Annals of Botany **107**: 1557–1566, 2011
doi:10.1093/aob/mcr045, available online at www.aob.oxfordjournals.org

ANNALS OF BOTANY
Founded 1887

PART OF A SPECIAL ISSUE ON EVOLUTION AND DEVELOPMENT

Alternate transcripts of a floral developmental regulator have both distinct and redundant functions in opium poppy

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Received: 4 August 2010 Returned for revision: 7 October 2010 Accepted: 24 January 2011 Published electronically: 7 March 2011

• *Background and Aims* The MADS-box transcription factor AGAMOUS (AG) is an important regulator of stamen and fruit identity as well as floral meristem determinacy in a number of core eudicots and monocots. However, its role outside of these groups has not been assessed explicitly. Examining its role in opium poppy, a basal eudicot, could uncover much about the evolution and development of flower and fruit development in the angiosperms.
• *Methods AG* orthologues were isolated by degenerate RT-PCR and the gene sequence and structure examined; gene expression was characterized using *in situ* hybridization and the function assessed using virus-induced gene silencing.
• *Key Results* In opium poppy, a basal eudicot, the *AGAMOUS* orthologue is alternatively spliced to produce encoded products that vary at the C-terminus, termed *PapsAG-1* and *PapsAG-2*. Both transcripts are expressed at high levels in stamens and carpels. The functional implications of this alternative transcription were examined using virus-induced gene silencing and the results show that *PapsAG-1* has roles in stamen and carpel identity, reflecting those found for *Arabidopsis AG*. In contrast, *PapsAG-2*, while displaying redundancy in these functions, has a distinctive role in aspects of carpel development reflected in septae, ovule and stigma defects seen in the loss-of-function line generated.
• *Conclusions* These results describe the first explicit functional analysis of an *AG*-clade gene in a basal eudicot; illustrate one of the few examples of the functional consequences of alternative splicing in transcription factors and reveal the importance of alternative transcription, as well as gene duplication, as a driving force in evolution.

Key words: Flower development, MADS-box, *Papaver somniferum*, alternative transcription, *AGAMOUS*, gene-silencing.

INTRODUCTION

A key means of generating morphological diversity is through duplication of critical regulatory genes and consequent diversification in the deployment of their function (Force *et al.*, 1999; Moore *et al.*, 2005). The permutations for diversity are amplified by the fact that such duplicated genes may interact with myriad other components and programmes in different ways. In *Arabidopsis*, a number of MADS-box transcription factors have been shown to be required for specifying floral organ identity, development and determinacy. Homologues of these genes have been postulated to regulate analogous processes in other angiosperms, while alterations in the number and function of such genes is thought to have contributed to the morphological diversity of angiosperm floral structures (Irish and Litt, 2005; Kaufmann *et al.*, 2005).

In *Arabidopsis*, the A*GAMOUS* (*AG*) MADS-box gene is required for determining stamen and carpel identity and floral determinacy. However, functional analyses of *AG*-related gene duplicates have suggested that there is some variation in the roles of such gene products in other species. For instance, the *AG* orthologue in antirrhinum is *FARINELLI* (*FAR*), mutations of which affect the stamens only (Causier *et al.*, 2005). Instead, *PLENA* (*PLE*), a paralogue of *AG* and *FAR*, is required in antirrhinum to condition stamen and carpel differentiation and determinacy (Bradley *et al.*, 1993; Causier *et al.*, 2005). Phylogenetic analyses suggest that the gene duplication giving rise to the *AG* and *PLE* paralogous lineages occurred in the ancestor of the core eudicots (Kramer *et al.*, 2004; Zahn *et al.*, 2006). An independent duplication event in the monocots gave rise to two *AG*-related genes in maize and rice. These two genes were suggested to have discrete functions in maize (Mena *et al.*, 1996). Genetic analyses in rice have confirmed the subfunctionalization of these duplicates with *OsMADS58* controlling carpel development and floral determinacy and *OsMADS3* controlling stamen identity (Yamaguchi *et al.*, 2006).

Little is known, though, of the potential roles of *AG*-related genes in the basal eudicots. In *Eschscholzia californica*, two *AG*-related genes have been identified, *EScaAG1* and *EScaAG2*, that have similar expression patterns in stamens and carpels, with *EScaAG1* being expressed at much higher levels, suggesting that *EScaAG2* may be non-functional (Zahn *et al.*, 2006). In *Aquilegia formosa*, a single *AG*-like gene has been identified and shown to be expressed in stamens, stamenodia and carpels (Voelckel *et al.*, 2010). In *Thalictrum dioecium*, *ThdAG1* is expressed in developing stamen and carpel primordia, while *ThdAG2* expression is limited to the developing

ovules, suggesting that these genes may have distinct roles (Di Stilio *et al.*, 2005). These genes were isolated by extensive degenerate RT-PCR sampling or from cDNA libraries generated from floral tissues. All of these expression analyses are generally consistent with a potential role for basal eudicot *AG* homologues in specifying reproductive identity. As of yet, though, functional analyses have not been carried out for any *AG*-related basal eudicot gene.

Opium poppy (*Papaver somniferum*) is a basal eudicot species in the order Ranunculales and family Papaveraceae. The Ranunculales is well supported as the sister group to all the other eudicots (Angiosperm Phylogeny Group, 2009). The opium poppy is largely recognizable by its distinctive fruit, the capsule, which is rich in opiate-containing laticiferous vessels, and so this fruit has been the subject of considerable physiological and biochemical studies (Kapoor, 1997). In addition, opium poppy is ideally suited to analyses of gene function, as loss of function of individual genes can be achieved through highly efficient virus-induced gene silencing (VIGS; Hileman *et al.*, 2005).

In a search for the MADS-box genes involved in fruit development in opium poppy a *PapsAG* locus was identified that undergoes alternative splicing to produce two abundantly expressed transcripts, *PapsAG-1* and *PapsAG-2*. These transcripts differ only in the encoded C-terminal domains. The functions conferred by these alternatively spliced forms were explored and each was shown to have some unique function in floral development. These observations have important ramifications for developing new models for the evolution of floral homeotic gene function.

MATERIALS AND METHODS

Isolation of PapsAG *genes*

RT-PCR reactions using the QVT1 and QVT2 degenerate forward primers with an oligo dT reverse primer (Hileman *et al.*, 2006) were used to isolate MADS-box genes from carpel and flower cDNA of *Papaver somniferum* (Persian White). Total RNA was extracted using Trizol reagent (Invitrogen, Cleveland, OH, USA) and converted to cDNA using SuperscriptIII (Invitrogen) as per manufacturers instructions. Isolated sequences were cloned into pCR4-TOPO sequencing vector (Invitrogen) and sequenced to identify *AG*-like clones for further analysis. A total of 23 and 18 sequenced clones for *PapsAG-1* and *PapsAG-2* were identified and used to generate consensus cDNA sequences.

Sequence and phylogenetic analyses

Translated sequences of *AG* orthologues were aligned using CLUSTAL_X (Thompson *et al.*, 1997) and alignments refined by hand using BioEdit (Hall, 1999). Sequences for *PapsAG-1* and *PapsAG-2* were deposited in Genbank with accession numbers GU123602 and GU123603.

Expression analyses using RT-PCR

Total RNA was extracted using the Trizol reagent (Invitrogen) and approx. 300 ng was used in 10-μL cDNA synthesis, reactions using Superscript^{TM}III reverse transcriptase (Invitrogen). For cDNA synthesis the poly(T) primer used was 5′-GACTCGAGTCGACATCGA$(T)_{17}$. Primers for testing expression of *PapsAG-1* and *PapsAG-2* were: AGbF 5′-TATGACTCTCGGAACTTTCTC-3′ forward primer with AG1R 5′-ACATAGAATAGACTCAGC-3′ and AG2R 5′-GT AATGTAGTCAAATCCAGATG-3′ reverse primers. Actin primers were ACT1: 5′-ATGGATCCTCCAATCCAGAC-3′ and ACT2: TATTGTGTTGGACTCTGGTG-3′. PCR consisted of cycles of 94 °C for 30 s, 53 °C for 45 s, 72 °C for 1 min, preceded by a 5-min denaturation at 94 °C and followed by an extension at 72 °C for 6 min; 30 cycles for *PapsAG-1* and *PapsAG-2* and 26 cycles for Actin.

Genomic DNA PCR

Genomic DNA was extracted from leaf tissue using established protocols (Aldrich and Cullis, 1993). This was used in PCR reactions with AGbF/AG2R and AGbF/AG1R primer combinations using 30 cycles of 94 °C for 1min, 53 °C for 2 min, 72 °C for 3 min, preceded by a 5-min denaturation at 94 °C and followed by an extension at 72 °C for 6 min. PCR products were cloned into the pCR4-TOPO sequencing vector (Invitrogen) and 20 (10 for each primer set) clones were sequenced, then aligned with the cDNA sequences to map the location of the intron and predict the splicing sites.

Expression analyses using in situ *hybridization*

Hybridizations were carried out as previously described (Drea *et al.*, 2005, 2007) with minor modifications. Gene-specific regions derived from the C-terminal domain and 3′-UTR of *PapsAG-1*and *PapsAG-2* sequences were used to generate digoxyenin-labelled RNA probes. PCR fragments amplified with AG1sF/AG2sF forward primers and AG1T7R/AG2T7R reverse primers were cleaned using a Qiagen PCR purification kit and used in an *in vitro* transcription reaction with dig-UTP and T7 RNA polymerase (Roche).

AG1F: 5′-GAAGATAGAAGACATCAAACC-3′
AG2F: 5′-ATGATGGCATTCTCTTTCAAG-3′
AG1T7R: 5′-GATCTAATACGACTCACTATAGGGAGTCA ACATAGAATAGACTCAGC-3′
AG2T7R: 5′-GATCTAATACGACTCACTATAGGGAGTAA TGTAGTCAATCC-AGATG-3′
T7 RNA polymerase sites are underlined. Probe lengths were 250 bp and 209 bp, respectively.

Virus-induced gene silencing

Gene-specific regions of *PapsAG-1* and *PapsAG-2*, as well as concatenated *PapsAG-1*/*PapsAG-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 three-to-five 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. Three constructs incorporating regions of *PapsAG-1* and/or *PapAG2* into the

TRV2 vector cut with *Xba*I and *Bam*HI were assembled as follows:

AG1F: 5′-GCTCTAGAATGATGGCATTCTCTTTCAAG-3′
AG2F: 5′-GCTCTAGAGAAGATAGAAGACATCAAACC-3′
AG1R: 5′-CGGGATCCACATAGAATAGACTCAGC-3′
AG2R: 5′-CGGGATCCGTAATGTAGTCAAATCCAGATG-3′

Primers AG1F and AG1R were used to amplify a fragment for the vigsAG1 construct; primers AG2F and AG2R used to amplify a fragment for the vigsAG2 construct.

PapsAG-2 was reamplified with AG2BAM and AG2XHO and ligated into a *Bam*HI- and *Xho*I-cut TRV2 vector already containing *PapsAG-1*.

AG2BAM: 5′-CGGGATCCCTCGAATCAGCCAATCAC-3′
AG2XHO: 5′-CCGCTCGAGGTAATGTAGTCAAATCCAG-3′

Constructs vigsAG1 and vigsAG2 contain 255 nt and 239 nt of 3′-UTR sequences specific to *PapsAG-1* and *PapsAG-2*; vigsAG-D contains 313 nt and 297 nt of the *PapsAG-1* and *PapsAG-2* 3′ sequences concatenated in the TRV2 vector. At least 100 young seedlings were infiltrated with each of the three constructs and with an empty vector construct (containing no insert) as previously described (Hileman *et al.*, 2005; Drea *et al.*, 2007) and flowers analysed for phenotypes and gene silencing using semi-quantitative RT-PCR.

Scanning electron microscopy

Plant material was fixed overnight in FAA (3·7 % formaldehyde, 5 % acetic acid, 50 % ethanol) and then transferred to 70 % ethanol. Samples were dehydrated in an ethanol series (80 %, 90 %, 100 % ethanol) before critical-point drying in a Bal-Tec 030 critical-point drier. Samples were coated in gold using a Polaron SC7640 sputter coater and analysed on a Hitachi S3000H scanning electron microscope equipped with digital image capture.

RESULTS

Isolation and analysis of AG *orthologues in* Papaver somniferum

Degenerate RT-PCR with QVT primers (Hileman *et al.*, 2006) was used to isolate *AG* orthologues from *Papaver somniferum* flowers and developing capsules. Products were cloned and sequenced and two distinct classes of cDNAs were identified, designated *PapsAG-1* and *PapsAG-2* (Fig. 1). These cDNAs were identical in sequence to nucleotide position 599 and then sequence diverged completely. In the predicted protein products this corresponded to complete identity as far as the stop codon of *PapsAG-1* with *PapsAG-2* encoding a further 24 amino acids beyond this point (Fig. 1B).

Alignment of the predicted products of *PapsAG-1* and *PapsAG-2* with C and D class MADS box proteins from core eudicots, basal eudicots and monocots (Fig. S1 in Supplementary Data, available online) confirmed that *PapsAG* is an *AG* orthologue. PapsAG lacks characteristic D-lineage synapomorphies (Dreni *et al.*, 2007) such as Q105 and H113 which are diagnostic for D-lineage MADS box proteins as compared with C-lineage sequences. In addition, both *PapsAG-1* and *PapsAG-2* encode the AG sequence motifs I and II (Fig. 1B), which are highly conserved in C class proteins (Kramer *et al.*, 2004). Another feature of C-lineage *AG*-like genes is the presence of an extra intron at the C-terminus corresponding to a splice site at the extreme 3′ end of the genes (Kramer *et al.*,

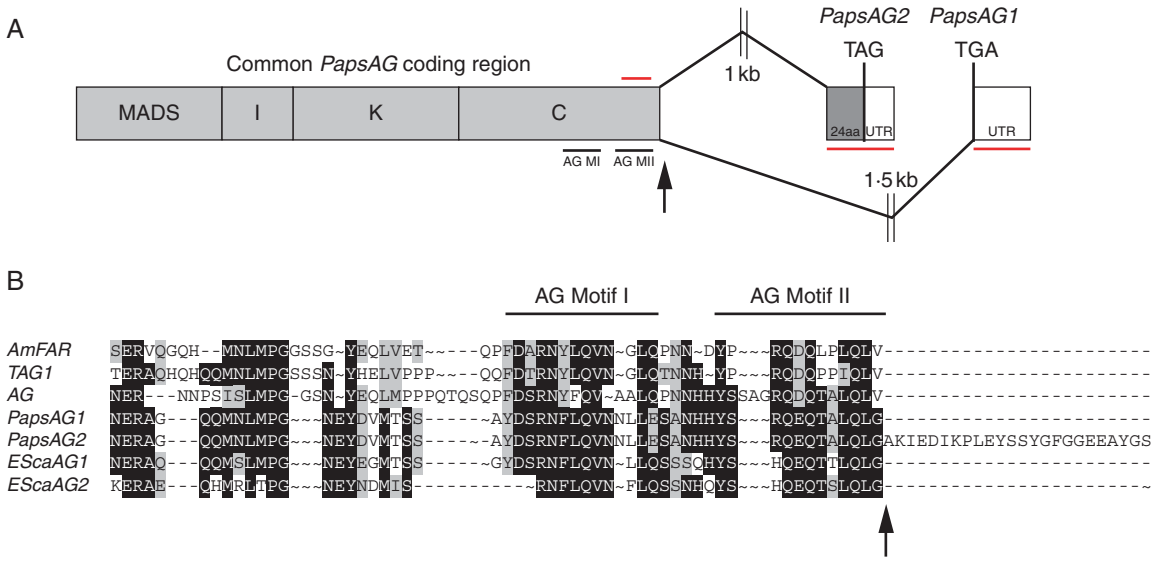

FIG. 1. Gene structure of the *Papaver somniferum AG* gene. (A) Schematic showing how alternative splicing at the 3′ end of the coding sequence generates two transcripts, *PapsAG-1* and *PapsAG-2* (not drawn to scale). Regions underlined in red are gene-specific fragments used for gene-silencing with VIGS and for *in situ* mRNA hybridization probes. The region overlined with a red bar indicates the presence of a forward primer outside of the region used in vigs lines for expression analysis. Black arrows indicate the splicing donor site. AG MI, AG Motif I; AG MII, AG Motif II. (B) Alignment of the C-terminal regions of translated PapsAG-1 and PapsAG-2 sequences with AG orthologues from tomato (TAG; Q40168), *Antirrhinum majus* (AmFAR; CAB42988), *Arabidopsis thaliana* (AtAG; P17839) and *Eschscholzia californica* (EScaAG1 and EScaAG2; AAZ53205 and AAZ53206). PapsAG-2 has a 24-amino-acid extension. The conserved AG motifs are indicated.

2004); further investigations were performed to determine if the *PapsAG-1* and *PapsAG-2* cDNAs were the result of alternative splicing at this site. Using a common forward primer and reverse primers specific for each of the putative 3′-UTRs, the corresponding genomic regions from *Papaver somniferum* DNA preparations were isolated. Analysis of the resulting sequences revealed that *PapsAG-1* follows the splicing pattern typical of *AG* orthologues and splices to a point approx. 1·5 kb downstream where it encounters the stop codon. *PapsAG-2*, on the other hand, proceeds to an alternative splice site approx. 1 kb downstream of the donor splice site and incorporates an extra 24-amino-acid coding region in the process (Fig. 1A, B). Examination of the corresponding genomic sequence showed that there were sequence motifs for donor and acceptor splice sites that matched consensus splice sites in plants (Fig. S2 in Supplementary Data; Brown and Simpson, 1998). Furthermore, the two proposed acceptor sites for *PapsAG-1* and *PapsAG-2* were the only two predicted splice acceptor sites (with confidence levels of 100 % and 94 %, respectively) using the NetPlantGene prediction tool (Hebsgaard *et al.*, 1996).

Alternative splicing affecting *AG* family members has been reported previously (Kitahara and Matsumoto, 2000; Lightfoot *et al.*, 2008), including a report in crocus where it affects the C-terminal region specifically (Tsaftaris *et al.*, 2005), but any functional consequences have not been assessed in these cases. Protein structure prediction using PredictProtein (Rost *et al.*, 2004) suggests that the 24-amino-acid extension encoded by *PapsAG-2* maintains a structural profile similar to that of the unextended version and is most likely involved in protein–protein interactions. In turn, the modifications of the *PapsAG-2* C-terminal domain protein sequence would suggest that it might form qualitatively distinct higher-order protein complexes as compared with *PapsAG-1*.

Expression analysis of PapsAG-1 *and* PapsAG-2

To examine the expression patterns of the two *PapsAG* transcripts semi-quantitative RT-PCR and *in situ* hybridizations were employed. Sepals, petals, stamens and carpels were dissected from stage P6 and P7 flowers (stages according to Drea *et al.*, 2007) and, using RT-PCR, both *PapsAG-1* and *PapsAG-2* showed expression in all whorls (Fig. 2B). Both genes were expressed more strongly in the two innermost whorls, as expected for *AG* orthologues. A more detailed analysis of expression patterns within the flower was conducted using *in situ* hybridization on flowers at stages P1, P3, P5 and P7 using probes derived from 3′-UTR sequences and so specific for each transcript. The expression patterns for both transcripts were identical with the possible exception of a somewhat broader expression domain of *PapsAG-2* in the petal (Fig. 2A, arrow). Expression of both transcripts was detected very early in the young floral meristem (P1) and through early to mid-stages of flower development was detectable in stamens and carpels (Fig. 2A, P3, P5 and P7). Later in flower development the stamen expression was restricted to the anthers (Fig. 2A, P7) as has been observed for *Arabidopsis AG* (Ito *et al.*, 2004). The expression in stamens and carpels and *PapsAG-2* expression in petals are maintained at stage P5 and both transcripts are detected in septae periphery and in primordial ovule at stages P6 and P7 (Fig. S3 in Supplementary Data). Control experiments using corresponding sense probes and a histone H4 antisense probe were performed (Fig. S4 in Supplementary Data).

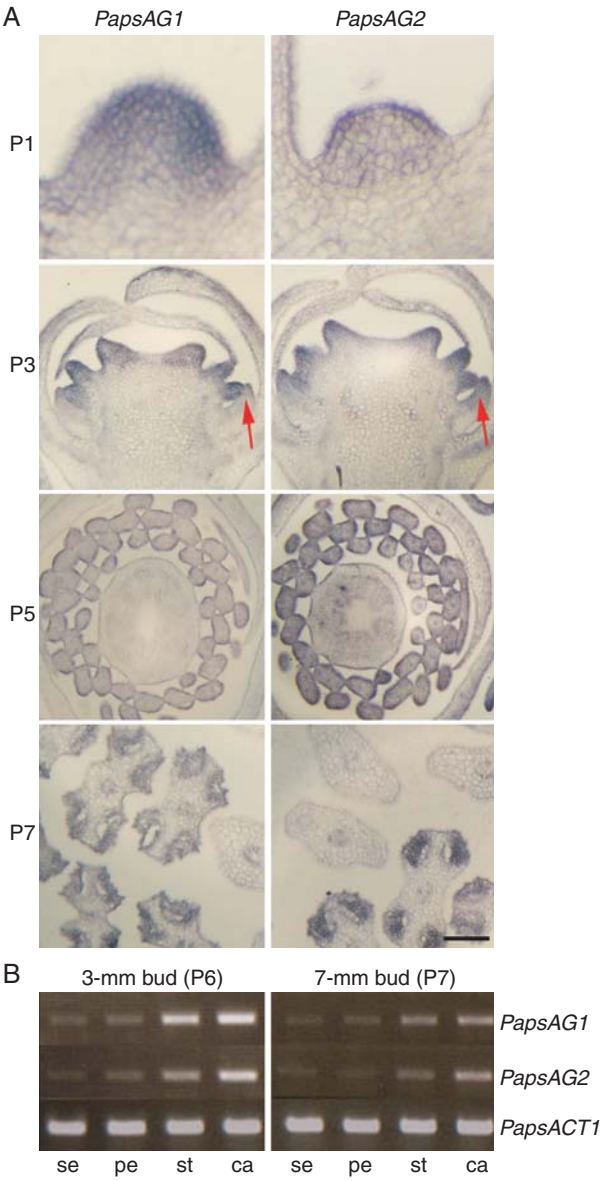

FIG. 2. Expression analyses of *PapsAG-1* and *PapsAG-2*. (A) *In situ* hybridization of *PapsAG-1* and *PapsAG-2* transcript-specific probes on young *P. somniferum* flowers showing similar expression patterns. P1 stage, Young meristem in longitudinal section before visible organ primordia appear; P3 stage, longitudinal section of developing flower bud; P5 stage, cross-section of older flower bud; P7 stage, cross-section through developing anthers with adjacent filament sections. The stages are as in Drea *et al.* (2007). Scale bar = 200 μm. (B) RT-PCR with *PapsAG-1* and *PapsAG-2* transcript-specific primers using cDNA from dissected floral organs (sepal, se; petal, pe; stamen, st; carpel, ca) of older bud stages (P6, 3-mm buds; P7, 7-mm buds). Amplification of the *P. somniferum ACTIN* gene, *PapsACT1*, was used as a control.

Functional dissection of AG *orthologues in* Papaver somniferum

Both *PapsAG-1* and *PapsAG-2* are expressed at high and comparable levels in the poppy flower, indicating that they

are potentially functional and do not represent pseudogenes. Given the considerable sequence similarity and nearly identical expression patterns of the *PapsAG-1* and *PapsAG-2* transcripts, it was essential to ascertain whether the encoded functions were similar or distinct. To investigate the functions of both transcripts' products, VIGS constructs were generated to silence each transcript individually and both transcripts simultaneously. At least 100 young seedlings were infiltrated with each of the three constructs as previously described (Hileman *et al.*, 2005; Drea *et al.*, 2007) and flowers analysed for phenotypes and gene silencing using semi-quantitative RT-PCR. Table 1 summarizes the phenotype frequencies and type for each of the three constructs. In the cases of vigsAG1 and vigsAG-D at least 50 % of the infiltrated plants produced abnormal flowers, whereas only approx. 12 % of vigsAG2 flowers were identified as having defects. This could be due to the possibility of both the more subtle functional roles of the *PapsAG-2* transcript and the increased difficulty in recognizing these more subtle defects in the flowers. Flowers were examined at two main stages of development: when the flower is pendant prior to anthesis just before its upright extension and flower opening, corresponding to stage P8 (Fig. 3), and at anthesis (Fig. 4). RNA was extracted from the inner two whorls of the flowers examined and RT-PCR was performed to test for presence of the TRV2 construct containing the correct sized insert and to test for down-regulation of the *PapsAG-1* and *PapsAG-2* transcripts (Fig. 6). Control experiments with TRV2 containing no insert (TRV2-E) were also carried out but no defects were observed (Fig. S5 in Supplementary Data).

PapsAG-1 *loss of function affects stamen and carpel whorls*

In wild-type *P. somniferum* flowers, the globular ovary is crowned by a radial arrangement of fused stigmatic rays containing papillae-lined pores for the capture of the copious pollen grains and conduction of pollen tubes to the ovules within (Fig. 4A, B). The multicarpellate gynoecium is attached to the pedicel by a thin gynophore, and lacks an obvious style. The gynoecium is paracarpous, in that the margins of the fused carpels form projections called septae rather than meeting at the centre of the ovary (Bernáth, 1998). The surrounding stamens are numerous and hypogynous. In contrast to the unfixed number of stamens and carpels, the perianth consists of two (or sometimes three) sepals and four petals. The flowers progress through a series of well-characterized stages (Drea *et al.*, 2007), and by stage 8, when the flower bud is pendant, all floral organs are distinct (Fig. 3A).

Flowers of infiltrated plants were opened manually and visually examined for any obvious defects at stage 8 and in open flowers. vigsAG1 flowers displayed a consistent phenotype consisting of partially transformed stamens – anthers appeared petalloid, more obviously at the extreme distal end, an open stigma where the rays have not converged centrally, and extended gynophores (Fig. 3B, C). Though the capsule is recognizable in general morphology, inside the developing capsule ectopic petals and/or sepals were observed in many cases (Fig. 3C). The effects on gynophore development were more apparent in mature flowers, in which the gynophore was quite elongated and resembled an extended pedicel (Fig. 4F). Examination of mature flowers also revealed the extent of the defects in ovule development where both aborted and unfertilized ovules were observed, particularly in the distal region of the capsule (Fig. 4D). Stigma defects produced either over-papillated or more naked surfaces (Fig. 4C, E). Overall it appeared that *PapsAG-1* affects both the stamen and carpel whorl identity and differentiation. The presence of ectopic outer-whorl tissues within the carpel of some vigsAG1 plants also suggests that the gene plays a role in determinacy.

PapsAG-2 *loss of function affects capsule development*

The effects of *PapsAG-2* VIGS were more difficult to identify presumably due to more subtle effects on flower development. Stage 8 flower buds were overtly normal in appearance and it was only on close examination of the developing capsules that defects in stigma arrangement and overall capsule shape were observed (Fig. 3E, F). Stamens appeared to be normal in all of these plants. In a subset of the defective flowers identified, the entire capsule was curved (Fig. 3E), which was reminiscent of the pedicel curvature observed in pendant opium poppy flowers at stage 8. The gynophores of these flowers, however, appeared to be normal in all cases. In mature flowers the stigma defects were more obvious. The radial arrangement of the

TABLE 1. *Summary of phenotypes identified using VIGS to silence* PapsAG1 *and* PapsAG2 *genes individually and both genes simultaneously*

	Phenotype			
vigs line	Whorl 3	Whorl 4	Number	Total
vigsAG1	Anthers partially transformed	Carpel (stigma and ovules) deformed, gynophores extended	51	51
vigsAG2	Normal	Stigma and ovule defects	12	
	Normal	Pedicel-like bend in carpel	4	16
vigsAG-D	Petalloid	Carpel petalloid or sepalloid with recurring flower inside	37	
	Petalloid	Severely sepalloid and hollow; some contain rudimentary ovules	23	
	Some petalloidy – anthers partially transformed	Carpels deformed	5	
	Morphologically normal but some partially green	Carpels deformed	4	69

Approximately 100 seedlings for each construct were infiltrated and screened for VIGS-induced phenotypes.

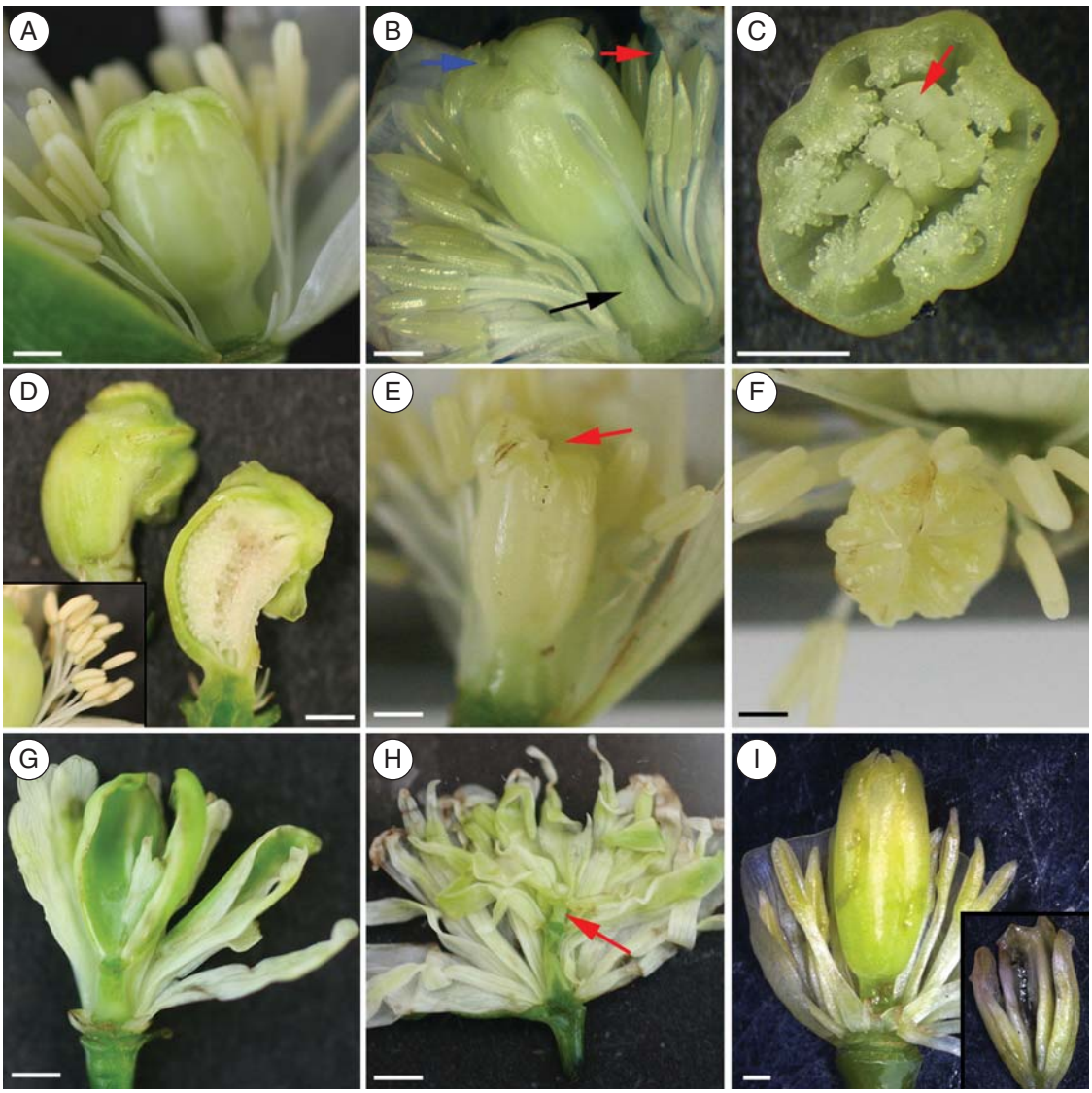

FIG. 3. Phenotypes of vigsAG1,vigsAG2 and vigAG-D plants at pendant flower stage: (A) wild-type poppy flower; (B) vigsAG1 flower showing extended gynophore (black arrow), partially transformed anthers (red arrow) and open stigma (blue arrow); (C) vigsAG1 capsule cut in transverse showing sepals developing inside (arrow); (D) vigsAG2 curved capsule (inset shows normal stamens from a wild-type flower); (E, F) vigsAG2 flower showing defective stigma (red arrow); (G, I) vigsAG-D flowers showing petaloid stamens and sepaloid carpel with petals inside (G), petaloid carpel with another flower inside with extra pedicel indicated (red arrow, H) and elongated sepaloid carpel which was completely empty inside (inset, I). Scale bars = 2 mm.

stigmatic rays, as observed in wild-type plants (Fig. 4A), was distorted (Fig. 4G) and when the capsule was opened it was seen that there were corresponding defects in ovule development (Fig. 4H) – ovules were very white and enlarged or underdeveloped. The stigmas of vigsAG2 plants were examined more closely using scanning electron microscopy. As was the case for vigsAG1, stigmas were occasionally overpapillate or contained naked surfaces (Fig. 4I, J). Papillae sometimes developed along the centre of the rays where no pores were forming and occasionally an ovule was found developing amongst the papillae (Fig. 4J). In other cases, the pores were reversed in orientation with the turn directed toward the centre of the radial surface, rather than around the stigma boundaries (Fig. 4I). Overall the effect of *PapsAG-2* was restricted to the innermost whorl and could be interpreted as being the result of a mild determinacy loss within the carpel.

Both PapsAG *transcripts act redundantly to confer floral determinacy and organ identity*

Silencing of both *PapsAG* transcripts simultaneously generated flowers with a dramatic phenotype involving a complete loss of stamen and carpel identity and a high incidence of loss of determinacy (Fig. 3G–I). vigsAG-D flowers consisted of stamens that were considerably or completely transformed into petals (Fig. 4A). Carpels were affected in three main ways: (1) individual carpels could be transformed into a sepal that enclosed developing petals (Fig. 3G), or (2) they could surround an empty interior (Fig. 3I) or (3) they could

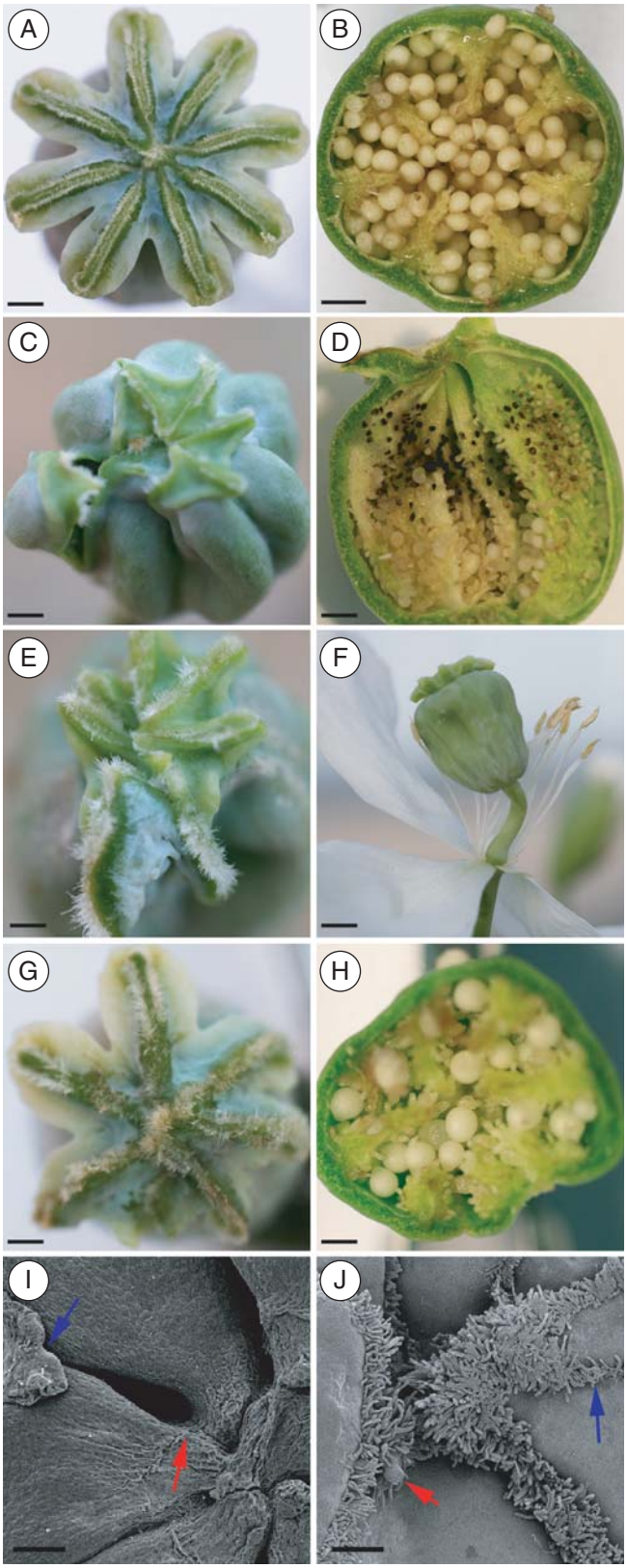

FIG. 4. Distinct effects of *PapsAG-1* and *PapsAG-2* gene silencing in capsule development: (A) wild-type stigma; (B) wild-type capsule cut open in transverse orientation showing ovules and septae; (C, E) vigsAG1 deformed stigmas; (D) vigsAG1 capsule cut in longitudinal orientation showing defective ovule development particularly at the distal end; (F) vigsAG1 open flower showing extended gynophores and modified anthers; (G, H) vigsAG2 stigma and open capsule cut in transverse orientation showing the deformed stigma and corresponding ovule defects within the capsule; (I, J) scanning electron micrographs of vigsAG2 stigmas, showing a retracted stigmatic ray (blue arrow) and a stigmatic pore in reverse orientation, i.e. it is directed towards the centre of the stigma (red arrow, I); disorganized stigma with ectopic papillae (blue arrow) and ovule (red arrow, J). Scale bars: (A–E, G, H) = 2 mm; (F) = 5 mm; (I) = 200 μm; (J) = 100 μm.

be transformed into petals (Fig. 3H). The identity of the transformation to petals or sepals was assigned based on visual identification (colour and form) and by scanning electron microscopy on the transformed tissues.

An internal examination of transformed carpels (region boxed in Fig. 5A) using scanning electron microscopy showed a completely smooth adaxial wall (Fig. 5B). Where internal structures were observed within these carpels, they consisted of some rudimentary ovule development on the adaxial surface where the integuments are only barely discernable (Fig. 5C) and/or short papillae at the distal end (Fig. 5D). These observations indicate that both *PapsAG-1* and *PapsAG-2* have a role in the specification of floral determinacy, as well as shared roles in determining aspects of floral organ identity. Of the vigsAG-D lines, 13 % displayed a weaker phenotype (Table 1) where there was a more partial transformation of stamens, recognizable carpels with some defects and a normal (unextended) gynophore (Fig. 5E–H). In these cases the carpels produced a smaller stigmatic ray area (Fig. 5E, F) or were misshapen (Fig. 5G, H). The existence of such phenotypes suggests that stamen and carpel identity can be uncoupled from the determinacy function.

DISCUSSION

PapsAG-1 *and* PapsAG-2 *are derived from an alternative splicing event*

Sequence and phylogenetic analyses of MADS box genes have shown the prevalence of ancient and recent gene duplications in establishing the repertoire of these genes in a number of extant angiosperms (Mena *et al.*, 1996; Kramer *et al.*, 2004; Yamaguchi *et al.*, 2006; Zahn *et al.*, 2006). Gene duplication can have various functional consequences such as neofunctionalization, subfunctionalization or pseudogenization (Drea *et al.*, 2006, 2007; Yamaguchi *et al.*, 2006; Dreni *et al.*, 2007) but a single gene can also potentially generate multiple forms through the production of alternative transcripts from the same locus.

Genomic analyses in rice and *Arabidopsis* suggest that 20 % of genes are alternatively spliced with intron retention being the most common consequence (Wang and Brendel 2006). It is not unusual in genes encoding proteins with modular structure where exon gain or loss can result in the acquisition or relinquishing of discrete functional modules such as the acquisition of target domains in organelle-localized gene products (Long *et al.*, 1996) or potential neofunctionalization through sequence modification in transmembrane regions (Drea *et al.*, 2006). The *FCA* gene, encoding an RNA-binding protein required for flowering, produces multiple transcript forms (Macknight *et al.*, 2002). These vary in

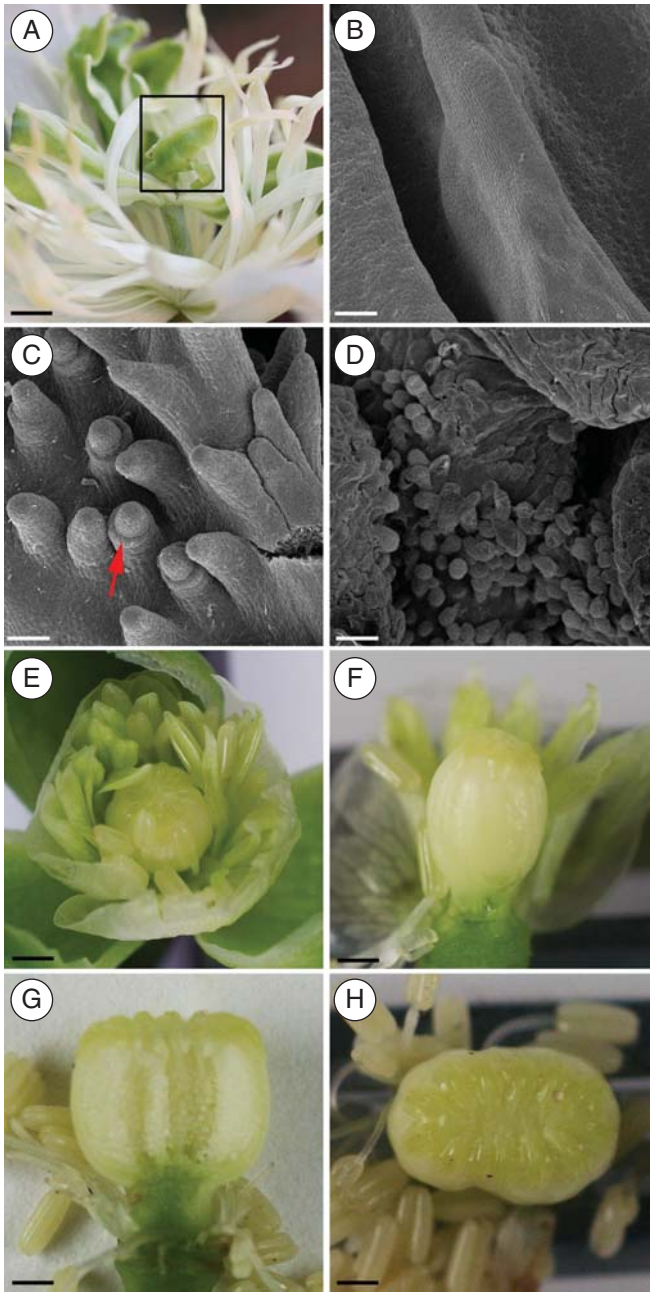

FIG. 5. Phenotypes of vigsAG-D plants: (A) strongly transformed vigsAG-D flower with no distinguishable stamens and rudimentary gynoecium (boxed); (B–D) scanning electron micrographs of adaxial transformed carpels (boxed in A) showing the lack of any ovule initiation (B), in some lines there are rudimentary ovules without developing integuments (red arrow, C) and some stigmatic papillae at the distal end (D). (E–H) Phenotypes of weaker vigsAG-D lines showing partially petaloid (E, F) or generally normal stamens (G, H) and recognizable carpels that show a reduced stigmatic ray area (E, F) or resemble fused carpels (G, H). Scale bars: (B–D) = 50 μm; (A, E–H) = 2 mm.

both spatial and temporal expression of the transcripts and in the abundance of the resulting proteins with subsequent effects on the timing of flowering responses. Alternative splicing and RNA processing within the MADS-box genes in general and the C-class genes in particular has been reported (Kitahara and Matsumoto 2000; Cheng *et al.*, 2003; Lee *et al.*, 2005; Lightfoot *et al.*, 2008) but the functional significance of these events has not yet been elucidated. Where alternative splicing of C-class genes was reported, the splicing site was not the extreme C-terminal splice junction (intron 8) except for the report in *Crocus* (Tsaftaris *et al.*, 2005), though the number and position of introns are generally conserved in orthologues from various species (Kramer *et al.*, 2004).

The two isoforms of the encoded *PapsAG* products differ only in the length of the C-terminus region. Divergence in the C-terminal domains encoded by various MADS-box genes has been deemed to be an important determinant of whether a gene duplicate is retained (Janssens *et al.*, 2008) and can affect the nature of the interactions with other MADS-box proteins in higher-order complexes (Geuten *et al.*, 2006). Since the analyses performed here were unable to discern any major differences in the expression patterns of the *PapsAG-1* and *PapsAG-2* transcripts, it is suspected that their distinct functions are likely to be due to differences in their protein–protein interactions or in transcriptional activation potential.

Conservation and diversification in AG *function*

This study shows that the *Papaver somniferum AG* orthologue is required to specify stamen and carpel identity as well as floral determinacy, similar to the role of *Arabidopsis AG*. However, loss of *AG* function in *Arabidopsis* results in homeotic conversions of stamens into petals and carpels into sepals (Bowman *et al.*, 1991). This is in contrast to the loss of *PapsAG* function in vigsAG-D plants, in which homeotic conversions of carpels to petals were observed in addition to other defects. This phenotype is more similar to the complete loss of *AG*-like gene function in *Antirrhinum plena farinelli* double mutants, which display homeotic conversions of both stamens and carpels into petaloid tissue (Davies *et al.*, 1999). This has been postulated to be due to misregulation of B-class MADS box genes in antirrhinum through disruption of *PLENA* and *FAR* interactions with a third whorl specific factor (Davies *et al.*, 1999). In *P. somniferum*, such a postulated third whorl factor would presumably differentially interact with the duplicated B class MADS box genes which each possess distinct functions (Drea *et al.*, 2007).

In *Arabidopsis*, the role of *AG* in floral determinacy has been associated with a higher level of *AG* activity as compared with that required for organ identity specification (Mizukami and Ma, 1995). Furthermore, analyses of partial loss-of-function *AG* alleles in *Arabidopsis* has provided support for the idea that the C-terminus of the K domain is required for distinct third and fourth whorl functions as well as correlating determinacy with increased gene function (Sieburth *et al.*, 1995). In other species, these two *AG* roles appear to have been subfunctionalized; for instance, in rice the *AG* orthologues *OsMADS3* and *OsMADS58* have distinct roles, with *OsMADS3* being required predominantly for stamen identity specification and *OsMADS58* being the main player in conferring floral determinacy (Yamaguchi *et al.*, 2006).

This study shows that *AG* activities in opium poppy are uncoupled through having a single gene encode two alternative transcripts encoding distinct proteins with different lengths of C-terminal domains. *PapsAG-1* mediates organ identity and

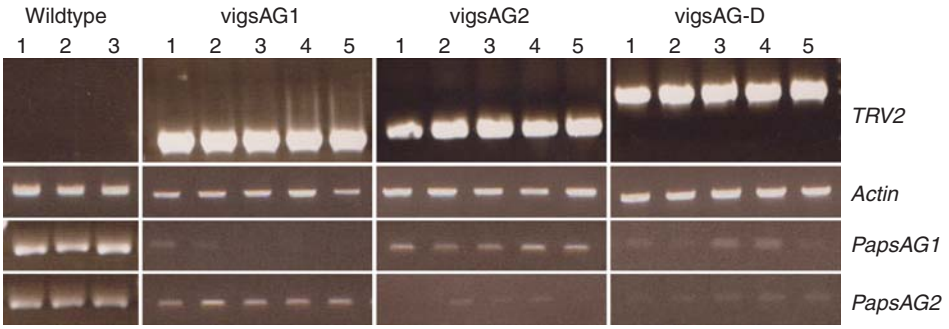

FIG. 6. RT-PCR of vigs lines. RT-PCR on RNA/cDNA extracted from the two innermost whorls (stamen and carpels) of pendant flowers from vigs lines for five representative plants transformed with each construct to test for the presence of the TRV construct and for reduced expression of the *PapsAG* transcripts. Three individual wild-type plants with tissue from the same stage (pendant) were included for comparison.

determinacy functions in both the stamen and the carpel whorls, whereas *PapsAG-2* function appears to be largely restricted to the carpel. This parsing of functions into two transcripts is distinct from gene duplication and subfunctionalization, and may reflect a lineage specific mechanism to encode distinct protein functions. A similar observation has been made for the multiple splice forms that are unique to arthropods and that have not been observed in vertebrate systems; nonetheless, the overall developmental function of these receptor isoforms is conserved (Schmucker and Chen, 2009). Though the *PapsAG* transcripts show some functional redundancy, the unique functions encoded by each transcript would account for the retention of both, in a manner similar to that postulated for the retention of duplicated, subfunctionalized genes (Force *et al.*, 1999; Moore and Purugganan, 2003; Moore *et al.*, 2005). As such, the production of alternative transcripts may provide another means to diversify gene function, and may reflect fine tuning of the types of multiprotein complexes that can be formed to mediate different developmental functions.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: amino acid alignment of AG orthologues from taxa across the angiosperms, with accompanying table giving sequence name and accession numbers of protein sequences used in alignment. Figure S2: detailed view of the 3′ end of the *PapsAG* transcripts aligned with corresponding genomic sequence. Figure S3: mRNA ISH of *PapsAG-1* and *PapsAG-2* at stages P5, P6 and P7. Figure S4: mRNA ISH with *PapsH4-1* and with sense probes for *PapsAG-1* and *PapsAG-2*. Figure S5: empty TRV2 vector control results at pendant and mature flower stages.

ACKNOWLEDGEMENTS

P.H. is funded by a BBSRC studentship and S.D. by the University of Leicester and a Royal Society Research Grant.

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