Development 134, 4157-4166 (2007) doi:10.1242/dev.013136

Functional analyses of genetic pathways controlling petal specification in poppy

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MADS-box genes are crucial regulators of floral development, yet how their functions have evolved to control different aspects of floral patterning is unclear. To understand the extent to which MADS-box gene functions are conserved or have diversified in different angiosperm lineages, we have exploited the capability for functional analyses in a new model system, *Papaver somniferum* (opium poppy). *P. somniferum* is a member of the order Ranunculales, and so represents a clade that is evolutionarily distant from those containing traditional model systems such as *Arabidopsis*, *Petunia*, maize or rice. We have identified and characterized the roles of several candidate MADS-box genes in petal specification in poppy. In *Arabidopsis*, the *APETALA3* (*AP3*) MADS-box gene is required for both petal and stamen identity specification. By contrast, we show that the *AP3* lineage has undergone gene duplication and subfunctionalization in poppy, with one gene copy required for petal development and the other responsible for stamen development. These differences in gene function are due to differences both in expression patterns and co-factor interactions. Furthermore, the genetic hierarchy controlling petal development in poppy has diverged as compared with that of *Arabidopsis*. As these are the first functional analyses of *AP3* genes in this evolutionarily divergent clade, our results provide new information on the similarities and differences in petal developmental programs across angiosperms. Based on these observations, we discuss a model for how the petal developmental program has evolved.

KEY WORDS: Petal identity, Homeotic genes, MADS-box genes, Poppy, *Papaver somniferum*

INTRODUCTION

Nearly all angiosperm flowers possess a perianth, a series of sterile organs that surround the reproductive organs – the stamens and carpels. In many flowering plant lineages, the perianth is differentiated into distinct outer organs called sepals, and inner organs called petals; this is termed a bipartite perianth. Petals are therefore defined as occupying the spatial position outside of the stamens but internal to the sepals, and are often large, showy and pigmented, and contain specialized reflective papillate epidermal cells (Endress and Matthews, 2006). The evolution of flowers with elaborated petals is likely to have increased fitness through facilitating pollinator interactions (Clegg and Durbin, 2003).

Our current understanding of the molecular mechanisms controlling petal-identity specification rests largely on functional analyses carried out in several core eudicot species, including *Arabidopsis thaliana* and *Antirrhinum majus* (Weigel and Meyerowitz, 1994). In *Arabidopsis*, for example, the *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) MADS-box genes are required to specify petal and stamen identity, and mutations in these genes result in homeotic transformations of petals into sepals and stamens into carpeloid structures (Bowman et al., 1991; Goto and Meyerowitz, 1994; Jack et al., 1992). The AP3 and PI gene products heterodimerize, and are likely to act in vivo as part of larger MADS-box protein complexes, in order to specify petal as well as stamen identity (Bowman et al., 1991; Goto and Meyerowitz, 1994; Honma and Goto, 2001; Jack et al., 1992; Pelaz et al., 2001; Riechmann et al., 1996).

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Accepted 7 September 2007

Although a considerable amount is known about the molecular mechanisms specifying petal identity in *Arabidopsis* and other core eudicot species, there is little functional evidence that homologs of these genes play similar roles in petal-identity specification outside of the core eudicots. It is generally accepted that a bipartite perianth with distinct petals evolved independently multiple times within the flowering plants (Drinnan et al., 1994; Takhtajan, 1991). However, exactly when such events occurred is still unresolved. Phylogenetic analyses have been used to suggest that transitions between a unipartite and bipartite perianth have occurred multiple times within the eudicots (Albert et al., 1998; Soltis et al., 2005; Zanis et al., 2003). These analyses, though, are equivocal in determining the direction of such evolutionary transitions. One possibility is that a bipartite perianth represents independent evolutionary events in core eudicots as compared with the Ranunculales (Fig. 1A). Alternatively, a bipartite perianth might have been ancestral, and was lost in only a few, derived, non-core eudicot lineages (Fig. 1B). Determining whether core eudicots and Ranunculales species possess similar or divergent developmental genetic mechanisms to condition petal identity would be valuable in assessing the merits of these two hypotheses.

A variety of studies have already been carried out to assess the roles of *AP3* homologs in core eudicot species. A duplication in the *AP3* gene lineage at the base of the core eudicots gave rise to the eu*AP3* and *TM6* lineages, which are characterized by having distinct C-terminal sequence motifs (Kramer et al., 1998). The sequence motifs in the *TM6* lineage genes are more similar to those of the paleo*AP3* genes, which are found in non-core eudicot angiosperms (Kramer et al., 1998). Genetic analyses of eu*AP3* genes in core eudicot species, such as of the *Arabidopsis AP3* gene, support the idea that these genes have a common function in petal-identity specification, as well as in stamen specification (de Martino et al., 2006; Jack et al., 1992; Schwarz-Sommer et al., 1992; Vandenbussche et al., 2004). By contrast, core eudicot *TM6* genes appear to have a more restricted role in conditioning stamen identity (de Martino et al., 2006; Rijpkema et al., 2006).

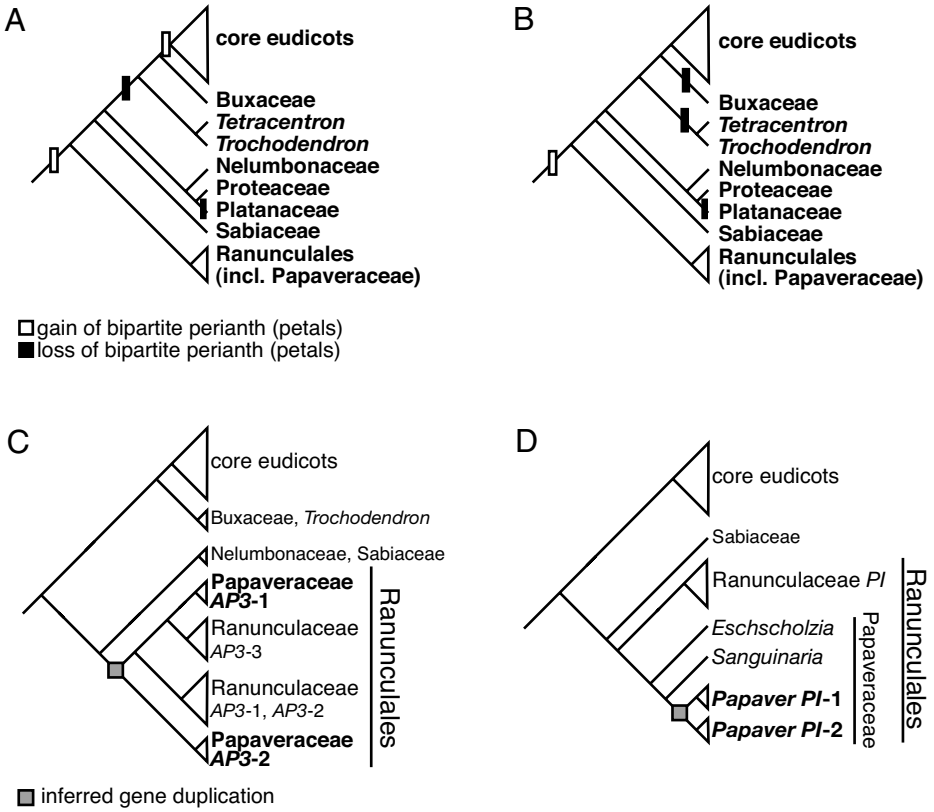

Fig. 1. Inferred evolutionary history of petals and MADS-box genes in the eudicots. (**A**,**B**) Alternative scenarios for the evolution of petals. (A) The evolution of a bipartite perianth occurred independently in the lineage leading to the Ranunculales as compared with the core eudicots. (B) The evolution of a bipartite perianth occurred prior to the radiation of the eudicots, with losses of this character in multiple lineages. (**C**,**D**) Summary of phylogenetic analyses. For comprehensive phylogenetic analyses, see Figs S1, S2 in the supplementary material. (C) The duplication of paleo*AP3*-like genes resulting in *PapsAP3-1* and *PapsAP3-2* occurred within the Ranunculales, pre-dating the divergence of the Ranunculaceae and Papaveraceae. (D) The duplication of *PI*-like genes occurred relatively recently in the Papaveraceae, leading to *PapsPI-1* and *PapsPI-2* in *P. somniferum*. Clades in bold represent those containing *Paps* genes described in this study.

The roles of paleo*AP3* genes in non-core eudicot angiosperms are somewhat unclear. A variety of expression analyses have been carried out that, in general, support the idea that paleo*AP3* genes have a conserved role in stamen identity specification, but their role in petal specification remains ambiguous. For instance, in many basal angiosperms, paleo*AP3* genes show strong and ubiquitous expression in stamens, but often inconsistent, weak or patchy expression in petal primordia (Kim et al., 2005; Kramer and Irish, 1999; Zahn et al., 2005). In non-grass monocots, expression of paleo*AP3* genes can be observed in developing petaloid organs in some taxa [e.g. in *Lilium longiflorum* (Tzeng and Yang, 2001)], but not in others [e.g. *Aparagus officinalis* (Park et al., 2003)]. Functional analyses in several monocot grasses have demonstrated that the paleo*AP3* genes in these species are required for the development of stamens and lodicules (Ambrose et al., 2000; Nagasawa et al., 2003; Xiao et al., 2003). As these grass species lack petals, such studies cannot directly assess the roles of paleo*AP3* genes in petal development. Furthermore, a chimeric *AP3* gene containing a non-core eudicot paleo*AP3* motif has been shown to be sufficient to rescue stamen, but not petal, identity in *Arabidopsis* (Lamb and Irish, 2003). By contrast, other studies have shown that ectopic overexpression of a monocot paleo*AP3* gene can rescue both petal and stamen development, suggesting that levels of paleo*AP3* gene expression might be important in determining developmental function (Whipple et al., 2004). Based on these observations, it has been suggested that the paleo*AP3* genes lack the capacity to fully specify petal identity, although they may play subsidiary roles in petal cell-type differentiation (Kramer and Irish, 1999; Kramer and Irish, 2000).

In order to explicitly and critically test the role of paleo*AP3* and *PI* homologs in the specification of petals in a non-core eudicot lineage, we have carried out functional analyses of paleo*AP3* and *PI*-like genes in the non-core eudicot *Papaver somniferum* (opium poppy). The Ranunculales, which includes *Papaver* species (the poppies), are well-supported as the sister group to all other eudicots (Angiosperm Phylogeny Group, 2003; Kim et al., 2004) (Fig. 1) and so are well-placed for comparative studies. *P. somniferum* is one of the oldest cultivated medicinal plants, and its continuing pharmaceutical importance as the source of a variety of morphinan alkaloids has made it one of the best-studied *Papaver* species (Chitty et al., 2006; Kapoor, 1995; Millgate et al., 2004; Zulak et al., 2007). Furthermore, functional genetic analyses can be rapidly carried out in *P. somniferum* using virus-induced gene silencing (Hileman et al., 2005). Our results provide the first functional evidence that petal identity can be conditioned by the action of a paleo*AP3* gene, and suggest that petal-identity specification in non-core eudicots relies on an independently derived, but similar, developmental program as that present in core eudicots. In addition, we show that there has been extensive functional diversification in the role of paleo*AP3* gene products in specifying petal and stamen identity in poppies.

MATERIALS AND METHODS

Isolation of *Paps* genes

RT-PCR reactions using the following degenerate primers were used to isolate poppy sequences from floral poly(A) RNA: PapsAP3DF [5′-AA(G/A)AAGCT(A/C)AAGA(A/G)CT(A/T/G)AC(A/T)(A/G)TTCT-3′] and PapsAP3DR [5′-ATC(T/G)T(G/C)TCC(T/C/A)(T/G/A)(T/G/C)CCT-(C/T)TGC(T/C)AT(C/T)TC-3′] for the *PapsAP3* genes; PapsPIDF [5′-AAGAGG(A/C)(A/G)AA(A/C)TGG(G/A)(T/A)T(G/C/T)(T/C/A)G/C)AA (G/A)A-3′] and PapsPIDR [5′-AG(A/G)(C/T/A)G(C/T)TT(A/G)TT-(G/A/T/C)TC(C/T)(A/G)C(C/T)TC(T/C/A)A-3′] for the *PapsPI* genes. Full-length sequences were obtained using RACE (rapid amplification of cDNA ends) according to the manufacturer's instructions (GIBCO-BRL, Carlsbad, CA).

Phylogenetic analyses

Nucleotide sequences of *AP3* and *PI* homologs were aligned using CLUSTALX and refined by hand using MacClade (Maddison and Maddison, 2000). Aligned sequences were used to generate maximum likelihood trees under the GRT + gamma model of molecular evolution, as implemented in GARLI (www.bio.utexas.edu/faculty/antisense/garli/Garli.html) (D. J. Zwickl, PhD thesis, University of Texas at Austin, 2006).

RT-PCR

Total RNA was extracted using Trizol (Invitrogen, Cleveland, OH) and approximately 300 ng was used in 10 μl cDNA synthesis reactions using Superscript III Reverse Transcriptase (Invitrogen). For cDNA synthesis, the poly(T) primer used was 5′-GACTCGAGTCGACATCGA(T)$_{17}$. Primers for testing expression of *PapsAP3-1*, *PapsAP3-2*, *PapsPI-1* and *PapsPI-2* were: PAP3-11, 5′-AAGAAATAAAGCCATGGAGG-3′ and PAP3-12, 5′-CTAAATACCGATTTTGGAGTC-3′; PAP3-21, 5′-AAGCTAAGGAACCACACTGA-3′ and PAP3-22, 5′-CACGCATGGTTCATAGATAT-3′; PPI-11, 5′-TTGCCAAACTACAACAAGTG-3′ and PPI-12, 5′-TAGCAGCTATGATCATGATC-3′; PPI-21, 5′-ACTCAAGAAAAATGGAAGAC-3′ and PPI-22, 5′-GCTTTTTATAAGTTCTTTGC-3′. Actin primers were: ACT1, 5′-ATGGATCCTCCAATCCAGAC-3′ and ACT2, 5′-TATTGTGTTGGACTCTGGTG-3′.

In situ hybridization

Hybridizations were carried out as previously described (Drea et al., 2005; Kramer and Irish, 1999) with minor modifications. Gene specific regions derived from the C-terminal domain and 3′ UTR of *PapsAP3-1*, *PapsAP3-2*, *PapsPI-1*, and *PapsPI-2* sequences were used to generate digoxygenin-labeled RNA probes.

Virus-induced gene silencing

Gene-specific regions of *PapsAP3-1*, *PapsAP3-2*, *PapsPI-1* and *PapsPI-2*, as well as concatenated *PapsAP3-1/PapsAP3-2* and *PapsPI-1/PapsPI-2* sequences were introduced into the TRV2 vector (Liu et al., 2002), transformed into *Agrobacterium* strain GV3101 and used to infiltrate poppy seedlings at the 3- to 5-leaf stage as previously described (Hileman et al., 2005). Individual resulting plants were assayed for the presence of the viral vector using RT-PCR as well as for any visible phenotype.

Scanning electron microscopy

Plant material was fixed overnight in 3.7% formaldehyde, 5% acetic acid, 50% ethanol (FAA) and then transferred to 70% ethanol. Samples were dehydrated in an ethanol series before critical-point drying. Samples were coated in gold and analyzed on a Zeiss ISI-SS40 scanning electron microscope equipped with digital image capture.

Yeast two-hybrid assays

PapsPI-1, PapsPI-2, PapsAP3-1 and PapsAP3-2 IKC domains were fused with the GAL4 DNA-binding domain in the pGBT9 vector and with the GAL4 activation domain in the pGAD424 vector (Clontech, Mountain View, CA). Two independent transformations for each vector combination were performed and five colonies per transformation were used for β-galactosidase liquid assays, using the protocol available at http://www.fhcrc.org/science/labs/gottschling/yeast/Bgal.html.

RESULTS

Identification of *P. somniferum AP3*-like and *PI*-like genes

We identified *P. somniferum* paleo*AP3*-like and *PI*-like genes using a degenerate RT-PCR approach. In referring to *P. somniferum* genes, we have used the abbreviation *Paps*, as attached to gene symbols, so as to distinguish them from genes of other species. Sequencing and Southern hybridization analyses showed that there are two paleo*AP3*-like (*PapsAP3-1* and *PapsAP3-2*) and two *PI*-like (*PapsPI-1* and *PapsPI-2*) genes in the *P. somniferum* genome (data not shown). Phylogenetic analyses of paleo*AP3*-like and *PI*-like genes from a variety of basal eudicot species are consistent with the hypothesis that in both cases the duplications occurred within the Ranunculales (Fig. 1C,D and see Figs S1, S2 in the supplementary material). The duplication resulting in paralogous paleo*AP3*-like genes appears to have occurred early in the radiation of the Ranunculales (Fig. 1C and see Fig. S1 and Table S2 in the supplementary material), whereas the duplication giving rise to poppy *PI*-like genes appears to have occurred more recently within Papaveraceae (Fig. 1D and see Fig. S2 and Table S2 in the supplementary material).

Both *PapsAP3-1* and *PapsAP3-2* encode characteristic paleo*AP3* PI-derived motifs (Kramer et al., 1998), but the *PapsAP3-1* predicted product lacks the consensus paleo*AP3* motif (Kramer et al., 1998) (Fig. 2A). This pronounced difference at the C-terminus is due to insertions/deletions between the *PapsAP3* genes that engender frameshifts beginning at the start of exon 6. As such, *PapsAP3-1* lacks pleisiomorphic sequence motifs, but has gained a novel C-terminus, whereas *PapsAP3-2* retains the ancestral character state of a paleo*AP3* C-terminal motif.

The *PapsPI-1* gene encodes a product containing the PI-motif as well as a sequence extension at the C-terminus (Fig. 2B). The *PapsPI-2* predicted product lacks the consensus PI-motif (Kramer et al., 1998) at the C-terminus. This appears to be due to a single nucleotide insertion in the 3′ coding region followed by a 2-nucleotide deletion 22 bp downstream, generating a stop codon and a truncated protein (Fig. 2B). Although this domain has been shown to be essential for protein function in the *Arabidopsis* PI protein (Lamb and Irish, 2003), the *Pisum sativum PI* gene also lacks this conserved domain but has been shown to be capable of rescuing the *Arabidopsis pi* mutant phenotype (Berbel et al., 2005).

Diversification in expression of *P. somniferum* genes

P. somniferum flowers contain two sepals, four petals, numerous hypogynous stamens, and generally 8 to 12 carpels, which produce the capsular fruit (Kapoor, 1995). The early development of floral organ primordia in *P. somniferum* can be characterized by a variety of morphological landmark stages (Fig. 3). These include stage P3 of development when the petal, stamen and carpel primordia are first clearly visible, and are enclosed by the surrounding sepal primordia. By stage P5, the anther and filament of the stamens are discernable and the capsule has become invaginated; ovules are distinguishable by stage P5. Between stages P7 and P8 the petals undergo accelerated growth and extend to fill the space bounded by the two sepals. This rapid growth of the petals continues to force the sepals apart when the bud opens just after anthesis. At later stages, *P. somniferum* buds (stage P8 and later) become pendant prior to anthesis, before extending upright as the flower opens owing to asymmetric growth of the pedicel. At maturity, each floral organ type is characterized by distinctive epidermal cell morphologies (Fig. 4A). Sepal cells are somewhat irregular, with numerous stomata present on the abaxial surface, and adaxial sepal epidermal cells being more domed. Petal cells are distinctively long and narrow, with prominent ridges on the adaxial surface that differ from the characteristic conical and papillar cells found in petals of many species. Stamen filaments possess elongated epidermal cells, whereas the epidermal cells of the anther are more irregularly shaped. The gynoecium results from a fusion of multiple carpels and possesses distinctive ridges; the epidermal cells between the ridges are relatively flat and are interspersed with stomata.

RT-PCR and in situ hybridization analyses showed that each of the *P. somniferum* genes had a distinct pattern of expression during floral development. *PapsAP3-1* expression was initially detected in petal and stamen primordia at stage P3, when these primordia are first

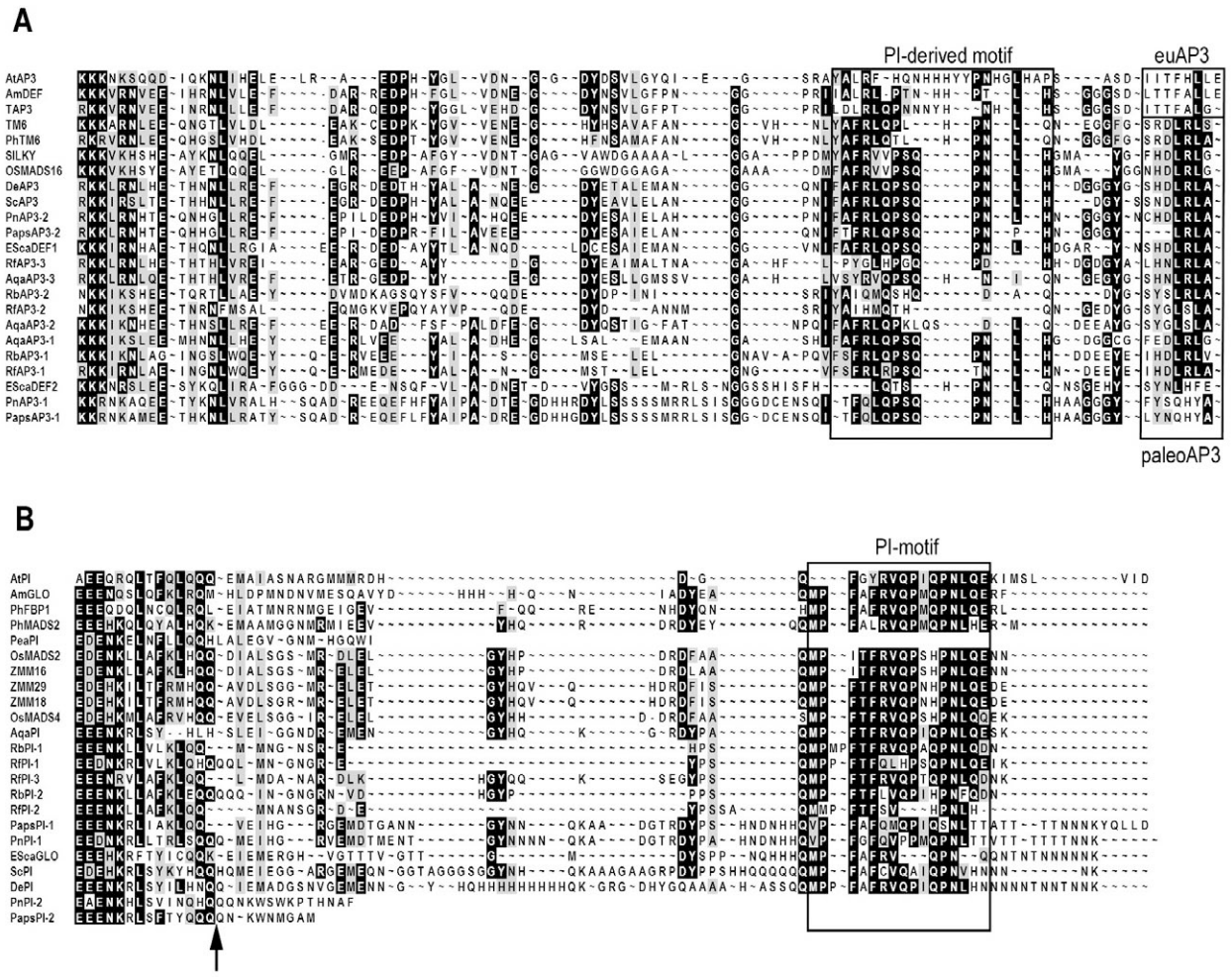

Fig. 2. Sequence alignment of *P. somniferum* AP3- and PI-like sequences. Alignment of C-terminal domains of predicted AP3 (**A**) and PI (**B**) proteins from core eudicots, monocots and basal eudicots. Conserved domains are boxed. Identical residues are highlighted in black and similar residues in gray. The arrow in B indicates the position of the insertion in PapsPI-2 that mediates a frameshift.

apparent (Fig. 3A). Expression of *PapsAP3-1* throughout the developing stamens and petals persisted through later stages of flower development (Fig. 3A,B). Weak expression of *PapsAP3-1* could be detected in sepal and carpel primordia at later stages of development (Fig. 3B). By contrast, *PapsAP3-2* expression was initially detected at stage P4 in stamen primordia, but was excluded from petal primordia (Fig. 3A). This pattern persisted until the P5 stage of flower development when *PapsAP3-2* expression was also detected in petal primordia (Fig. 3A). *PapsAP3-2* expression appeared to be more ubiquitous at later stages of development, in that low levels of expression could be detected in sepal and carpel primordia through stage P8 (Fig. 3B). The sequence and expression analyses indicate that *PapsAP3-2* is more representative of paleo*AP3*-like genes of other Ranunculales species (Kramer et al., 2003; Kramer and Irish, 2000; Shan et al., 2006), suggesting that lack of early and ubiquitous petal-specific expression is ancestral in this clade.

PapsPI-1 was expressed throughout the petal and stamen primordia from stage P3 onwards throughout floral development, an expression pattern that appeared similar to that of *PapsAP3-1* (Fig. 3A). *PapsPI-2* expression was detected early in P3 buds in petals, stamens and carpels (Fig. 3A), and remained largely restricted to the stamen and carpel whorls and at lower levels throughout later stages of floral development (Fig. 3A,B).

Loss-of-function phenotypes demonstrate a distinct function in floral organ identity for each *P. somniferum* gene

To assess the functional roles of the *AP3*-like and *PI*-like genes in *P. somniferum*, we used tobacco rattle virus (TRV)-mediated, virus-induced gene silencing (VIGS) (Hileman et al., 2005). Gene-specific regions of *PapsAP3-1*, *PapsAP3-2*, *PapsPI-1* and *PapsPI-2*, as well as concatenated *PapsAP3-1/PapsAP3-2* and *PapsPI-1/PapsPI-2* sequences, were introduced into the TRV2 vector (Liu et al., 2002) for silencing. We screened approximately 500 infiltrated seedlings for each of the six constructs, with 0.5-8% of resulting plants containing the transformation vector and showing a phenotype (see Table S1 in the supplementary material). Transcript levels of silenced genes were considerably reduced (Fig. 6), although not completely eliminated, consistent with our previous observations that TRV-VIGS is an effective tool to downregulate gene expression in *P. somniferum* (Hileman et al., 2005). Silencing of an individual gene did not affect the expression of the other *AP3*-like or *PI*-like genes (Fig. 6), indicating that silencing was gene-specific.

The range of phenotypes in silenced plants was distinct for each construct. Reduction in *PapsAP3-1* expression resulted in homeotic transformations of petals to more sepaloid organs (Fig. 4B), whereas stamens were unaffected. This transformation was evident at the

cellular level, with both adaxial and abaxial second-whorl cells showing sepaloid characteristics (Fig. 4B). Conversely, silencing of *PapsAP3-2* expression resulted in stamens that showed variable transformations to carpeloid structures containing ovules, whereas the petals remained completely unaffected (Fig. 4C). The VIGS phenotypes indicate that *PapsAP3-1* and *PapsAP3-2* control distinct developmental pathways. When both *PapsAP3-1* and *PapsAP3-2* were silenced simultaneously, both petals and stamens showed homeotic transformations (Fig. 4D, Fig. 5). The range of petal phenotypes in these plants was similar to that of the *PapsAP3-1*-silenced lines, but the transformation of stamens to carpels was generally much more severe than in the *PapsAP3-2*-silenced lines alone. These results suggest that *PapsAP3-1* and *PapsAP3-2*, although having discrete functions, are also redundant in regulating stamen development. Furthermore, one target of their transcriptional action is *PapsPI-1*, as its expression was downregulated in the carpeloid stamens of plants silenced for both *PapsAP3-1* and *PapsAP3-2* (Fig. 6).

VIGS of *PapsPI-1* resulted in flowers with homeotic transformations of stamens to carpeloid organs and petals possessing sepaloid characteristics (Fig. 4E). By contrast, silencing of *PapsPI-2* produced no visible phenotype (data not shown). However, the transformation of stamens to carpeloid organs observed in the *PapsPI-1*-silenced lines was less severe than when both *PapsPI* genes were silenced (Fig. 4F), suggesting that *PapsPI-2* might play a redundant role in stamen development. Coordinate silencing of *PapsPI-1* and *PapsPI-2* also resulted in downregulation of *PapsAP3-1* expression in carpeloid stamens, whereas *PapsAP3-2* expression was less severely affected (Fig. 6).

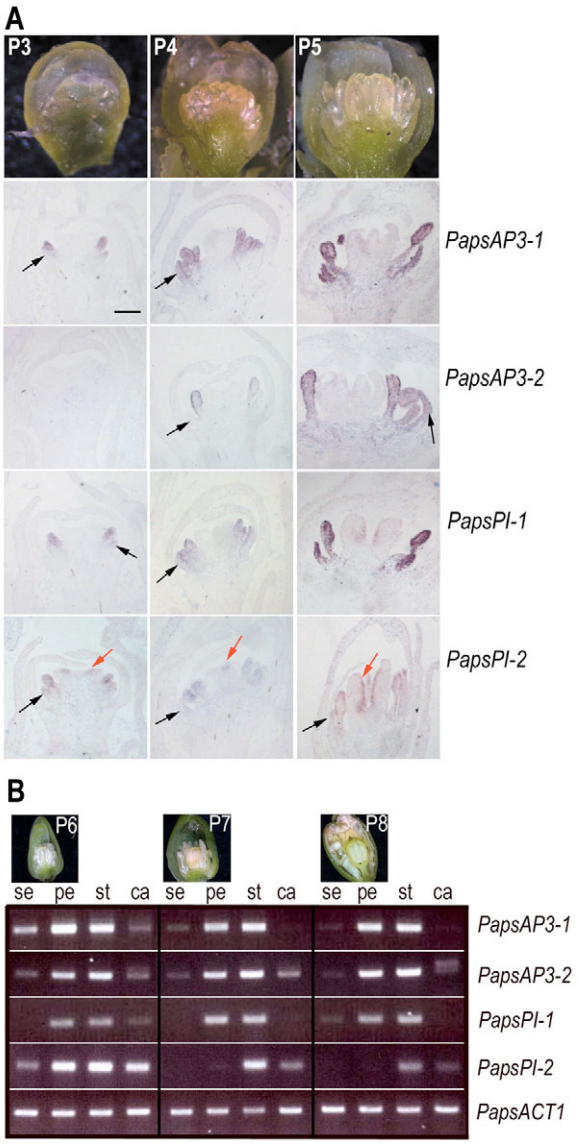

Fig. 3. Expression analyses of *PapsAP3* and *PapsPI* genes. (**A**) mRNA in situ hybridization analyses for *PapsAP3-1*, *PapsAP3-2*, *PapsPI-1* and *PapsPI-2* during P3 to P5 stages of poppy flower development. Light micrographs of representative stages are shown. Black arrow, petal primordia; red arrow, carpel primordia. *PapsAP3-1* is expressed in petal and stamen primordia commencing at stage P3, and is maintained in these organs through later stages. This is in contrast to *PapsAP3-2* expression, which is initially restricted to the stamen primordia; later, *PapsAP3-2* expression expands to include developing petals at stage P5. *PapsPI-1* expression commences in petal and stamen primordia at stage P3, and is maintained in this pattern through later stages. *PapsPI-2* expression is weak but detectable in petal, stamen and carpel primordia at stage P3; by stage P5, expression is predominantly in stamens and on the adaxial side of the carpels. Scale bar: 200 μm, for all panels. (**B**) RT-PCR analyses of *PapsAP3* and *PapsPI* expression during stages P6 to P8 of poppy flower development (as shown above). At each stage, sepals (se), petals (pe), stamens (st) and carpels (ca) were removed separately for analysis. The *P. somniferum* actin gene (*PapsACT1*) was used as an amplification control.

Conservation and diversification in *P. somniferum* protein-protein interactions

Various lines of evidence have suggested that homodimerization of the paleo*AP3*-like gene products was ancestral (Winter et al., 2002), and that in core eudicots the ability of *AP3* gene products to homodimerize has been lost (Riechmann et al., 1996; Schwarz-Sommer et al., 1992). To determine the dimerization capabilities of the *P. somniferum* gene products, we carried out yeast two-hybrid assays on pairwise combinations of *P. somniferum* proteins (Table 1). *PapsPI-2* did not dimerize with any of the tested proteins, although this does not obviate the possibility that it interacts with other MADS-box gene products to effect its function. This is supported by the observation that silencing of *PapsPI-2* enhances the silenced phenotype of *PapsPI-1* (Fig. 4F), pointing to the possibility that both *PapsPI* gene products participate in common protein complexes. *PapsAP3-2* was able to homodimerize, supporting the idea that it has retained the ancestral character state. Furthermore, *PapsAP3-1* and *PapsAP3-2* were able to heterodimerize with *PapsPI-1*. This heterodimerization could serve to delimit *PapsPI-1* expression to the petal and stamen whorls as a consequence of autoregulatory feedback control, as has been shown for *Arabidopsis* and *Antirrhinum* (Honma and Goto, 2000; Trobner et al., 1992). Our results are consistent with this model, because *PapsAP3-1* and *PapsAP3-2* are required to maintain expression of *PapsPI-1* but not *PapsPI-2* (Fig. 6). This also implies that evolutionary changes in *AP3* lineage gene expression can drive alterations in the domains of *PI* gene expression, without having to invoke secondary changes in *PI* regulation.

DISCUSSION

Petal and stamen specification in *P. somniferum*

We have examined the functions of *AP3*-like and *PI*-like genes in *P. somniferum* in order to define their roles in specifying different floral organ identities. Our data clearly show that paleo*AP3* and *PI* homologs play key roles in the specification of petals in a non-core

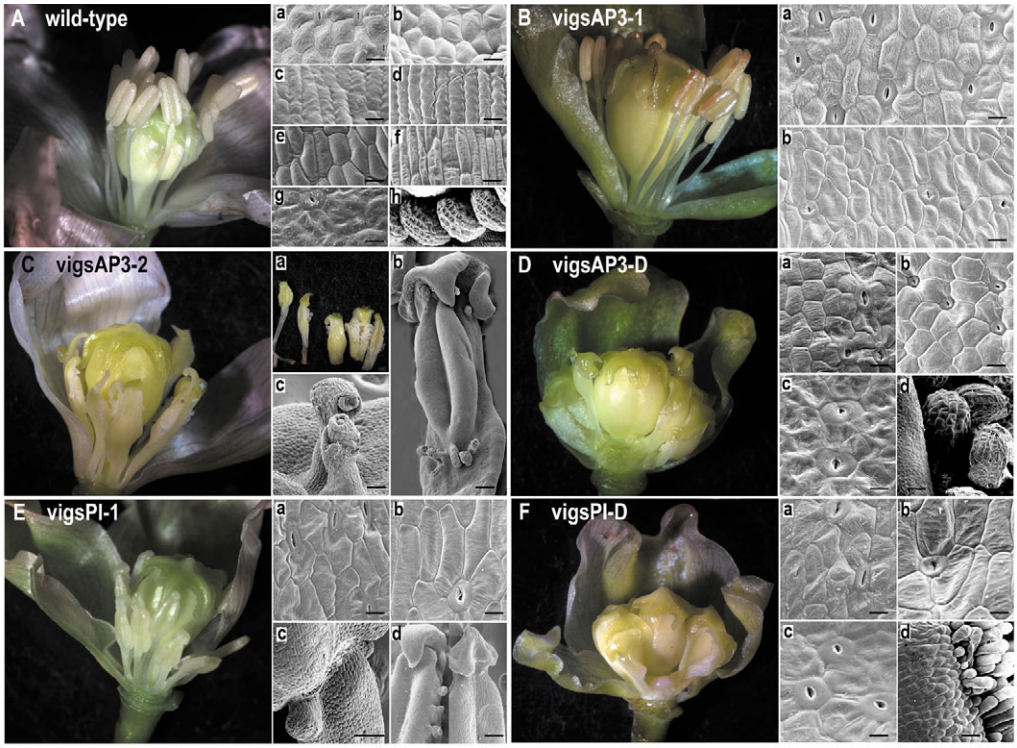

Fig. 4. Functional analyses of *PapsAP3* and *PapsPI* genes using virus-induced gene silencing. (**A**) Wild-type poppy flower. Scanning electron micrographs (SEMs) of abaxial (a) and adaxial (b) sepals; abaxial (c) and adaxial (d) petals; anther (e) and filament (f) of stamens; carpel wall (g) and ovules (h). (**B**) vigsAP3-1 (silenced for *PapsAP3-1*) flower showing homeotic transformation of petals into sepaloid organs. SEM of abaxial (a) and adaxial (b) sepaloid petals. (**C**) vigsAP3-2 flower showing variable transformations of stamens to carpeloid structures. Range of carpeloidy in stamens (a). SEM of carpeloid stamen with stigmatic ray overlying anther tissue (b) and showing the presence of ectopic ovules (c). (**D**) vigsAP3-D (vigsAP3-1/AP3-2) flower showing a strong homeotic transformation of petals and stamens. SEM of abaxial (a) and adaxial (b) sepaloid petals; abaxial surface (c) and ectopic ovules of carpeloid stamens (d). (**E**) vigsPI-1 flower displaying homeotic transformations in petals and stamens. SEM of abaxial (a) and adaxial (b) sepaloid petals; emerging ovule at junction of anther and filament of carpeloid stamen (c) and examples of stigmatic tissue overlying the anther of carpeloid stamens (d). (**F**) vigsPI-D (vigsPI-1/PI-2) flower showing strong homeotic transformations of petals and stamens. SEM of abaxial (a) and adaxial (b) sepaloid petals; abaxial surface (c) and ovules of carpeloid stamens (d). Scale bars: 30 μm in Aa-f, Ba,b, Da,b, Ea,b, Fa,b; 10 μm in Ag, Dc, Fc; 100 μm in Ah, Cc, Dd, Ec,d, Fd; 300 μm in Cb.

eudicot species. We have shown that the *AP3* and *PI* lineage genes have undergone extensive functional diversification as a result of both recent and more ancient gene duplication events. Whereas *PapsPI-1* is required to specify both petal and stamen identity, *PapsAP3-1* functions primarily in the specification of petals, and *PapsAP3-2* functions primarily in the specification of stamens. The differences in *PapsAP3-1* and *PapsAP3-2* functions can be ascribed in part to differences in their expression (Fig. 3). Differences in *PapsAP3-1* and *PapsAP3-2* functions also are presumably owing to differences in protein–co-factor interactions (Table 1), which are likely to reflect the C-terminal motif differences between the two *PapsAP3* gene products, as variations in these C-terminal motifs have been implicated in functional diversification in other systems (de Martino et al., 2006). Together, these data support the idea that *PapsAP3-1* and *PapsAP3-2* play qualitatively different roles in floral development.

Fig. 5. Functional analysis of *PapsAP3* genes. Wild-type (right) and vigsAP3-D (left) mature flower buds. A dramatic homeotic conversion of petals and stamens is seen in the vigsAP3-D bud.

Our analyses in *P. somniferum* also illuminate aspects of the regulatory hierarchy controlling petal and stamen development in this species. *PapsAP3-1*, which is required for petal specification, is redundantly regulated by both *PapsPI* genes in stamens, but not in petals; similarly, the *PapsAP3* genes are redundantly required for *PapsPI-1* expression in stamens, but not petals (Fig. 5). These observations suggest that the mechanisms controlling *PapsAP3* and *PapsPI* gene expression in petals versus stamens are distinct, and reflect whorl-specific differences in regulation. This could in turn reflect differences in autoregulatory feedback control; we have shown that the *PapsAP3-2* gene product can homodimerize, whereas *PapsAP3-1* and *PapsAP3-2* were able to heterodimerize with *PapsPI-1*. One possibility is that *PapsAP3-2*, which is expressed and functions predominantly in stamens, can act via larger multimeric MADS-box protein complexes to regulate the expression of

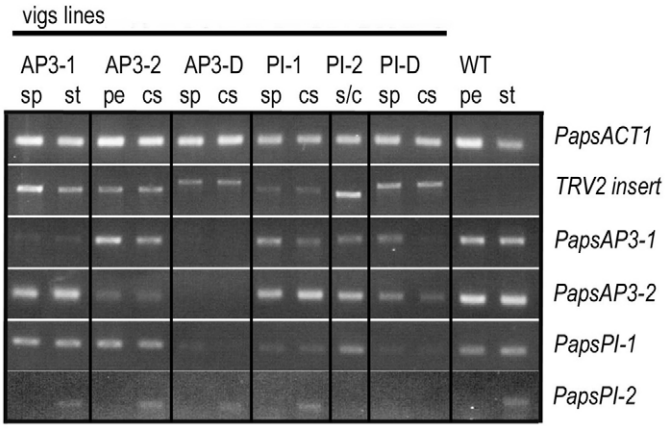

Fig. 6. Expression analyses of VIGS lines. Petals (pe), sepaloid petals (sp), stamens (st), carpeloid stamens (cs) and pooled stamens and carpels (s/c) were dissected out and used for RT-PCR analyses. Expression of *PapsACT1*, *PapsAP3-1*, *PapsAP3-2*, *PapsPI-1* and *PapsPI-2* in each of the six VIGS backgrounds and in wild-type flowers is shown. RT-PCR was also used to demonstrate the presence of the TRV2 vector insert in each transformation. *PapsACT1* served as an amplification control.

PapsAP3-1 and *PapsPI-1* in those organs. By contrast, none of the *PapsAP3* or *PapsPI* genes appears to play a role in auto- or cross-regulatory interactions in maintaining gene expression in the petals, pointing to an alternative regulatory strategy. In *Arabidopsis*, the *AP1* MADS-box gene plays just such a role in regulating *AP3* and *PI* expression in the second whorl (Hill et al., 1998; Ng and Yanofsky, 2001); however, orthologs of *Arabidopsis AP1* do not exist in Ranunculales (Litt and Irish, 2003), suggesting that other gene functions, potentially *AP1* paralogs, play the equivalent regulatory role in *P. somniferum*.

Subfunctionalization and adaptive change of *PapsAP3* genes

Gene duplications can provide the raw material for the evolution of new morphologies, either through neofunctionalization (the evolution of a novel function in one gene copy) or through subfunctionalization (the partitioning of ancestral function), with the possibility of further adaptive changes to one or both paralogs (Force et al., 2005; Force et al., 1999; Ohno, 1970). Why duplicated genes persist in genomes at higher frequencies than expected, possibly allowing for subsequent changes in protein function, remains an important question of genome evolution. The immediate retention of duplicate genes appears to depend, in part, on the biological roles of the encoded proteins. Genes encoding products that function as key regulatory components, such as transcription factors, as well as those that participate in large multiprotein complexes [e.g. MADS-domain proteins (Honma and Goto, 2001)], appear to be preferentially maintained owing to the requirement for a stoichiometric balance with other components in the pathway (Birchler and Veitia, 2007; Evangelisti and Wagner, 2004). The selective retention of duplicate *AP3* gene lineages, both at the base of the core eudicots (Kramer et al., 1998) and in Ranunculales (this work), might reflect the requirement for their functions in a developmentally and biochemically complex regulatory system.

We have provided two scenarios for the evolution of a bipartite perianth containing petals (Fig. 1A,B). Under both of these evolutionary scenarios, the ancestor of eudicots is considered to have possessed a bipartite perianth with distinct petals, and this condition was inherited in the Ranunculales. We therefore argue that the combinatorial function of *PapsAP3-1* and *PapsAP3-2* to specify a bipartite perianth with distinct petals was inherited from a eudicot ancestor with a single paleo*AP3* homolog capable of specifying both petals and stamens. This is also consistent with our phylogenetic analyses, which indicate that the ancestral eudicot possessed a single paleo*AP3* gene (see Fig. S1 in the supplementary material). Subsequent to the duplication in the paleo*AP3* lineage in Ranunculales, the two resulting paralogs became subfunctionalized such that in poppy, *PapsAP3-1* retained the ancestral subfunction of specifying petal identity, and *PapsAP3-2* retained the ancestral subfunction of specifying stamen identity. Interestingly, whereas *PapsAP3-2* does not seem to retain any residual role in petal specification, *PapsAP3-1* does retain some ability to direct the development of stamens, as evidenced by the stronger transformation of stamens to carpeloid organs in the coordinate silencing of *PapsAP3-1* and *PapsAP3-2*, as compared with silencing of *PapsAP3-1* alone (Fig. 4).

The subfunctionalization of *PapsAP3-1* and *PapsAP3-2* is also evident in their patterns of gene expression and co-factor interactions. Although they have overlapping patterns of expression in both petals and stamens, *PapsAP3-2* is expressed predominantly in stamens, and only comes to be expressed in petals at later stages of development. It is also clear that adaptive changes in protein-protein interactions have occurred during the evolution of *PapsAP3-1* and *PapsAP3-2*. The proteins encoded by *PapsAP3-1* and *PapsAP3-2* can heterodimerize with *PapsPI-1*, but *PapsAP3-2* can also homodimerize. The ability to heterodimerize as well as homodimerize has been observed for paleo*AP3* gene products from lily, a monocot (Tzeng et al., 2004). Thus, it seems likely that the ancestral eudicot paleo*AP3* gene possessed both capabilities, which have subsequently been parsed in the *PapsAP3-1* and *PapsAP3-2* genes. This diversification in function presumably reflects the differences in C-terminal motifs found in *PapsAP3-1* as compared with *PapsAP3-2*, as these motifs have been found to play crucial roles in protein-protein interactions and function (Lamb and Irish, 2003; Tzeng et al., 2004). Therefore, subfunctionalization of *PapsAP3-1* and *PapsAP3-2* is likely to have occurred through evolutionary shifts in expression, in interaction with other protein partners, and through changes in their binding affinity for

Table 1. Summary of yeast two-hybrid assays with PapsAP3 and PapsPI

	PapsAP3-1BD	PapsAP3-2BD	PapsPI-1BD	PapsPI-2BD	BD
PapsAP3-1AD	–	–	++	–	–
PapsAP3-2AD	–	+	++	–	–
PapsPI-1AD	++	++	–	–	–
PapsPI-2AD	–	–	–	–	–
AD	–	–	–	–	–

AD, Gal4 activation domain; BD, Gal4 binding domain.
++, Strong interaction; +, weak interaction; –, no interaction.

downstream cis-regulatory sequences of target genes in the respective petal and stamen developmental pathways. The differences in *PapsAP3-1* and *PapsAP3-2* functions are likely to reflect an early diversification event in the Ranunculales. A similar *AP3* subfunctionalization event has been postulated to have occurred in *Aquilegia*, another member of the Ranunculales, based on disparate expression patterns of the three *Aquilegia AP3*-like genes (Kramer et al., 2007). However, this hypothesis has yet to be functionally tested.

In contrast to the roles of *PapsAP3* genes during flower development, our genetic analyses of *PapsPI* genes indicates that these duplicates have not parsed their functions in a whorl-specific manner. *PapsPI-1* appears to have retained the majority of function, based on its robust expression levels, protein interaction capabilities, and the strong homeotic phenotype observed for *PapsPI-1*-silenced plants. *PapsPI-2* could potentially be on the way to becoming a pseudogene; our VIGS studies indicate that it possesses only a redundant role in specifying petal and stamen identity, it is expressed at low levels, and has accumulated mutations that result in the loss of conserved C-terminal motifs. Duplication of *PI* genes appears to have occurred multiple times across the Ranunculales. Some lineages such as *Clematis* and Papaveraceae possess two *PI* copies, whereas other Ranunculales such as *Aquilegia* have only one *PI* gene (see Fig. S2 in the supplementary material). Silencing of the single *PI* homolog present in *Aquilegia* results in a phenotype similar to that we have described for silencing of *PapsPI-1*. However, the homeotic transformations observed for silencing of *Aquilegia PI* are difficult to ascribe to silencing of this locus alone, as these plants show coordinate downregulation of *AP3* homologs as well (Kramer et al., 2007). As such, it remains to be tested whether the function we have ascribed to *PapsPI-1* represents a common role for *PI*-like genes across the Ranunculales.

The evolution of petal-identity pathways

Our data provide the first functional evidence that paleo*AP3* genes play critical homeotic roles in petal specification. Based on these results, in conjunction with a number of other observations, we propose a model to explain the evolution of the petal-identity pathway. The role of *AP3*-like and *PI*-like genes appears to be conserved in regulating stamen development across angiosperms, and presumably reflects the ancestral role of such genes in specifying male reproductive identity, a function that is likely to have been conserved in gymnosperms where such genes are expressed in male cones (Mouradov et al., 1999; Sundstrom et al., 1999). During the early diversification of the angiosperms, we propose that, concomitant with the evolution of the angiosperm bisexual axis, the pattern of expression of paleo*AP3* genes broadened. This is consistent with the broad and variable patterns of paleo*AP3* gene expression seen in a variety of basal angiosperm species (Kim et al., 2005). However, because no functional data exist for any basal angiosperm species, it is unclear to what extent this diversification in expression is responsible for directing petaloid identity.

We suggest that the ancestral role for *AP3*-like and *PI*-like genes in the angiosperms might have been in specifying regional identity, as opposed to organ identity. True petals are defined as occupying the second whorl (or inner perianth) and having a narrow base, distinctive pigmentation and conical reflective epidermal cells (Endress, 2006; Martin et al., 2002; Weberling, 1989). A number of species possess second-whorl structures that do not display the morphological characteristics of petals (e.g. tepals and lodicules) and, conversely, there are species that possess organs that display a petaloid morphology outside of the second whorl (e.g. heterotopic petaloidy). Therefore, the development of true petals depends on two separable processes: second-whorl regional specification and appropriate morphological differentiation.

AP3- and *PI*-like gene functions have been examined in monocot grasses, which lack petals. In addition to specifying stamen identity, these genes are also required for the specification of a grass-specific organ, the lodicule (Ambrose et al., 2000; Nagasawa et al., 2003; Whipple et al., 2007). Lodicules appear to correspond to second-whorl structures in that they occupy a region of the flower outside of the stamens but internal to the other non-reproductive organs of the grass flower, but otherwise are not homologous with petals in that they do not display the morphological characteristics of a petal, although they do have a distinctive morphology (Clifford, 1988; Dahlgren et al., 1985; Whipple et al., 2007). These data from grasses demonstrate that *AP3/PI*-like genes can condition the specification of non-petaloid second-whorl structures. Furthermore, analyses of *AP3/PI* gene expression in the monocot *Asparagus officinalis*, which possesses a perianth composed of two whorls of undifferentiated tepals, has shown that *AP3* and *PI* orthologs are expressed in inner-whorl, but not outer-whorl, organs (Park et al., 2004; Park et al., 2003), suggesting a role in regional specification. Similarly, silencing of a *PI* ortholog in *Aquilegia* suggests that it is required for the specification of inner, but not outer whorls of petaloid organs (Kramer et al., 2007). The genetic requirement for *AP3/PI* gene functions in specifying core eudicot petals (de Martino et al., 2006; Jack et al., 1992; Schwarz-Sommer et al., 1992; Vandenbussche et al., 2004), non-core eudicot petals (this work), and lodicules (Ambrose et al., 2000; Nagasawa et al., 2003; Whipple et al., 2007), suggests a common role for these genes in specifying a spatially limited regional domain within the flower. As such, this might facilitate multiple instances of re-recruitment of *AP3*-like genes to direct a petal developmental program in the second whorl as petals are evolutionarily lost and regained across angiosperms.

Consistent with this idea, our data support a model whereby different and independent gene duplication events have allowed for the recruitment of *AP3*-dependent gene functions for specifying petal identity. In the core eudicots, the eu*AP3/TM6* gene duplication event has allowed for the subfunctionalization (through shifts in both expression and dimerization capabilities) of the resulting gene copies. In basal eudicots, a distinct gene duplication event has resulted in the *PapsAP3-1* and *PapsAP3-2* genes that have also parsed their functions. Regardless of whether petals arose independently in the core eudicots as compared with the Ranunculales, or whether petals in the core eudicots and Ranunculales are homologous (Fig. 1), we have shown that distinct gene duplication events have allowed for the differential recruitment of gene functions to specifying petal identity in these different angiosperm lineages.

We thank Jalean Petricka, Emily Hood, Jenny Yamtisch, Kristina Gremski and Takudzwa Shumba for technical assistance; Iain Dawson and members of the Irish laboratory for discussion and comments throughout the course of this work; and Gunter Wagner and John Carlson for their comments on the manuscript. This work was supported by grants #IOB-0110731 and IOB-0516789 from the National Science Foundation to V.F.I. and by a Yale University Brown fellowship to S.D.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/23/4157/DC1

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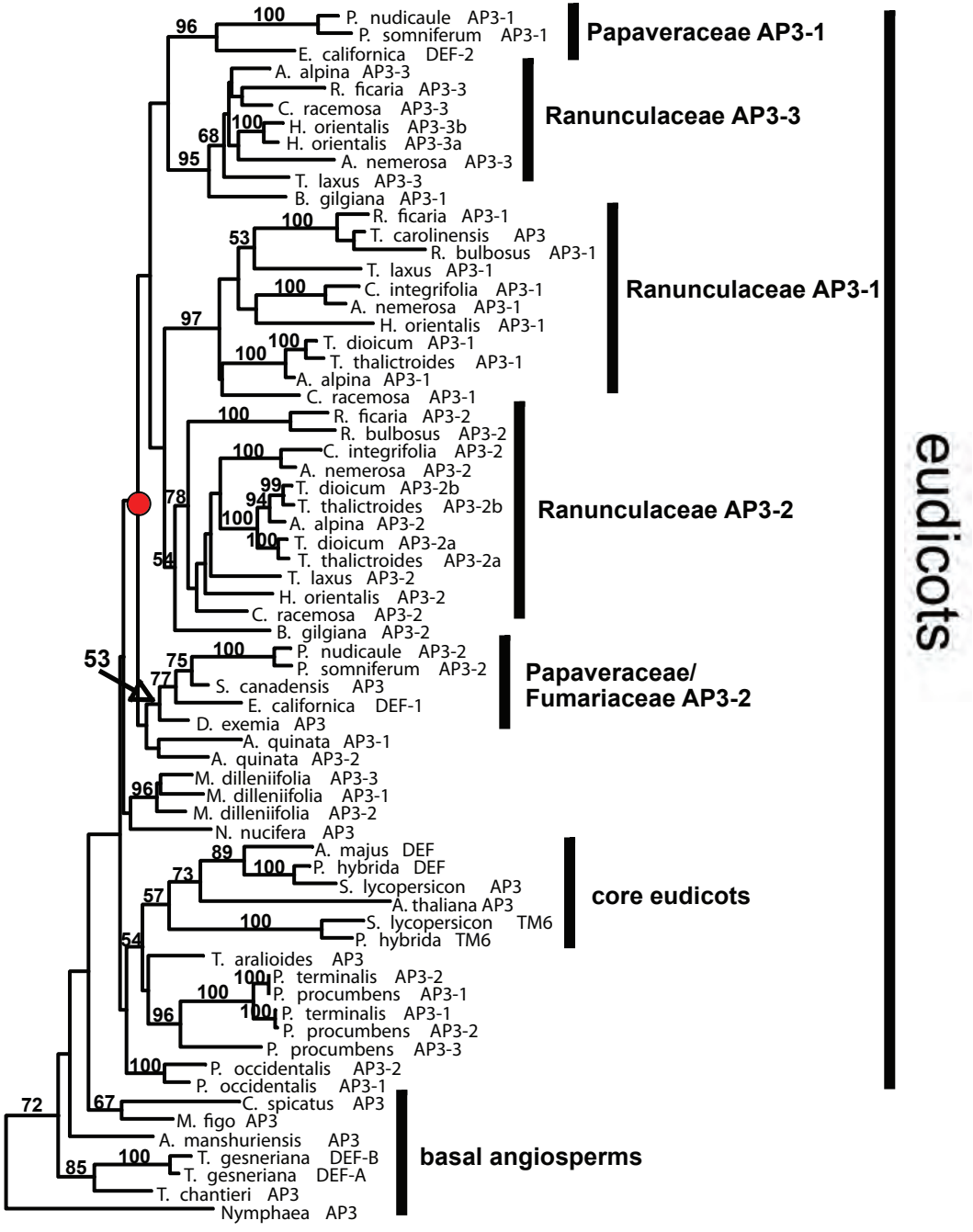
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Papaveraceae AP3-1
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C. racemosa AP3-3
H. orientalis AP3-3b
H. orientalis AP3-3a
A. nemerosa AP3-3
T. laxus AP3-3
B. gilgiana AP3-1
Ranunculaceae AP3-3
R. ficaria AP3-1
T. carolinensis AP3
R. bulbosus AP3-1
T. laxus AP3-1
C. integrifolia AP3-1
A. nemerosa AP3-1
H. orientalis AP3-1
T. dioicum AP3-1
T. thalictroides AP3-1
A. alpina AP3-1
C. racemosa AP3-1
Ranunculaceae AP3-1
R. ficaria AP3-2
R. bulbosus AP3-2
C. integrifolia AP3-2
A. nemerosa AP3-2
T. dioicum AP3-2b
T. thalictroides AP3-2b
A. alpina AP3-2
T. dioicum AP3-2a
T. thalictroides AP3-2a
T. laxus AP3-2
H. orientalis AP3-2
C. racemosa AP3-2
B. gilgiana AP3-2
Ranunculaceae AP3-2
P. nudicaule AP3-2
P. somniferum AP3-2
S. canadensis AP3
E. californica DEF-1
Papaveraceae/
Fumariaceae AP3-2
D. exemia AP3
A. quinata AP3-1
A. quinata AP3-2
M. dilleniifolia AP3-3
M. dilleniifolia AP3-1
M. dilleniifolia AP3-2
N. nucifera AP3
A. majus DEF
P. hybrida DEF
S. lycopersicon AP3
A. thaliana AP3
S. lycopersicon TM6
P. hybrida TM6
core eudicots
T. aralioides AP3
P. terminalis AP3-2
P. procumbens AP3-1
P. terminalis AP3-1
P. procumbens AP3-2
P. procumbens AP3-3
P. occidentalis AP3-2
P. occidentalis AP3-1
C. spicatus AP3
M. figo AP3
A. manshuriensis AP3
T. gesneriana DEF-B
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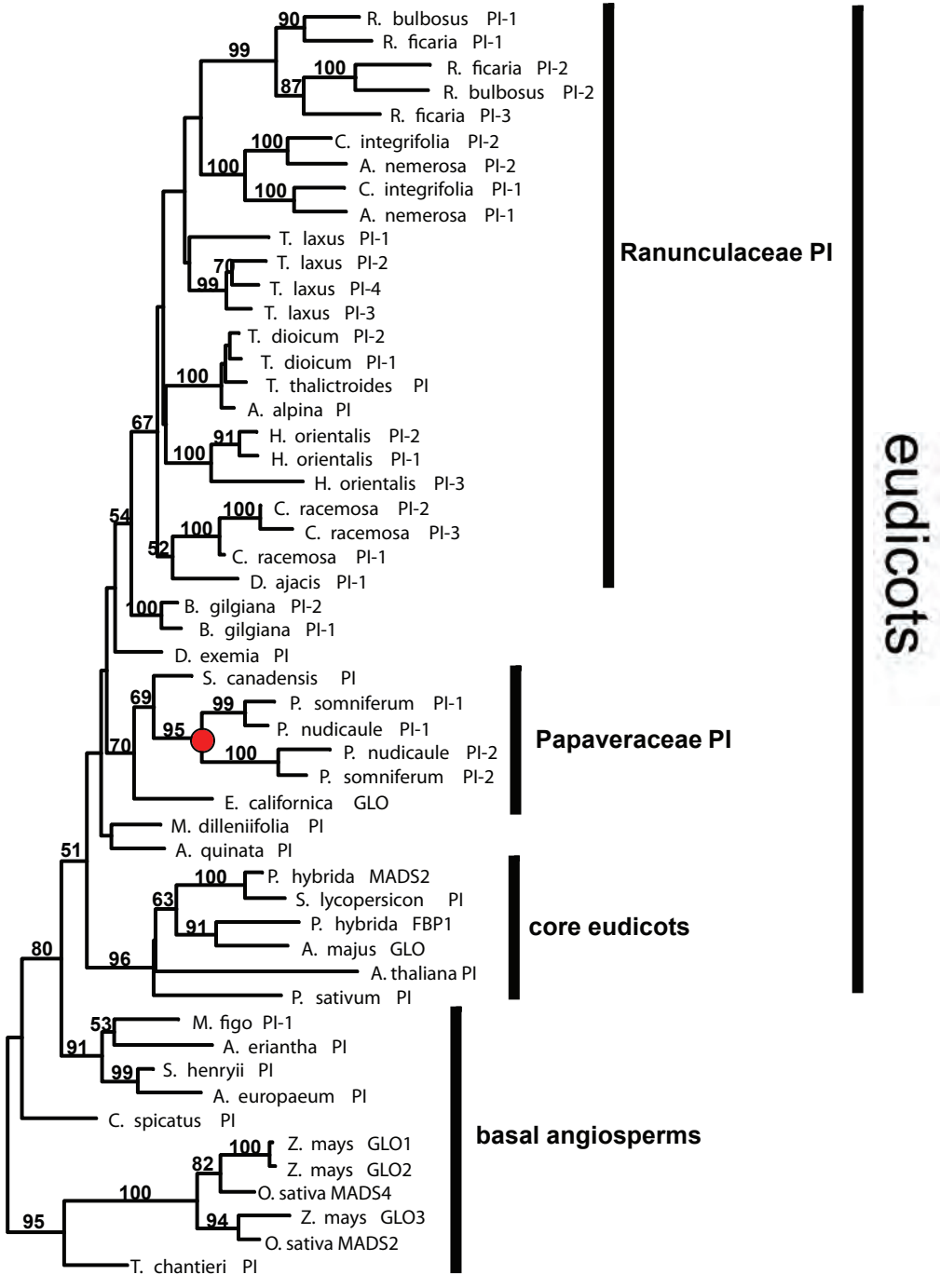
T. chantieri AP3
Nymphaea AP3
basal angiosperms
eudicots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A. nemerosa PI-2
C. integrifolia PI-1
A. nemerosa PI-1
T. laxus PI-1
T. laxus PI-2
T. laxus PI-4
T. laxus PI-3
T. dioicum PI-2
T. dioicum PI-1
T. thalictroides PI
A. alpina PI
H. orientalis PI-2
H. orientalis PI-1
H. orientalis PI-3
C. racemosa PI-2
C. racemosa PI-3
C. racemosa PI-1
D. ajacis PI-1
B. gilgiana PI-2
B. gilgiana PI-1
D. exemia PI
S. canadensis PI
P. somniferum PI-1
P. nudicaule PI-1
P. nudicaule PI-2
P. somniferum PI-2
E. californica GLO
M. dilleniifolia PI
A. quinata PI
P. hybrida MADS2
S. lycopersicon PI
P. hybrida FBP1
A. majus GLO
A. thaliana PI
P. sativum PI
M. figo PI-1
A. eriantha PI
S. henryii PI
A. europaeum PI
C. spicatus PI
Z. mays GLO1
Z. mays GLO2
O. sativa MADS4
Z. mays GLO3
O. sativa MADS2
T. chantieri PI
Ranunculaceae PI
Papaveraceae PI
core eudicots
basal angiosperms
eudicots

Table S1. Summary of VIGS lines generated for each construct

	vigsAP3-1	vigsAP3-2	vigsAP3-D	vigsPI-1	vigsPI-2	vigsPI-D
Phenotype	27	19	13	12	0	4
Petal transformation	27	0	13	12	0	4
Stamen transformation	0	19	13	12	0	4

Approximately 500 plants were screened for each construct during the course of 30 independent VIGS experiments; the numbers of plants with visible phenotypes are indicated.

Table S2. Genes used for phylogenetic analyses

Gene (GenBank accession number)	Species
AP3 (AAL49893); *PI* (BAA06465)	*Arabidopsis thaliana*
PeaPI (AAW29099)	*Pisum sativum*
DEF (CAA44629); *GLO* (CAA48725)	*Antirrhinum majus*
PhDEF (X69946); *PhTM6* (AAS46017); *PhFBP-1* (M91190), *PhMADS-2* (AY370521)	*Petunia hybrida*
TAP3 (ABG73412); *TM6* (ABG48621); *TPI* (ABG73411)	*Solanum lycopersicon*
PloAP3-1 (AAO26529); *PloAP3-2* (AAO26530)	*Platanus occidentalis*
PpAP3-1 (ABF56135); *PpAP3-2* (ABF56136); *PpAP3-3* (ABF56137)	*Pachysandra procumbens*
PtAP3-1 (AAC42585); *PtAP3-2* (AAC42586)	*Pachysandra terminalis*
TraAP3 (DQ453774)	*Trochodendron aralioides*
MdAP3-1 (AAR87670); *MdAP3-2* (AAR87671); *MdAP3-3* (AAR87672); *MdPI* (AAR87673)	*Meliosma dilleniifolia*
NnAP3 (ABE11602)	*Nelumbo nucifera*
PnAP3-1 (AAC42588); *PnAP3-2* (AAC42589); *PnPI-1* (AAC42570), *PnPI-2* (AAC42571)	*Papaver nudicaule*
PapsAP3-1 (EF071992); *PapsAP3-2* (EF071993); *PapsPI-1* (EF071994), *PapsPI-2* (EF071995)	*Papaver somniferum*
EScaDEF-1; *EScaDEF-2*; *EScaGLO*	*Eschscholzia californica*
ScAP3 (AAD31696); *ScPI* (AAD31699)	*Sanguinaria canadensis*
DeAP3 (AAC42590); *DePI* (AAC42572)	*Dicentra exemia*
AkqAP3-1 (AAT46097); *AkqAP3-2* (ABC02398); *AkqPI* (AAT46101)	*Akebia quinata*
AqaAP3-1 (AAO26497); *AqaAP3-2* (AAO26498); *AqaAP3-3* (AAO26499); *AqaPI* (AAO26500)	*Aquilegia alpina*
AnnAP3-1 (AAO26489); *AnnAP3-2* (AAO26490); *AnnAP3-3* (AAO26491); *AnnPI-1* (AAO26493); *AnnPI-2* (AAO26495)	*Anenome nemerosa*
CliAP3-1 (AAO26518); *CliAP3-2* (AAO26519); *CliPI-1* (AAO26520), *CliPI-2* (AAO26521)	*Clematis integrifolia*
CirAP3-1 (AAO26510); *CirAP3-2* (AAO26511); *CirAP3-3* (AAO26512); *CirPI-1* (AAO26513); *CirPI-2* (AAO26514)	*Cimicifuga racemosa*
DaPI (AAC42577)	*Delphinium ajacis*
HoAP3-1 (AAO26522); *HoAP3-2* (AAO26523); *HoAP3-3a* (AAO26524); *HoAP3-3b* (AAO26525); *HoPI-1* (AAO26526), *HoPI-2* (AAO26527), *HoPI-3* (AAO26528)	*Helleborus orientalis*
RbAP3-1 (AAC42591); *RbAP3-2* (AAD31697); *RbPI-1* (AAC42574); *RbPI-2* (AAC42575)	*Ranunculus bulbosus*
RfAP3-1 (AAC42569); *RfAP3-2* (AAD31698); *RfAP3-3* (AAO26531); *RfPI-1* (AAC42573); *RfPI-2* (AAD31700)	*Ranunculus ficaria*
ThdAP3-1 (AAW78033); *ThdAP3-2a* (AAW78034); *ThdAP3-2b* (AAW78035); *ThdPI-1* (AAW78031), *ThdPI-2* (AAW78032)	*Thalictrum dioicum*
ThtAP3-1 (AAO26534); *ThtAP3-2a* (AAO26535); *ThtAP3-2b* (AAO26536); *ThtPI* (AAO26537)	*Thalictrum thalictroides*
TrcAP3 (AY162904)	*Trautvettaria carolinensis*
TrlAP3-1 (AAO26538); *TrlAP3-2* (AAO26540); *TrlAP3-3* (AAO26542); *TrlPI-1* (AAO26544), *TrlPI-2* (AAO26546), *TrlPI-3* (AAO26548), *TrlPI-4* (AAO26550)	*Trollius laxus*
BgAP3-1 (AAO26505); *BgAP3-2* (AAO26506); *BgPI-1* (AAO26508), *BgPI-2* (AAO26509)	*Berberis gilgiana*
ZmGLO-1 (CAC33848), *ZmGLO-2* (CAC33849), *ZmGLO-3* (CAC33850)	*Zea mays*
OsMADS-2 (Q40702), *OsMADS-4* (Q40703)	*Oryza sativa*
TgDEFA (AB094965); *TgDEFB* (AB094966)	*Tulipa gesneriana*
TacAP3 (AAF73935); *TacPI* (AAF73942)	*Tacca chantieri*
ChsAP3 (AAF73930); *ChsPI* (AAF73939)	*Chloranthus spicatus*
MfAP3 (AAC42592); *MfPI-1* (AAC42578)	*Michelia figo*
ArePI (AAR87680)	*Aristolochia eriantha*
AmAP3 (AAV65053)	*Aristolochia manshuriensis*
AePI (AAF73936)	*Asarum europaeum*
SahPI (AAR87679)	*Saruma henryii*
NymAP3 (AAR87709)	*Nymphaea* sp.