3 and PI proteins contain highly conserved protein domains. The MADS- and K-box are both involved in protein dimerization (Riechmann *et al*., 1996), and are linked together by a lesser conserved part, the I-region and 'finished' by a variable C-terminal. Transcription of *AP3* and *PI* in *Arabidopsis* is observed throughout development of petals and stamens, but they are not transcribed in non-flower tissues (Sommer *et al*., 1990; Jack *et al*., 1992; Tröbner *et al*., 1992; Goto and Meyerowitz, 1994; Serrano-Cartagena *et al*., 2000). The expression pattern of *AP3* and *PI* genes in flowers of lower dicots differs to that of higher dicots, indicating that the function of the genes may be different in this group of angiosperms (Kramer *et al*., 1998; Kramer and Irish, 1999, 2000). This finding has been connected with the theory that petals have evolved several times within the angiosperms (Takhtajan, 1991). In other higher dicots *AP3* or *PI* genes are also transcribed in seedlings (Munster *et al*., 2001), leaves (Southerton *et al*., 1998;Yu *et al*., 1999; Munster *et al*., 2001) and roots (Yu *et al*., 1999; Munster *et al*., 2001), and *StDEF4* (*Solanum tuberosum* Walp.) is specifically expressed in vascular bundles leading to the petals and stamens (Garcia-Maroto *et al*., 1993), suggesting a more complex gene function.

This paper reports on the expression pattern of an *AP3/DEF* and a *PI/GLO* gene (named *EhAP3* and *EhPI*) in *Eranthis hyemalis* (L.) Salisb. (Ranunculaceae). The flower perianth of *Eranthis* consists of five to eight yellow tepals, also described as yellow sepals, and five to ten tubular-shaped nectar containing honey-leaves, also described as petals (Hiepko, 1995). The stamens are numerous and there are between two and ten carpels. Basal leaves and flowers initiate from a tuberous rhizome and three green cauline leaves arranged in a whorl form an involucre around the developing flower (Hiepko, 1995). In Ranunculaceae, nectar-producing organs are supposed to be derived from stamens (Hiepko, 1995), and are named honey-leaves ('Honigblätter'; Prantl, 1887). Transcription studies are performed using reverse transcribed *in situ* PCR (RT–ISPCR) to obtain information about the expression of B-class genes at the single cell level.

MATERIALS AND METHODS

Isolation of AP3 *and* PI *cDNAs*

Total RNA was extracted from flowers, leaves, stems and roots using FastRNA Kit-Green (BIO 101, Carlsbad, USA) and genomic DNA was extracted from leaves using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). First strand cDNA synthesis was carried out in a 50 µl reaction with MuLV reverse transcriptase, RNase inhibitor, buffer II, $MgCl_2$, dNTPs and a poly$(T)_{16}$ primer, following the MuLV Reverse Transcriptase protocol (Applied

* For correspondence. E-mail martins@bot.ku.dk

TABLE 1. *Previously published B-class genes retrieved from the GenBank database*

	Genes
Previously published species	
Antirrhinum majus L	*AmDEF* (16017), *AmGLO* (16023)
Arabidopsis thaliana Schur	*AtPI* (42128), *AtAP3* (166607)
Delphinium ajacis Ledeb.	*DaPI* (3170477)
Dicentra eximia Torr.	*DePI* (3170467), *DeAP3* (3170503)
Gerbera × *hybrida*	*GhGGLO1* (4218172), *GhGDEF2* (4218170)
Medicago sativa L.	*MsNMH7* (1870205), *MsNGL9* (13095570)
Papaver nudicaule L.	*PnPI-1* (3170463), *PnAP3-1* (3170499)
Ranunculus ficaria L.	*RfPI-1* (3170469), *RfAP3-1* (3170461)
Ranunculus bulbosus Costa	*RbPI-1* (3170471), *RbAP3-1* (3170505)
Sanguinaria canadensis L.	*ScPI* (4883905), *ScAP3* (4883899)
Silene latifolia Britten & Rendle	*SlM2* (602901), *SlM3* (602903)
Gnetum gnemon L.	*GgM13* (5019463)
From this work	
Eranthis hyemalis (L.) Salisb.	*EhPI* (16304399), *EhAP3* (16304397)

Genbank GI numbers in parentheses.

Biosystems, Foster City, USA). To amplify *AP3* and *PI* genes, the following primer sequences (according to IUPAC rules) were used in the following PCR reaction: *AP3*/*PI* MADS-box sequence: 5′-atmcagathaagagratmgagaa-3′/5′-athgagatmaaragrathgagaa-3′ and *AP3*/*PI* K-box sequence: 5′-cagattttgctcaagaccgcgca-3′/5′-agagcatgttcaatggggataagc-3′. The primer sequences amplify a fragment of approx. 400 bp and were designed from existing *AP3* and *PI* sequences from six ranunculid species: *Ranunculus ficaria*, *R. bulbosus*, *Delphinium ajacis*, *Papaver nudicaule*, *P. californicum* and *Dicentra eximia*.

PCR was performed in 100 µl reactions containing buffer II, $MgCl_2$, dNTPs, primers and Amplitaq Gold, according to the Amplitaq Gold protocol (Applied Biosystems). Amplification began with an activation step of 10 min at 95 °C, followed by 40 cycles of 40 s denaturing at 95 °C, 40 s annealing at 45 °C, and 1 min extension at 72 °C. Amplified products were analysed on a 2 % nusieve agarose gel. Bands of approx. 400 bp were cloned using the cloning PCR-Script kit (Stratagene, La Jolla, USA) and clones were analysed based on fragment length and sequenced. Sequencing reactions (1/2 reactions in 20 µl volumes) were carried out using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit with Amplitaq DNA Polymerase, FS (Applied Biosystems) and 5 × sequencing buffer for dilution of BigDye Ready Reaction Premix (Applied Biosystems). Before gel separation the sequence reaction products were cleaned using the DyeEx Spin Kit (Qiagen). Fragments were separated on a 5 % Long Ranger gel (FMC Bioproducts, Rockland, USA) on an ABI 377 Prism DNA Sequencer (Applied Biosystems). ABI Prism 377 Collection software version 2·1 was used to evaluate the sequences.

Phylogenetic analysis

Alignment of published *AP3* and *PI* cDNA sequences was done by hand using GeneDoc (Nicholas and Nicholas, 1997). Previously published B-class genes were retrieved from the GenBank database (see Table 1). To avoid many missing characters in the matrix only the sequence covering the MADS-box, I-region and part of the K-box was used in the analysis (alignment available at: www.bot.ku.dk/staff/boj/martin/ap3pi.txt). The *Gentum gnemon* gene *GgM13* was used as outgroup because it is the closest relative of the B-class genes (Becker *et al*., 2000). The most parsimonious trees were generated using the PAUP* 4·0b2a package (Swofford, 1999). Characters were treated as missing when nucleotide data were not available. Phylogenetic trees were found using the heuristic search algorithm, 1000 replicate run, and random stepwise addition of sequences under the TBR (tree–bisection–reconnection) algorithm for branch swapping. Jackknife values greater than 50, generated from a 10 000 replicate run using Parsimony Jackknifer version 4·22 (Farris *et al*., 1996), were assigned to nodes.

Tissue preparation and fixation

Plant material of *Eranthis hyemalis* was taken from the Botanical Garden, University of Copenhagen, Denmark. Flowers were fixed in FAA [5·5 % acetic acid, 66 % EtOH (96 %), 20 % H_2O, 8·5 % paraformaldehyde (24·5 %)], placed in vacuum for 30 min and left at 5 °C overnight followed by a 20 min dehydration in 70 % EtOH. All material was embedded in ParaPlast, sectioned at 10 µm and placed on silane-coated *in situ* PCR slides (Applied Biosystems). Shortly before RT–ISPCR, sections were deparaffinized in Histoclear 2 × 30 min, followed by 10 min 1 : 1 Histoclear/100 % EtOH and 2 × 10 min in 100 % EtOH. Slides were kept in 100 % EtOH until reverse transcription (usually for less than 1 h).

RT–PCR optimization

Prior to RT-ISPCR, amplification of both *EhAP3* and *EhPI* transcripts was optimized with specific primers (see below) in solution. Optimization was done with total RNA templates of different flower developmental stages and also

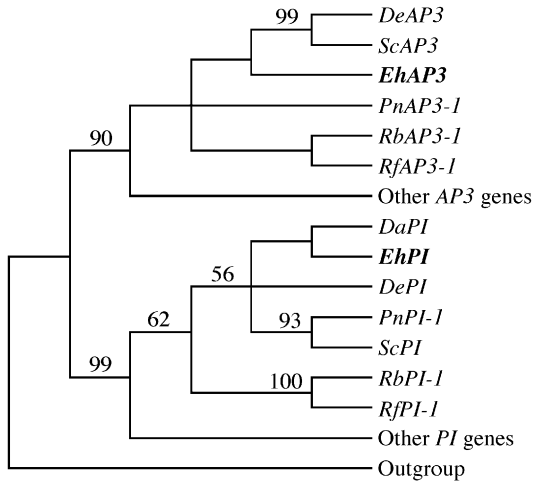

FIG. 1. Phylogenetic analysis of B-class genes from different dicot species. The *Eranthis* genes (bold) group with other ranunculid *AP3* and *PI* genes. Numbers above branches indicate jackknife values > 50. The cladogram is based on seven trees of equal length containing 295 parsimony informative characters (length 1622; consistency index = 0·42, retention index = 0·50). Other *AP3* genes: *AtAP3*; *AmDEF*; *GhGDEF2*; *MsNMH7*; and *SlM3*. Other *PI* genes: *AtPI*; *AmGLO*; *GhGGLO1*; *MsNGL9*; and *SlM2*. Outgroup: *GgM13* (see Materials and Methods).

on leaf, stem and root templates. RNA from flowers also covers tissue from the tuberous rhizome, because flowers initiate from this organ. The *EhAP3* (264 bp) and *EhPI* (289 bp) gene transcripts were sequenced on an ABI 377 Prism DNA Sequencer (Applied Biosystems). The *EhAP3* and *EhPI* primer pairs are designed and tested so they do not amplify genomic DNA, as the reverse primers span exons 3 (A–B) and 4 (B–C) in the K-box of the B-class genes (Sundström *et al*., 1999). However, the forward primer does amplify one copy of single stranded genomic DNA during each thermal cycling giving a maximum background signal from 30 copies in each nucleus.

Reverse transcribed in situ *PCR*

RT–ISPCR was performed according to Johansen (1997) with the following modifications: the 50 µl RT-mix contained 2000 U ml^{-1} MuLV reverse transcriptase (Applied Biosystems) and 200 U ml^{-1} RNase inhibitor (Applied Biosystems) in 50 mM KCL, 10 mM Tris–HCl, 5 mM $MgCl_2$, 1 mM of each dNTP and 5 µM $poly(T)_{16}$ (Applied Biosystems). As a negative control, one section on each slide was not reverse transcribed. Reactions were reverse transcribed for 10 min at room temperature followed by 20 min at 42 °C in the GeneAmp *in situ* apparatus. The slides were kept on ice for 5 min and then disassembled before the 40 µl PCR mix was added: 200 U ml^{-1} AmpliTaq DNA polymerase in 40 mM KCL, 8 mM Tris–HCl, 2·5 mM $MgCl_2$, 0·02 mM of each dNTP (with 10 % DIG-11-dUTP) and 0·8 µM of the two *EhAP3* or *EhPI* specific primers: *EhAP3*: 5′-cgaagaaccggaattgtgaa-3′, 3′-tacgacaaagaatcggagaagg-5′, and *EhPI*: 5′-caaacaggcaggttacctactcc-3′, 3′-ctaaggcacctgaagggtga-5′. PCR amplification began with a 3 min denaturing step at 94 °C followed by a 30 cycle two-step PCR of 1 min denaturing at 94 °C and 1 min at 60 °C.

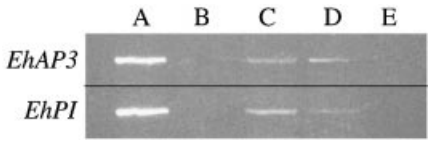

FIG. 2. Amplification of *EhAP3* and *EhPI* on total RNA from different tissues of *Eranthis* using solution RT-PCR analysis. A, Flower and tuberous rhizome; B, roots; C, leaves; D, stems; E, DNA template.

Detection of products

Primary antibodies conjugated to alkaline phosphatase were used to detect the *EhAP3* and *EhPI* RT-PCR products. Blocking was done in 1 % BSA-C (Arion, Wageningen, The Netherlands) in PBS for 1 h after which 100 µl of a BSA-C/PBS mix containing 800 µl ultra pure water, 100 µl BSA-C, 100 µl PBS and 10 µl of the alkaline phosphatase labelled anti-DIG antibody (Roche, Mannheim, Germany) was applied to each section. After 1 h slides were placed under running water for 30 min and detection of alkaline phosphatase was carried out for 8–10 min using one NTB/BCIP ready-to-use tablet (Roche). Mounting was done in Mowiol (Sigma-Aldrich, Steinheim, Germany).

RESULTS AND DISCUSSION

Isolation and phylogenetic analysis of Eranthis *B-class genes*

The *AP3*- and *PI*-specific primers amplify a fragment of approx. 400 bp. Although the primer sequences were designed from existing *AP3* and *PI* sequences from six species of Ranunculiflorae (see Materials and Methods) they also worked in amplification of B-class genes in several monocot species (unpubl. res.). Analysis of the *Eranthis* clones (17 *AP3* clones and 14 *PI* clones) showed only amplification of B-class genes suggesting that these primers are highly *AP3*- and *PI*-gene specific.

The *Eranthis AP3* and *PI* gene sequences contain the K-box conserved motifs (*AP3* amino acids = H/QYExM and *PI* amino acids = KHExL), characteristic of the B-class genes (Kramer *et al*., 1998). A simplified phylogenetic analysis of B-class genes shows that as expected, the *Eranthis* genes (named *EhPI* and *EhAP3*) are more closely related to *PI* and *AP3* genes from other ranunculid species than to B-class genes from more advanced dicot species (Fig. 1).

RT–PCR and RT-ISPCR expression analysis

Analysis of the *EhAP3* and *EhPI* transcript in different plant organs of *Eranthis* was done with simple solution RT–PCR experiments on total RNA extractions. RT–PCR products of the *EhAP3* and *EhPI* transcripts isolated from *Eranthis* flowers are present in both leaves and stems but not in roots (Fig. 2).

In situ transcription analysis of *EhAP3* and *EhPI* from *Eranthis* is shown in Fig. 3. The expression pattern of *EhAP3* and *EhPI* in the flower of *Eranthis* is not only

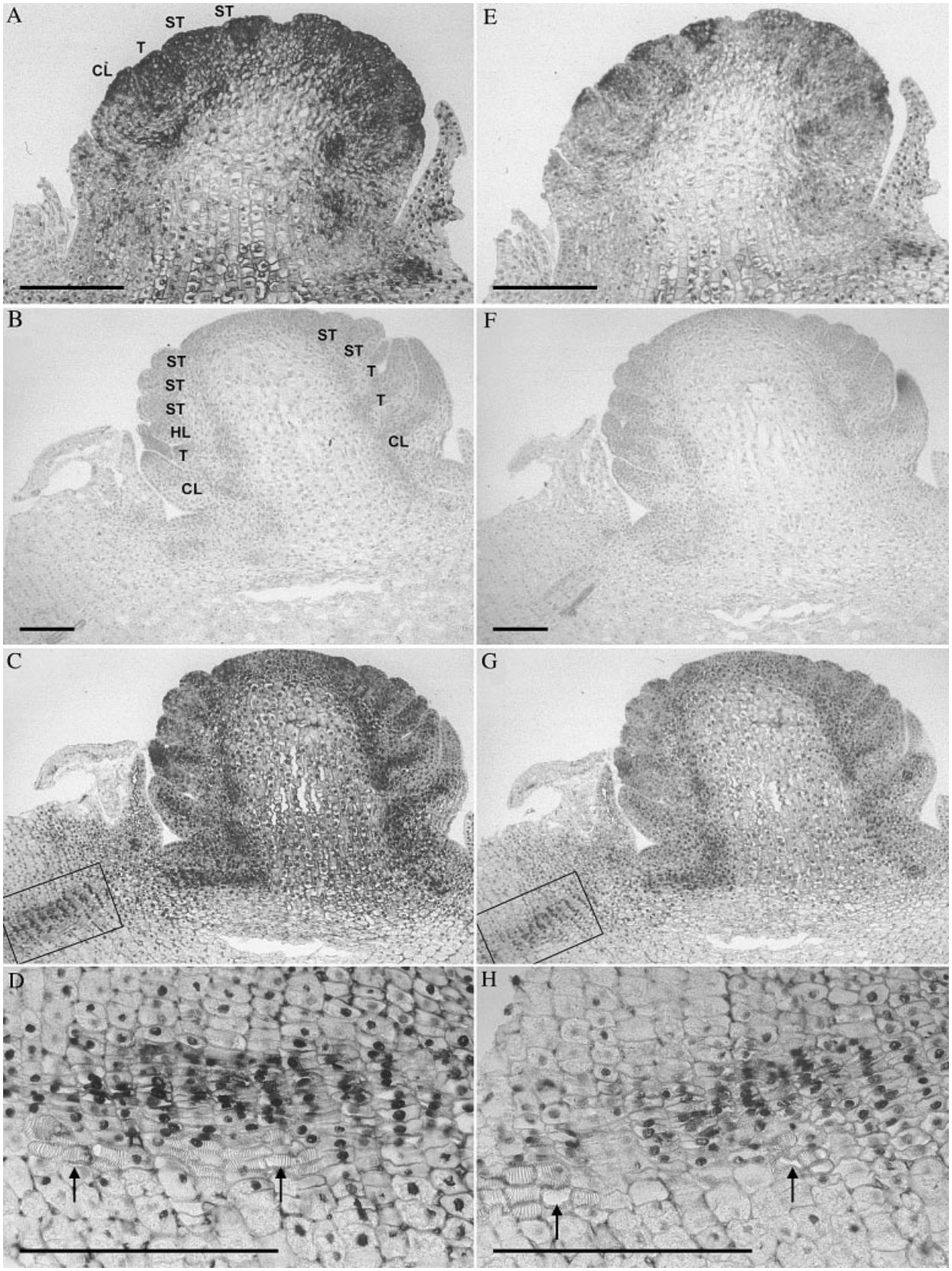

FIG. 3. Localization of *EhAP3* (A–D) and *EhPI* (E–H) transcripts in *Eranthis* using reverse transcribed *in situ* PCR. Cauline leaves and floral primordia are starting to appear in A and E. Honey-leaves and stamens are distinguished in C and G. Negative control in B and F (see Materials and Methods). Magnification of the vascular bundles in tissue of tuberous rhizome indicated by boxes in C and G are shown in D and H. Labels on A apply also to E and those on B to C, F and G. CL, Cauline leaf; T, tepal; HL, honey leaf; ST, stamen. Arrows in D and H indicate vessel members. Bar = 0·1 mm.

confined to tepals, honey-leaves and stamens but the genes are also transcribed in the cauline leaves surrounding the developing flower (Fig. 3A, C, E, G). *EhAP3* and *EhPI* are both transcribed in developing vascular tissue, which is seen in the tuberous rhizome, through the young flower stem and in the bundles leading to the young floral organ primordia (Fig. 3C, D, G, H). In sections of more developed flowers, *EhAP3* and *EhPI* transcripts are concentrated in the cauline leaves, tepals, honey-leaves and in stamens restricted to the sporogeneuos tissue, but transcription in the vascular bundles is not detectable (not shown). It appears as if *EhAP3* is transcribed more than *EhPI*, but this may simply reflect differences in efficiency of the two IS–PCR reactions. Overall, the transcription pattern of *EhAP3* and *EhPI* in *Eranthis* is similar, which suggests that the protein products also form heterodimers as in flowers of *Arabidopsis* (*AP3* and *PI*) and *Antirrhinum* (*DEF* and *GLO*) (Davies *et al*., 1996; Riechmann *et al*., 1996). However, as *EhAP3* and *EhPI* transcripts are not confined only to tepals, honey-leaves and stamens, as might be expected if the B-class gene transcription pattern applies to the ABC-model, it is unlikely that these genes only act as tepal, honey-leaves and stamen identity genes in *Eranthis*.

Northern blot analysis has shown that transcripts of the *PI* genes *ZMM18/29* (*Zea mays* L.), *EgrEGM2* (*Eucalyptus grandis* Maiden) and the *AP3* gene *GhGDEF2* (*Gerbera hybrida*) were also detectable in leaves (Southerton *et al*., 1998; Yu *et al*., 1999; Munster *et al*., 2001). It is interesting that B-class genes from these species and also *EhAP3* and *EhPI* from *Eranthis* are transcribed in leaves because transcription of floral identity genes in leaves of *Arabidopsis* is inhibited by other regulatory genes (Serrano-Cartagena *et al*., 2000). This suggests that inhibition of floral identity gene transcription in leaves is not preserved throughout angiosperms and it is therefore likely that B-class genes may have other functions besides acting as petal and stamen identity genes as in *Arabidopsis* (Jack *et al*., 1992; Goto and Meyerowitz, 1994).

Transcription of MADS-box genes in developing vascular tissue has been observed in several plant species (Decroocq *et al*., 1999; Sundström *et al*., 1999; Alvarez-Buylla *et al*., 2000). However, specific expression of B-class genes in vascular bundles is only reported from the *AP3* gene *StDEF4* (*Solanum tuberosum*), where the transcript was detected in vascular bundles leading to petals and stamens (Garcia-Maroto *et al*., 1993). Kramer and Irish (1999) studied B-class gene transcription and antibody localization in flowers of two ranunculid species but did not mention B-class gene expression in vascular bundles apart from anti-PI antibody staining in the vascular tissue at the base of petals in the flower of *Dicentra eximia* Torr. However, it does appear from *in situ* hybridization studies on flowers of *Papaver nudicaule* L. (Fig. 2B, C, J, I, P and T in Kramer and Irish, 1999); that both *PnAP3* and *PnPI-1* are transcribed in what is probably developing vascular strands leading to petals and stamens. This is similar to the present observations in *Eranthis*, except that expression in developing vascular bundles is also observed in the young flower stem and tuberous rhizome. This may suggest that B-class genes play a role in development of vascular tissue. If B-class genes are involved in development of vascular bundles, expression should also be detectable in roots of *Eranthis*. Although several solution RT–PCR experiments were performed on RNA from *Eranthis* roots, neither *EhAP3* nor *EhPI* transcripts were detected. Whether this could be due to inhibition of transcripts as seen in leaves of *Arabidopsis* (Serrano-Cartagena *et al*., 2000) requires further investigation.

Although transcription of B-class genes is generally restricted to petals and stamens in angiosperms, future research into B-class genes should aim to elucidate their function in tissues other than floral tissue. One way to explore this would be to perform transformation studies by ectopic expression or co-supression of B-class genes in species where these genes are transcribed in other organs and apparently have additional functions besides determination of petal and stamen identity.

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