New Phytologist

The MIK region rather than the C-terminal domain of AP3-like class B floral homeotic proteins determines functional specificity in the development and evolution of petals

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Received: *15 November 2007*
Accepted: *3 January 2008*

Summary

• In core eudicots, euAP3-type MADS-box genes encode a PISTILLATA (PI)-derived motif, as well as a C-terminal euAP3 motif that originated from a paleoAP3 motif of an ancestral APETALA3 (AP3)-like protein through a translational frameshift mutation. To determine the functional and evolutionary relevance of these motifs, a series of point mutation and domain-swap constructs were generated, involving *CsAP3*, a paleo*AP3*-type gene from the basal angiosperm *Chloranthus spicatus* encoding a truncated paleoAP3 motif, and *AtAP3*, a eu*AP3*-type gene from the core eudicot *Arabidopsis thaliana*.
• The chimeric constructs were expressed in *A. thaliana* under the control of the *AP3* promoter or the CaMV 35S promoter in an *ap3* mutant or wild-type background, respectively.
• Significant recovery of *AP3* function was obtained in both complementation and ectopic expression experiments whenever the region upstream of the C-terminal motifs (MIK region) from *A. thaliana* was taken, even when the PI-derived motif and the truncated paleoAP3 motif of CsAP3 substituted for the corresponding sequences from AtAP3. However, no or very weak complementation or gain-of-function was seen when the MIK region was from CsAP3.
• Our data suggest that changes in the MIK region rather than mutations in the C-terminal domain were of crucial importance for the evolution of the functional specificity of euAP3-type proteins in stamen and petal development.

Key words: *APETALA3*, basal angiosperm, *Chloranthus spicatus*, *CsAP3*, euAP3 motif, paleoAP3 motif, perianth evolution.

New Phytologist (2008) **178**: 544–558

doi: 10.1111/j.1469-8137.2008.02382.x

Introduction

Studies on MADS-box genes involved in flower development have provided greater insights into the evolution of flowers (Theissen *et al.*, 2000; Ng & Yanofsky, 2001; Soltis *et al.*, 2007; Theissen & Melzer, 2007). A simple conceptual framework explaining the development of floral organ identity is the widely known ABC model (Coen & Meyerowitz, 1991). According to this model, class B floral organ identity (or homeotic) genes are required for the development of petals (together with class A genes) and stamens (together with class

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C genes) (Coen & Meyerowitz, 1991). In the eudicotyledonous model plant *Arabidopsis thaliana*, the class B genes are represented by *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), which are not only necessary, but within a floral context also sufficient for specifying petal and stamen identity (Jack *et al.*, 1992; Goto & Meyerowitz, 1994; Krizek & Meyerowitz, 1996).

AP3-like and *PI*-like genes have already been identified in almost every group of flowering plants, including basal angiosperms (Theissen *et al.*, 1996, 2000; Riechmann & Meyerowitz, 1997; Kramer *et al.*, 1998; Alvarez-Buylla *et al.*, 2000; Berbel *et al.*, 2001; Becker & Theissen, 2003; Kim *et al.*, 2004). They encode MIKC-type transcription factors containing an N-terminal MADS domain (the 'M' in MIKC) involved in DNA binding and protein dimerization; an I domain ('I'); a K domain ('K') that is also involved in protein–protein interactions, possibly through the formation of coiled coils; and a C-terminal domain ('C') involved in higher order complex formation, at least in the case of some MIKC-type proteins (Kaufmann *et al.*, 2005).

It has been demonstrated that in core eudicots the expression of *AP3*-like and *PI*-like genes in petal and stamen primordia is in good agreement with a conserved function of these genes in specifying petal and stamen identity (Bowman *et al.*, 1989; Sommer *et al.*, 1990; Jack *et al.*, 1992; Trobner *et al.*, 1992; Vanderkrol *et al.*, 1993; Goto & Meyerowitz, 1994). However, more variable expression patterns in noncore eudicots suggest that the function of *AP3*-like and *PI*-like genes may not be strictly conserved throughout the angiosperms (Kramer & Irish, 2000; Kanno *et al.*, 2003; Park *et al.*, 2004; Kim *et al.*, 2005; Whipple *et al.*, 2007). This may apply especially to the class B gene function in specifying petal identity (Lamb & Irish, 2003; Kramer & Zimmer, 2006), possibly because petals originated several times independently (either from bracts or from stamens) in diverse angiosperm lineages (Eames, 1961; Bierhorst, 1971; Takhtajan, 1991; De Craene, 2007).

Although core eudicots comprise approx. 60% of all angiosperm species, the organization of flowers is fairly constant throughout this clade, with floral organs typically arranged in distinct whorls (Endress, 1994; De Craene *et al.*, 2003). By contrast, noneudicotyledonous angiosperms (including basal angiosperms and monocots) comprise only 25% of angiosperm species, but display enormous floral diversity characterized by variable numbers of floral organs arranged in whorls or spirals, and a lack of differentiation into calyx and corolla (Endress, 1994; De Craene *et al.*, 2003). Therefore, it might be revealing to study the floral organ identity genes from noneudicots, and especially basal angiosperms, to determine when and how class ABC gene functions have been established during the evolutionary history of the angiosperms (Alvarez-Buylla *et al.*, 2000; Theissen *et al.*, 2000; Soltis *et al.*, 2007).

Phylogenetic studies have shown that two major duplication events took place within the *AP3*/*PI*-like genes. The first event probably occurred in a common ancestor of all extant angiosperms after the divergence from gymnosperms *c.* 200–300 Myr ago, and led to distinct *AP3* and *PI* gene lineages (Purugganan, 1997; Kramer *et al.*, 1998; Theissen *et al.*, 2000; Aoki *et al.*, 2004; Kim *et al.*, 2004). The basal paleo*AP3* lineage generated in that way underwent another major duplication event close to the base of core eudicots. Thus two classes of *AP3*-like genes originated, termed eu*AP3*-type genes and *TOMATO MADS BOX GENE 6* (*TM6*) lineage genes (Kramer *et al.*, 1998).

Despite their generally quite high sequence similarity, the different lineages of *AP3*- and *PI*-like genes are characterized by diagnostic sequence elements found in the C-terminal region of the encoded proteins (Kramer *et al.*, 1998). *PI*-like genes encode a short hydrophobic region known as the PI motif. This motif evolved into a PI-derived motif in the *AP3* lineage after the split from the *PI* lineage. Paleo*AP3* genes have an additional motif downstream of the PI-derived motif, named the paleoAP3 motif, which is an ancestral feature because it is already present in some AP3/PI-like proteins of gymnosperms (Winter *et al.*, 2002a). Within the core eudicots, the *TM6*-like genes have retained the paleoAP3 motif, while a translational frameshift mutation transformed this motif into the new euAP3 motif in the eu*AP3* lineage (Vandenbussche *et al.*, 2003; Kramer *et al.*, 2006).

According to the evolutionary history of *AP3*-like genes described above, almost all angiosperms contain at least one gene encoding a paleoAP3 motif (paleo*AP3*-type gene or *TM6*-like gene). A remarkable exception is the lineage that led to *A. thaliana*, in which the *TM6*-like gene was lost (Lamb & Irish, 2003). In addition, the core eudicot species examined so far have at least one eu*AP3*-type gene. While the class B floral homeotic genes examined so far are all of the eu*AP3*-type, the *TM6*-like genes that have been examined (in petunia (*Petunia hybrida*) and tomato (*Lycopersicon esculentum*)) have functions restricted to stamen development (de Martino *et al.*, 2006; Rijpkema *et al.*, 2006). This function in specifying male organ identity probably reflects the ancestral role of *AP3*/*PI*-like genes already established in gymnosperms (Winter *et al.*, 1999).

The different functions of eu*AP3*-type (class B) and *TM6*-like genes in core eudicots are based to a considerable extent on different expression patterns (de Martino *et al.*, 2006; Rijpkema *et al.*, 2006). Whether the C-terminal motifs (the PI-derived motif, and the euAP3 and paleoAP3 motifs) from the different lineages are functionally equivalent is a matter of controversy. Lamb & Irish (2003) showed that a chimeric protein, in which the PI-derived motif and paleoAP3 motif of the paleo*AP3*-type gene *DeAP3* from the basal eudicot *Dicentra eximia* replaced the PI-derived motif and euAP3 motif of *A. thaliana AP3*, was unable to fully rescue *ap3-3* mutants. The ability of the chimeric construct to replace *AP3* was moderate in the third whorl of the flower, while the second (petal) whorl was not rescued at all, suggesting that the PI-derived motif and paleoAP3 motif have a stamen-promoting activity but do not support petal development in *A. thaliana* (Lamb & Irish, 2003). It was speculated that the acquisition of the euAP3 motif may have played a role in the origin of petals in

(a)

		PI-derived motif	paleoAP3 motif
Chloranthus spicatus	CsAP3	GGGHVFAFRL-QPI---QPN----LH–DNG---YGTH-----	
Asparagus officinalis	AspoDEF	GGSHVYAFRV-QPS---QPN----LH–GMG---CGPHDLRLA	
Lilium longiflorum	LillMADS1	GASHLYEFRV-QPS---QPN----LH–GMG---YGSHDLRLA	
Michelia figo	MicfAP3	GGA----------HILH–DTG---FGIHDLRLA	
Magnolia praecocissim	MagpMADS7	GGV----------HSLH–DTG---FGIHDLRLA	
Nymphaea sp.	NymsAP3	SDAQPCPVRV-QSSH---PN----LH–ERG---YGCHDLILG	
Oryza sativa	OsMADS16	AAADMFAFRV-VPS---QPN----LH-GMAY--GGNHDLRLG	
Triticum aestivum	TriaMADS51	LAADMYAFRV-VPS---QPN----LH-GMAY--GGSHDLRLG	
Zea mays	ZeamSilky1	APPDMYAFRV-VPS---QPN----LH-GMA---YGFHDLRLG	
Akebia quintata	AkeqAP32-2	GGSHVFAFRL-QQS---QPN----LQ-SGGE--FGMNDLRLA	
Dicentra eximia	DeAP3	GGQNIFAFRL-QPS---QPN----LH-DGGG--YGSHDLRLA	
Ranunculus ficaria	RanfAP3	NGVFSFRLR---PS---QTN----LH-NDEE--YEIHDLRLA	
Trautvetteria carolinensis	TracAP3	GVAPHIYSFRLHPS---QPN----LH-EDEE--YEIHGLRLA	
Petunia hybrida	PethTM6	GVHNLYAFRL-QTLH---PN----LQ-NGGG--FGSRDLRLA	
Nicotiana tabacum	NtTM6	GVHNLYAFRL-QTLH---PN----LQ-NGGG--FGSRDLRLA	
Hydrangea macrophylla	HydmTM6	RVPNSYAFGL-QPSH---PN----LHHGGGG--YGSHDLRLA	
Antirrhinum majus	AntmDEF	GGPRIIALRLPTNHH---PT----LHSGGG--SDLTTFALLE	
Arabidopsis thaliana	AtAP3	EGSRAYALRFHQNHHHYYPNHG--LHAPSA--SDIITFHLLE	
Petunia hybrida	PethGP	GGHRILALRLQPNHHQ--PNHHHLH--SGGGSDITTFALLE	
		PI-derived motif	euAP3 motif

(b)

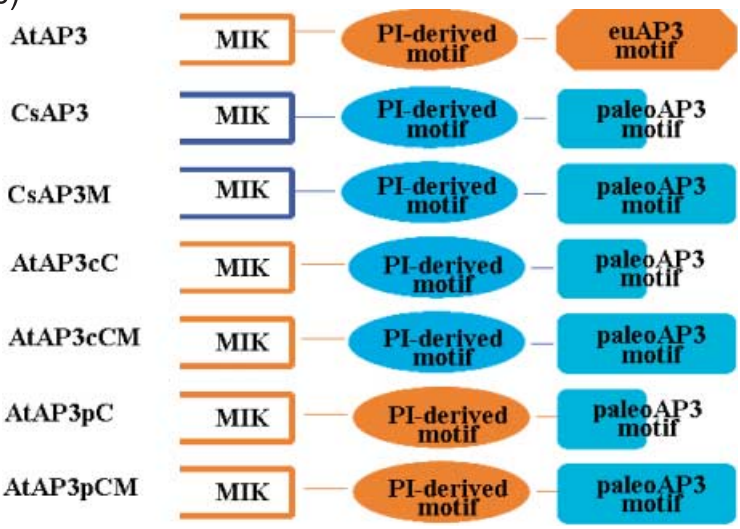

Fig. 1 (a) Amino acid sequences of the C-terminal motifs of AP3-like proteins from different species. (b) The *AP3* constructs used in transformation experiments and yeast two-hybrid analyses. The part in orange represents the region of the *Arabidopsis thaliana* AtAP3 protein; the part in blue represents the region of the *Chloranthus spicatus* CsAP3 protein.

core eudicots, an event that may have been independent of petal origin in other clades (Lamb & Irish, 2003). However, Whipple *et al.* (2004) reported that an *AP3*-like gene (*SILKY1*) from maize (*Zea mays*) encoding a paleoAP3 rather than a euAP3 motif can rescue *ap3* mutants from *A. thaliana* even with respect to petal development, suggesting that a paleoAP3 motif suffices for both petal and stamen development in *A. thaliana*.

Chloranthus spicatus (Chloranthaceae) is an evergreen subshrub with perfumed small flowers which is endemic to Southern China and belongs to the basal angiosperms *sensu lato* (Qiu *et al.*, 1999; Zanis *et al.*, 2002). It has flowers that are composed of just a stamen and a carpel (Endress, 1987; Doyle *et al.*, 2003). As there is evidence that the flowers at the base of extant angiosperms ('ancestral flowers') already had an undifferentiated perianth (reviewed by Theissen & Melzer, 2007), the simplicity of the flowers of *C. spicatus* very probably represents a state that was achieved by reduction (Li *et al.*, 2005). *Chloranthus spicatus* thus represents an interesting model with which to explore an early, drastic modification of flowers.

Previously, cloning of the *CsAP3* gene from *C. spicatus* was reported (Kramer *et al.*, 1998; Li *et al.*, 2005). As the gene is exclusively expressed in stamen primordia, any function, such as specifying organ identity, is probably restricted to stamen development. Remarkably, the conceptual CsAP3 protein has only a partial paleoAP3 motif (Fig. 1a), which is truncated because of a point mutation from guanine (G) to thymine (T) at position 658 in the cDNA sequence; this causes the change

from an otherwise highly conserved aspartic acid (D) codon (GAG) to a premature stop codon (TAG) (Kramer *et al.*, 2003; Litt & Irish, 2003; Stellari *et al.*, 2004; Li *et al.*, 2005).

Such a finding raises the intriguing question as to whether the truncation of an AP3-like protein is somehow linked to the reduction of the flowers of *C. spicatus*, specifically the absence of a perianth. It has been speculated that in Chloranthaceae a full paleoAP3 motif might only be required for petal and not for stamen development (Li *et al.*, 2005). Integrity of the motif might thus have become dispensable after the loss of the perianth within the Chloranthaceae, and hence there might have been no selection against the premature stop codon.

To determine the functional capacity of *CsAP3* we have now expressed different versions of it in *A. thaliana* wild-type and *ap3* mutant plants. To determine the functional importance of the PI-derived motif and paleoAP3 motif we have swapped these domains between AP3 from *A. thaliana* (henceforth termed AtAP3) and CsAP3, and determined to what extent the chimeric proteins can substitute for AtAP3 in heterologous expression and complementation experiments. To explore the functional relevance of the truncation of the CsAP3 motif in CsAP3, our experiments included constructs in which the precocious stop codon was mutated into a sense codon to yield protein versions with full-length paleoAP3 motifs. In addition, we tested the interaction of proteins employing the yeast two-hybrid system. Our data suggest that the MIK region is more important than the C-terminal domain of AP3-like floral homeotic proteins for functional specificity in the development and evolution of petals and stamens.

Materials and Methods

Isolation of *CsAP3* and *CsPI*

cDNA of the *CsAP3* gene was cloned employing 3′ rapid amplification of cDNA ends (3′-RACE) essentially as described by Li *et al.* (2005). Similarly, a 3′ partial cDNA sequence of the *PI*-like gene of *C. spicatus* (*CsPI*) was obtained by 3′-RACE using the *PI* gene-specific primer B1 (5′-AACAGGCAGG-TSACCTAYTC-3′). A 5′ partial cDNA sequence of *CsPI* was obtained using primers P3PI1 (5′-CCCTTCARRTGCCTGA-GSTC-3′) and SPI1 (5′-CCCTTCARRTGCCTGAGSTC-3′) and the 5′-RACE kit (Invitrogen, Carlsbad, CA, USA). The full-length cDNA of *CsPI* was obtained by restriction digestion and recombination of 3′-RACE and 5′-RACE fragments as previously described (Shan *et al.*, 2006).

Plasmid construction

A series of cDNAs, including *CsAP3*, *AtAP3cC* (*AtAP3*-MIK + *CsAP3*-C), *AtAP3pC* (*AtAP3*-MIKC$_{\text{PI-derived motif}}$ + *CsAP3*-C$_{\text{paleoAP3 motif}}$), *CsAP3M*, *AtAP3cCM* and *AtAP3pCM*, representing wild-type or chimeric genes, were generated on plasmid constructs (Fig. 1b). In the constructs of *CsAP3M*, *AtAP3cCM* and *AtAP3pCM*, the stop codon TAG in the C-terminal region of *CsAP3* was modified into GAG to generate a complete paleoAP3 motif, as compared with the constructs of *CsAP3* (wild-type), *AtAP3cC* and *AtAP3pC*, which encode an incomplete paleoAP3 motif. *5D3*-driven *AtAP3* was constructed as a positive control (Lamb & Irish, 2003; Whipple *et al.*, 2004).

The cauliflower mosaic virus (CaMV) *35S* promoter (Benfey & Chua, 1990) was fused to the cDNAs to drive nearly ubiquitous expression of all the transgenes in a wild-type background. Furthermore, to avoid ectopic expression of these transgenes, in another series of experiments the Arabidopsis *AP3* promoter *5D3* (Hill *et al.*, 1998) was used to drive expression of the transgenes in whorls 2 and 3 of developing Arabidopsis flowers in the *ap3-3* mutant background. The promoter sequence was amplified by PCR from DNA extracted from leaves of wild-type *Arabidopsis thaliana* (L.) Heynh. ecotype *Col*-0 using primers AtAP3pro5RA (5′-TCGCAT-GCTCCCGGCCGCCAT-3′) and AtAP3pro3A (5′-CAAAGA GAGAAGAATATGGCG-3′); these bind from position –1087 to +1 relative to the translation start codon ATG of the *AtAP3* gene.

Arabidopsis thaliana transformation and genotyping

The plasmid constructs described above were transformed into wild-type Landsberg erecta or heterozygous *AP3/ap3-3* *A. thaliana* plants, respectively, by the floral dip method (Barraclough *et al.*, 1998; Whipple *et al.*, 2004). The seeds of transgenic plants were selected on solid 0.5× MS medium containing 50 mg l^{-1} kanamycin and 50 mg l^{-1} rifampicin, and were genotyped by PCR with transgene-specific primers. Homozygous *ap3-3* transformants were isolated using a dCAPS marker designed with the dCAPS finder program, with at least one intronic primer to amplify only the endogenous gene (Neff & Chory, 1998; Lamb & Irish, 2003; Whipple *et al.*, 2004). After genotyping, we checked the copy number of transgenes by Southern blotting to select for single-insert lines. Then the homozygous mutant plants containing single transgenic cDNAs were analysed. At least 106 flowers taken from at least 10 plants were examined for each genotype, with each independent transgenic line represented by at least 298 flowers from at least 22 plants. Flowers between flowers 5 and 20 on the primary inflorescence were scored (Lamb & Irish, 2003). If more than half of the flowers we examined showed full rescue, strong rescue, medium rescue or weak rescue (as defined in Table 1, and shown in Fig. 2), the transformants were scored as fully rescued, strongly rescued, medium rescued or weakly rescued, respectively.

Northern blot analysis

Total RNA was extracted from *A. thaliana* leaves of transgenic plants for ectopic-expression analysis, using Trizol reagent

Table 1 Summary of the rescue phenotypes

	Phenotypes					
Name of plants	Mutant	Weak	Medium	Strong	Full	No. of plants
Landsberg erecta	0	0	0	0	10 (100%)	10
ap3-3	10 (100%)	0	0	0	0	10
5D3::AtAP3	0	0	7 (23.3%)	11 (36.7%)	12 (40.0%)	30
5D3::CsAP3	0	13 (46.4%)	15 (53.6%)	0	0	28
5D3::AtAP3cC	0	0	15 (29.4%)	17 (33.3%)	19 (37.3%)	51
5D3::AtAP3pC	0	0	7 (23.3%)	11 (36.7%)	12 (40.0%)	30
5D3::CsAP3M	0	11 (39.1%)	14 (60.9%)	0	0	23
5D3::AtAP3cCM	0	0	10 (25.6%)	12 (30.8%)	17 (43.6%)	39
5D3::AtAP3pCM	0	0	5 (22.7%)	8 (36.4%)	9 (40.9%)	22

Full rescue: petals indistinguishable from wild-type *Arabidopsis thaliana* flowers, stamens often short, and pollen produced.
Strong rescue: white but often short petals, and mosaic stamens.
Medium rescue: no rescue of petals, and mosaic or filament-like stamens.
Weak rescue: no rescue of petals, and carpeloid stamens.
Mutant: no petals and stamens.

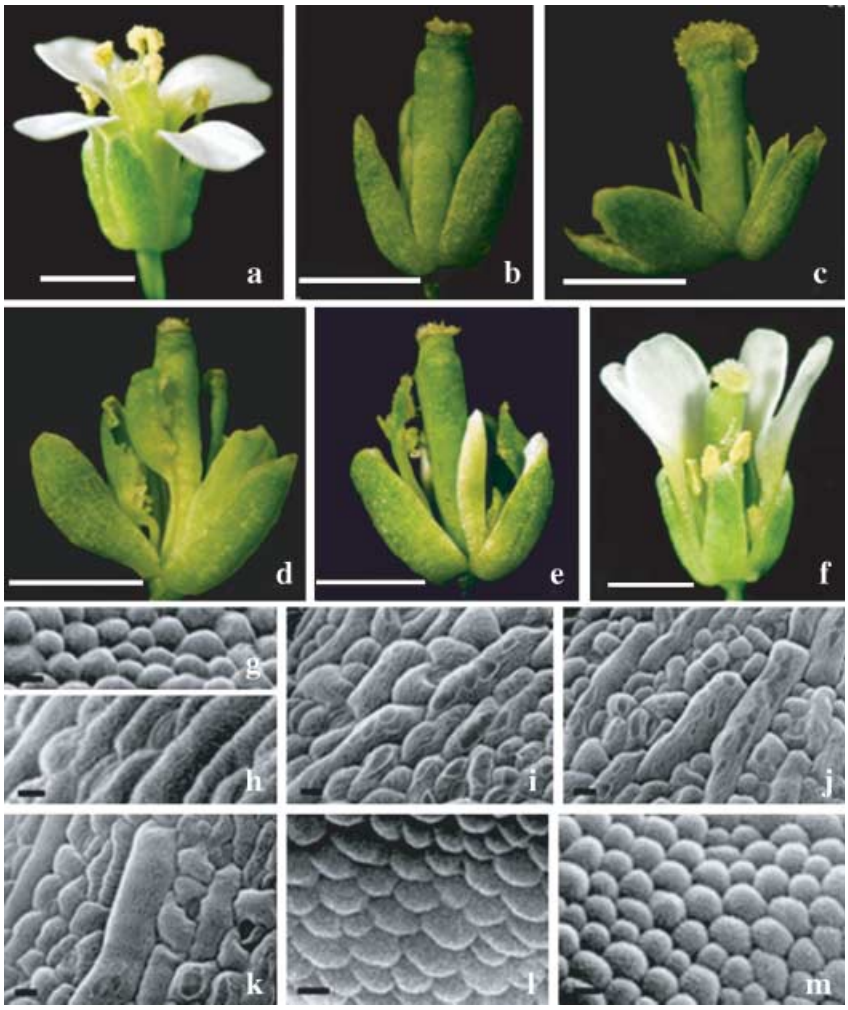

Fig. 2 Calibration of the degree of rescue of transgenic flowers. (a) Wild-type flower; (b) *ap3-3* mutant flower; (c–f) *ap3-3* homozygous mutant flowers of transgenic plants with different degrees of rescue: (c) and (d) showed weak to medium rescue, (e) strong rescue, and (f) full rescue. (g) Petal epidermis of a wild-type *Arabidopsis thaliana* flower. (h) Sepal epidermis of a wild-type *A. thaliana* flower. (i) Petal epidermis of the flower shown in (b). (j) Petal epidermis of the flower shown in (c). (k) Petal epidermal cells of the flower shown in (d). (l) Petal epidermal cells of the flower shown in (e). (m) Petal epidermal cells of the flower shown in (f). Bars: (a–f) 1 mm; (g–m) 10 μm.

(Invitrogen). Twenty-five μg of total RNA was loaded and separated on 1.0% formaldehyde agarose gel in each lane, and subsequently transferred to a HyBond-N^+ nylon membrane (Amersham Biosciences, Little Chalfont, UK) (Lü *et al.*, 2007). Gene-specific 3′ end fragments of a series of expression constructs were labelled with ($^{-32}$P) dCTP using Prime-a-Gene® label (Promega, Madison, WI, USA). Prehybridization and hybridization were performed as previously described (Lü *et al.*, 2007).

Reverse transcriptase–polymerase chain reaction (RT-PCR) and quantitative real-time RT-PCR analysis

Using Trizol reagent (Invitrogen), total RNA was extracted from the inflorescences of the wild-type *A. thaliana*, the *ap3-3* mutant plants and the rescued plants of *ap3-3* homozygotes carrying sets of transgenic constructs under control of the *5D3* promoter. First-strand cDNA was synthesized with Superscript™ III Reverse Transcriptase (Invitrogen) in a 20-μl reaction volume. For RT-PCR analysis, the PCR conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min; then 10 min of extension at 72°C. For real-time PCR analysis, triplicate quantitative assays were performed using the QuantiTect® SYBR® Green PCR kit with a Rotor-Gene 3000 (Corbett Research, QIAGEN, Hilden, Germany) detection system and software according to the manufacturer's instructions. Nested PCR conditions were 15 min at 95°C, followed by 40 cycles of 30 s at 94°C, 30 s at 56°C and 30 s at 72°C. Specific primer pairs were designed with the help of BEACON DESIGNER 4 software (Premier Biosoft International, Palo Alto, CA, USA). These primers included ATAP3ReTi-F2 (5′-CATACATGAGCTGG AACTAA-3′), ATAP3RETI-R2 (5′-ATGGTTCTGGTGG AAACGAAG-3′), CSAP3RETI-F (5′-CATCGACTGGA GGGGATAGAC-3′) and CSAP3RETI-R (5′-CTGGATGG GCTGCAAGCGAAAA-3′). In each experiment, two standard curves were applied for the relative quantification of the cDNA copies. The amplification of *Actin* was used as an internal control and to normalize all data. Each sample was analysed three times to determine reproducibility.

Scanning electron microscope analysis

All flowers collected from the transgenic *A. thaliana* or wild-type plants were immediately fixed with FAA (5 : 6 : 89 formalin : acetic acid : 50% ethanol). Then these flowers were dried and coated as described previously (Xu *et al.*, 2005), and observed with a Hitachi S-800 scanning electron microscope (SEM).

Yeast two-hybrid assays

Yeast two-hybrid assays were performed using the GAL4-based MATCHMAKER Two-Hybrid System (Clontech Mountain View, CA, USA). *Saccharomyces cerevisiae* strain AH109, GAL4 activation domain (AD) expression vector pGADT7 and GAL4 DNA-binding domain (DNA-BD) expression vector pGBKT7 were used. Full-length cDNAs of *CsAP3*, *AtAP3*, *AtPI* and the chimeric constructs *AtAP3pC* and *AtAP3 pCM* were amplified with primers generating *Nco*I or *Nde*I restriction enzyme digestion sites just before the start codon and a *Bam*HI site at the 3′ end. *Eco*RI and *Bam*HI sites were introduced to generate MADS-box-deleted versions of *CsAP3*, *CsPI*, *AtAP3*, *AtPI*, *AtAP3pC* and *AtAP3 pCM* for cloning into pGADT7 and pGBKT7. All constructs were verified by restriction enzyme analyses and sequencing. The yeast strain AH109 was transformed with the above constructs according to the manufacturer's protocol for the small-scale LiAc yeast transformation procedure. Confirmation of the transformants and interaction analyses were performed as previously described (Shan *et al.*, 2006). The transformants co-transformed with plasmids encoding AP3 and PI from *A. thaliana* without MADS domains were used as a positive control (Yang *et al.*, 2003), and the transformants containing plasmids pGADT7 and pGBKT7 were used as a negative control.

Results

Functionality of CsAP3, AtAP3 and chimeric proteins in *ap3-3* mutants of *Arabidopsis thaliana*

To determine whether the coding regions of the eu*AP3*-like gene *AtAP3* and the paleo*AP3*-type gene *CsAP3* have different functional capacities, we created a number of domain-swap and point mutation constructs (Fig. 1b) and introduced them into *A. thaliana* plants.

In our first series of experiments, *5D3*, the full-length promoter of *AtAP3*, was used to drive transgene expression in the wild-type expression domain of *AtAP3*, that is, developing whorl 2 and whorl 3 primordia of *A. thaliana* flowers. The phenotypes of transgenic lines were assayed in a homozygous *ap3-3* mutant background to determine whether the transgenes were able to compensate for the absence of wild-type AtAP3 protein. The *ap3-3* mutant is supposed to carry a null allele of *AtAP3* caused by a stop codon in the MADS box (Jack *et al.*, 1992).

For all constructs, a number of independent transformants were identified showing a range of phenotypes. The degree of rescue of the *ap3* mutant phenotype was categorized as 'no rescue', 'weak rescue', 'strong rescue' and 'full rescue', as shown in Fig. 2(c–f). Wild-type and mutant *ap3-3* flowers are shown for comparison in Fig. 2 (a and b, respectively). Flowers of fully rescued plants were similar to wild-type flowers. Their petals had the shape of wild-type petals but were somewhat smaller and stamens were not fully extended but could produce fertile pollen grains (Fig. 2f). Moreover, the epidermal cells of rescued petals (Fig. 2m) in these plants were characteristically rounded, thus resembling those of the wild type (Fig. 2g). In flowers of strongly rescued plants, the third-whorl

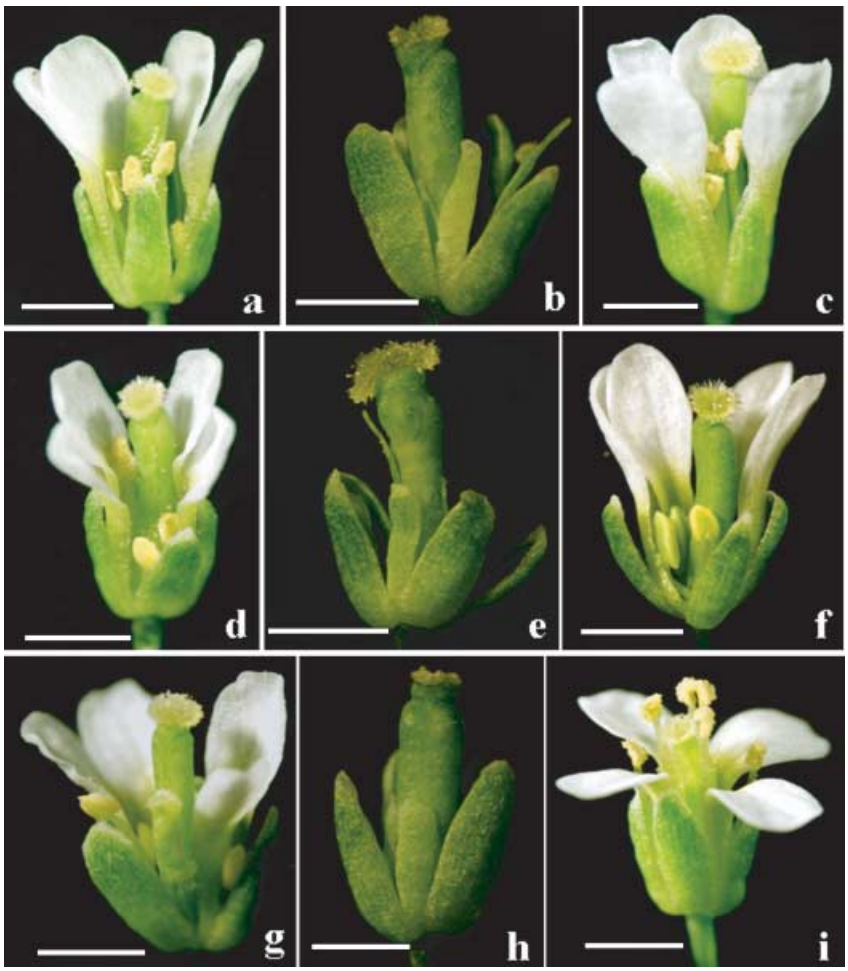

Fig. 3 Complementation of *Arabidopsis thaliana* B-class mutants. (a–g) Representative flowers of transgenic plants homozygous for *ap3-3* and carrying the following constructs are shown: (a) *5D3*::*AtAP3*, (b) *5D3*::*CsAP3*, (c) *5D3*::*AtAP3cC*, (d) *5D3*::*AtAP3pC*, (e) *5D3*::*CsAP3M*, (f) *5D3*::*AtAP3cCM*, and (g) *5D3*::*AtAP3pCM*. (h) Flower of an *ap3-3* mutant; (i) flower of an *A. thaliana* wild-type plant. Bars, 1 mm.

floral organs were mosaic organs between carpel and stamen; the second-whorl organs were similar to petals, but as small as sepals (Fig. 2e). The epidermal petal cells of strongly rescued plants were more similar to those of wild-type petals than sepals (Fig. 2i). Medium to weak complementation was also often seen in transgenic lines, in which neither stamens nor petals were completely rescued (Fig. 2c,d). In none of the lines was there a rescue of stamen identity without a similar level of petal rescue.

As a positive control, transgenic plants (*5D3*::*AtAP3*; *ap3-3*/*ap3-3*) expressing the wild-type *AtAP3* cDNA were assayed for their ability to rescue the *ap3-3* mutant. Most of the *5D3*::*AtAP3* transgenic lines showed full (40.0%) or strong (36.7%) rescue (Fig. 3a; Tables 1, 2). By contrast, only medium to weak rescue of stamen development, but no complementation of petal development, was obtained with *5D3*::*CsAP3* (Fig. 3b; Tables 1, 2), demonstrating that the *AP3*-like gene of *C. spicatus* can weakly substitute for the *AtAP3* gene of *A. thaliana* during stamen development, but not at all during petal development. To determine whether the truncated paleoAP3 motif (YGTH) is responsible for the functional insufficiency of *CsAP3* in our assay, a full-length paleoAP3 motif (YGTHDLCLG) was reconstituted by mutating the precocious stop codon of *CsAP3* into a sense codon. However, when the mutated cDNA *CsAP3M* (Fig. 1b) was expressed under the control of the *5D3* promoter (Fig. 4b), the transgenic *ap3-3* mutant plants showed again only weak rescue of the floral organs compared with that observed with the *5D3*::*AtAP3* constructs. The second-whorl floral organs remained green and generally sepal-like in appearance (Fig. 3e; Table 2). Also, although some mosaic organs were observed, no stamens were produced (1.66 ± 1.04; Table 2; Fig. 3e). Thus complementation of *ap3-3* mutants by *5D3*::*CsAP3M* was almost as poor as that of *5D3*::*CsAP3* in the *5D3*::*CsAP3*; *ap3-3* plants; only the third-whorl mutant phenotype was partially ameliorated, and the organs of the second whorl remained sepaloid.

To investigate which part of the *CsAP3* sequence is responsible for this insufficiency in mutant complementation, we tested a number of domain-swap constructs. Complementation was good when within the AtAP3 sequence the euAP3 motif was substituted by the 'repaired', full-length paleoAP3 motif of CsAP3 (construct *5D3*::*AtAP3pCM*; Fig. 1b). More than 70% of *5D3*::*AtAP3pCM* transgenic lines showed complete or strong complementation (Fig. 3g; Table 1). Rescue of organ

Table 2 Organ numbers of transgenic plants containing *AP3*-like constructs under the control of the *5D3* promoter

	Whorl 1	Whorl 2		Whorl 3			Whorl 4
Name of plants	S	S	P	St	O	C	C
Landsberg erecta	4 (0)	4 (0)	0 (0)	0 (0)	5.74 (0.62)	0 (0)	2 (0)
ap3-3	4 (0)	4 (0)	0 (0)	0 (0)	0.56 (0.85)	5.43 (0.85)	2 (0)
5D3::CsAP3	4 (0)	4 (0)	0 (0)	0 (0)	1.82 (1.06)	4.18 (1.06)	2 (0)
5D3::CsAP3M	4 (0)	4 (0)	0 (0)	0 (0)	1.66 (1.04)	4.34 (1.04)	2 (0)
5D3::AtAP3	4 (0)	0 (0)	4 (0)	2.84 (1.55)	1.92 (1.54)	1.25 (1.18)	2 (0)
5D3::AtAP3cC	4 (0)	0 (0)	4 (0)	3.68 (1.92)	1.74 (1.77)	0.54 (0.88)	2 (0)
5D3::AtAP3cCM	4 (0)	0 (0)	4 (0)	2.77 (1.54)	2.19 (1.60)	1.04 (1.21)	2 (0)
5D3::AtAP3pC	4 (0)	0 (0)	4 (0)	3.18 (1.80)	2.11 (1.68)	0.70 (0.93)	2 (0)
5D3::AtAP3pCM	4 (0)	0 (0)	4 (0)	2.65 (1.51)	2.43 (1.46)	0.92 (1.02)	2 (0)

S, sepal; P, petal; S-P, sepal-petal mosaic; St, stamen and stamen-like (stamen-shaped, but infertile and often immature-looking anther); O, filaments and mosaic organs; C, carpeloid organs. The numbers indicate the average number of organs of that type found in that genotype. Standard deviations are in parentheses.

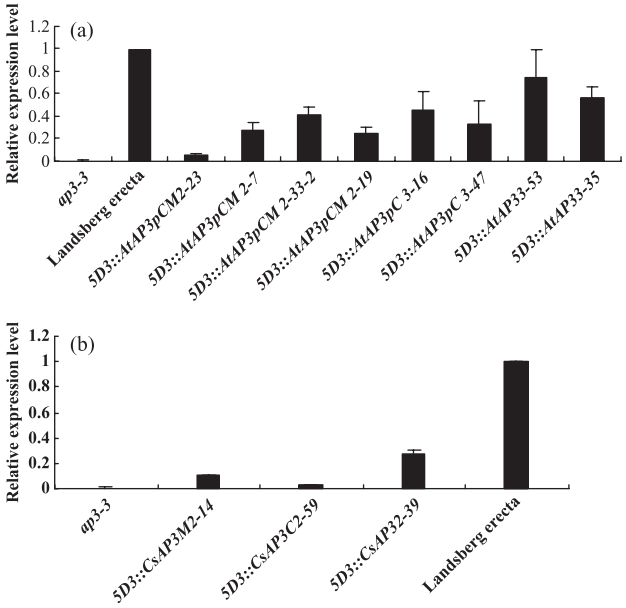

Fig. 4 Quantitative real-time PCR analysis. (a) Expression analyses of *5D3*::*AtAP3*, *5D3*::*AtAP3pC* and *5D3*::*AtAP3pCM* in the *ap3-3 Arabidopsis thaliana* mutant. The figure shows the relative expression level of the genes for *ap3-3* homozygous mutant, Wild-type Landsberg erecta, *5D3*::*AtAP3pCM2-23*, *5D3*::*AtAP3pCM2-7*, *5D3*::*AtAP3pCM2-33-2*, *5D3*::*AtAP3pCM2-19*, *5D3*::*AtAP3pC3-16*, *5D3*::*AtAP3pC3-47*, *5D3*::*AtAP33-53* and *5D3*::*AtAP33-35*. (b) Expression analyses of *5D3*::*CsAP3* and *5D3*::*CsAP3M* in the *ap3-3* mutant. *ap3-3*, homozygous mutant; *5D3*::*CsAP3M2-14*, *5D3*::*CsAP32-59*, *5D3*::*CsAP32-39*, Wild-type Landsberg erecta plants.

identity defects by *5D3*::*AtAP3pCM* was parallel in the third whorl and in the second whorl. In the third whorl only a few carpel-like organs remained (not more than 1.25 ± 1.8) and most were rescued into stamen or mosaic organs, and this was also the case in the second whorl, where most organs had petaloid characteristics (Table 2).

Interestingly, rescue was similarly complete even when the truncated rather than the full-length paleoAP3 motif was used to substitute the euAP3 motif (*5D3*::*AtAP3pC*) (Fig. 3d; Tables 1, 2). Excellent complementation was even still achieved when not only the truncated or full-length paleoAP3 motif but also the PI-derived motif of CsAP3 was used to substitute the corresponding sequences of AtAP3 (constructs *5D3*::*AtAP3cC* and *5D3*::*AtAP3cCM*) (Fig. 3c,f; Table 1).

To exclude the possibility that the different degrees of phenotype rescue are exclusively a function of the amount of transgene expression rather than transgene sequence, transgene expression was determined by quantitative real-time RT-PCR analysis (Fig. 4a,b). Transgene expression was found to be *c.* 5–80% of that of *AtAP3* in wild-type plants, and there was a strong correlation between transgene expression and degree of rescue only for different transformants that contained the same construct (Fig. 4a,b), and not when different constructs were compared. For example, lines *5D3*::*AtAP3pCM2-23* (no rescue), *5D3*::*AtAP3pCM2-7* (strong rescue) and *5D3*:: *AtAP3pCM2-33-2* (full rescue) showed 5, 35 and 50% of the level of expression of *AtAP3* in wild-type plants, respectively (Fig. 4a). However, the specific sequence of the transgenes, and not only the amount of transgene expression, was a decisive factor determining the phenotype of transgenic plants. For example, lines *5D3*::*CsAP32-39* (weak rescue; Fig. 4b) and *5D3*::*AtAP3pCM2-19* (strong rescue; Fig. 4a) showed similar amounts of transgene expression.

Overexpression of *CsAP3*, *AtAP3* and chimeric cDNAs in wild-type *A. thaliana* plants

In addition to the complementation experiments, *CsAP3*, *AtAP3* and all the chimeric constructs were also transformed into wild-type *A. thaliana* plants. In this second set of transformation experiments, the *35S* promoter was employed

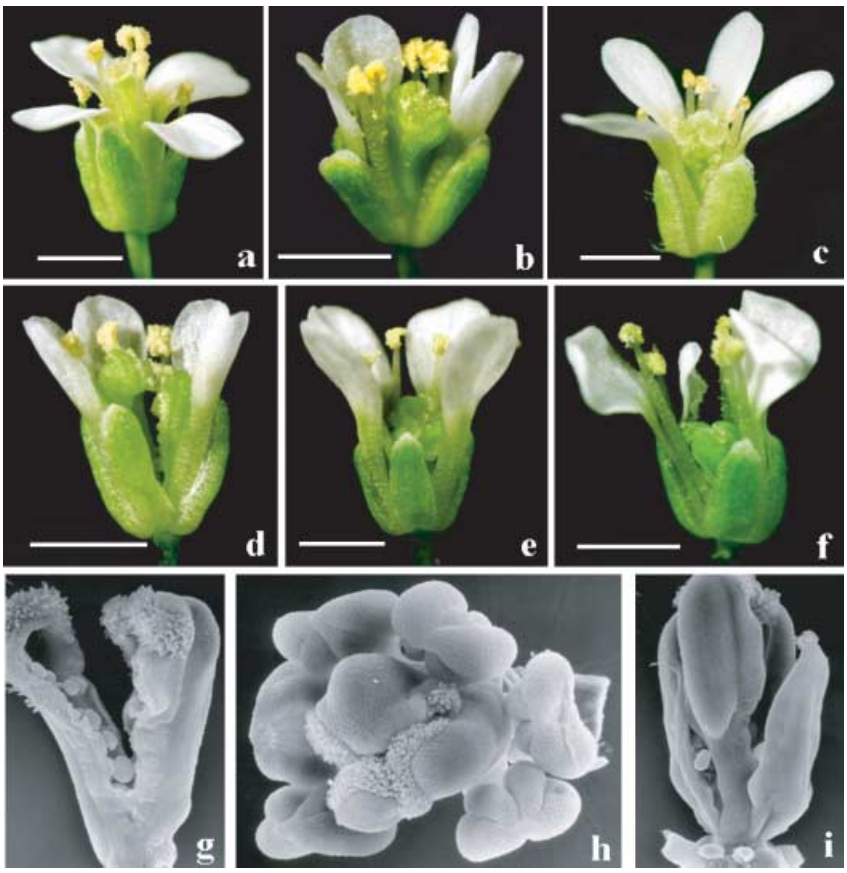

Fig. 5 Phenotypic changes produced by ectopic expression of *AtAP3* and the chimeric constructs *AtAP3cC*, *AtAP3cCM*, *AtAP3pC* and *AtAP3pCM* in *Arabidopsis thaliana*. (a) Wild-type flower. (b–f) Phenotypes of the fourth whorl floral organs in transgenic flowers: (b) the carpel is bent, (c) the carpel is dehisced, (d–f) carpels are transformed into mosaic tissues between carpel-like and stamen-like structures. (g) Scanning electron microscopy (SEM) of the fourth whorl organs shown in (d). (h) SEM of the third and fourth whorl organs shown in (e). (i) SEM of the fourth whorl organs shown in (f). Bars, (a–i) 1 mm.

Table 3 Summary of the phenotypes of all transgenic lines with a series of *AP3*-like constructs under the control of the *35S* promoter

Genotype	No. of transgenic plants	No. of plants with aberrant fourth whorl organs
35S::*AtAP3*	48	34 (64.8%)
35S::*CsAP3*	30	0
35S::*CsAP3M*	33	0
35S::*AtAP3cC*	50	31 (62.0%)
35S::*AtAP3cCM*	36	24 (66.7%)
35S::*AtAP3pC*	44	28 (63.6%)
35S::*AtAP3pCM*	54	34 (63.0%)

to drive nearly ubiquitous expression of the transgenes. As a positive control, transgenic plants expressing wild-type AP3 (*35S*::*AtAP3*) were generated and their ability to cause ectopic phenotypes was assessed. As wild-type *A. thaliana* plants express some *PI* (henceforth termed *AtPI*) in the fourth floral whorl, in particular transformation of carpels in the fourth floral whorl into stamen-like organs could be expected, as previously described (Krizek & Meyerowitz, 1996).

At least 30 *A. thaliana* transgenic plants for each construct were analysed (Table 3). Ectopic expression of *AtAP3*, *AtAP3pC*, *AtAP3cC* and *AtAP3pCM* in *A. thaliana* was confirmed by northern blot analysis, which demonstrated that the accumulation levels of transcripts in different lines were consistent with morphological alterations (Supplementary Material Fig. S3). Ectopic expression of *CsAP3M* and *CsAP3* driven by the *35S* promoter was detected by quantitative real-time RT-PCR analysis (Supplementary Material Fig. S1).

All phenotypic changes observed were restricted to fourth-whorl floral organs. Small changes in fourth-whorl development resulted in bent carpels or carpels with crevices at the top (Fig. 5b,c), while more serious developmental alterations led to organs with reduced stigmatic tissues, poorly developed valves (Fig. 5d–f), and mosaic tissues between carpels and stamens (Fig. 5g–i) (Krizek & Meyerowitz, 1996).

Whenever the MIK region was taken from *A. thaliana*, > 60% of transformants displayed obvious alterations in the fourth floral whorl (Table 3). This was irrespective of whether the paleoAP3 motif was truncated (constructs *35S*::*AtAP3pC*, *35S*::*ATAP3cC*) or full-length (*35S*::*AtAP3cCM*, *35S*::*AtAP3pCM*), or whether the PI motif was from *A. thaliana* (*35S*::*AtAP3pC*, *35S*::*AtAP3pCM*) or from *C. spicatus* (*35S*::*ATAP3cC*, *35S*::*AtAP3cCM*).

By contrast, when the MIK region was taken from *C. spicatus* (*35S*::*CsAP3*, *35S*::*CsAP3M*), no deviations from the wild-type phenotype were observed (Table 3), indicating

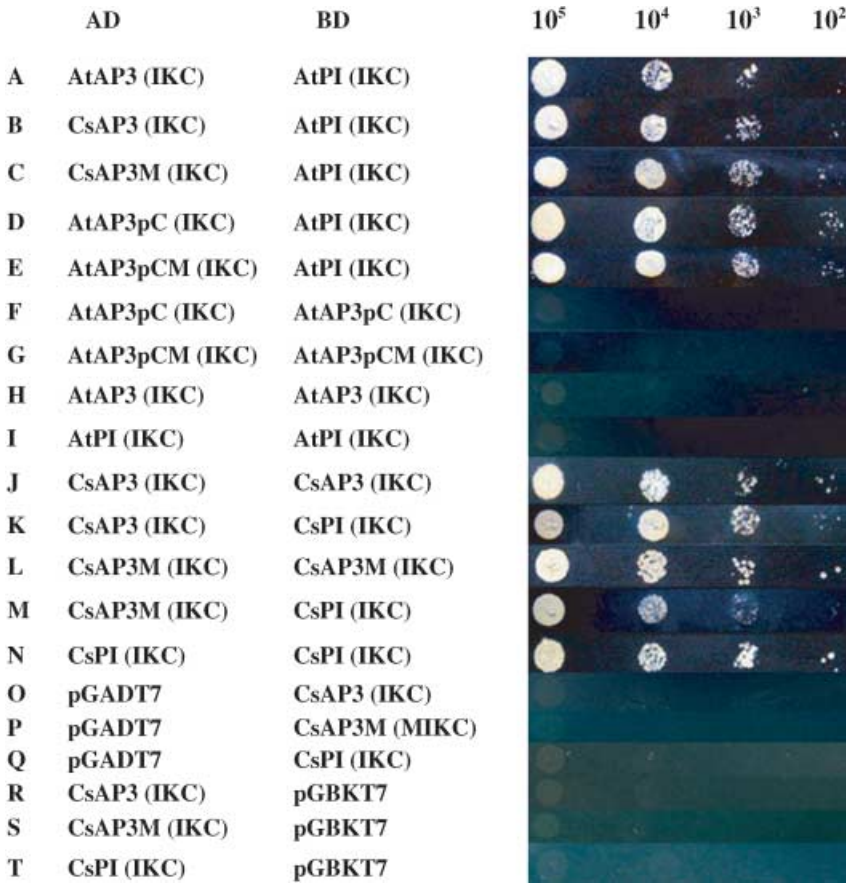

Fig. 6 Yeast two-hybrid assays. Interaction patterns of MADS-deleted AtPI, CsPI and AP3-like proteins including AtAP3, CsAP3 and the chimeric proteins AtAP3pC, AtAP3pCM and CsAP3M are shown. Serial dilutions of 105–102 AH109 cells containing different plasmid combinations were grown on the selective medium SD-LTHA + 5 mM 3-AT. L, leucine; T, tryptophan; H, histidine; A, adenine; 3-AT, 3-amino-1, 2, 4-triazole.

that sequences in the region upstream of the PI-derived motif rather than in the C-terminal domain itself are required for the ectopic gain-of-function in the fourth floral whorl.

Analysis of protein–protein interactions between AP3-like proteins and PI-like proteins

One remarkable feature of the B-class proteins of higher eudicots is the obligate heterodimerization of AP3-like proteins with PI-like proteins (Trobner *et al.*, 1992; Riechmann *et al.*, 1996). In *A. thaliana*, for example, coexpression of AtAP3 and AtPI is required for nuclear localization, suggesting that only AtAP3-AtPI heterodimers, and not AtAP3-AtAP3 or AtPI-AtPI homodimers, are able to enter the nucleus of the cell (McGonigle *et al.*, 1996). Consistent with this, sequence-specific DNA binding requires AtAP3-AtPI heterodimers (Riechmann *et al.*, 1996).

In order to investigate the conservation of the dimerization specificity of these proteins, we tested their interaction using yeast two-hybrid assays. It was of particular interest to determine whether any shortcomings in the heterologous expression experiments described above could be attributed to inabilities of the wild-type or mutated AP3-like proteins to heterodimerize with the endogeneous AtPI.

In our experiments, full-length MIKC structures and MADS-domain-deleted IKC regions were tested for interaction. While dimerization could not be observed for MIKC structures (data not shown), the MADS-deleted AtAP3 and AtPI sequences formed heterodimers as expected (Fig. 6a) (Yang *et al.*, 2003). Also, just like the MADS-deleted *A. thaliana* AtAP3 and AtPI proteins (IKC) (Fig. 6h,i), the truncated AP3-like proteins and PI-like proteins (IKC) failed to form homodimers (Fig. 6f,g). As negative controls, we demonstrated that transformants co-transformed with the fusion plasmid containing the MADS-deleted CsAP3 protein and the pGADT7 or the pGBKT7 free vector did not grow on the selective medium (Fig. 6o,r); nor did the transformants containing the MADS-deleted CsAP3M or CsPI protein and the pGADT7 or the pGBKT7 free vector (Fig. 6p,q,s,t).

As shown in Fig. 6, all the MADS-deleted versions of the AP3-like proteins investigated in this work (Fig. 1b) can form heterodimers with the MADS-deleted AtPI protein. As the specificity of heterodimerization is largely based on the sequences of the I domain and K domain (Riechmann *et al.*, 1996; Kaufmann *et al.*, 2005), the full-length (MIKC) sequence is also very likely to be able to form such heterodimers. Importantly, heterodimerization with AtPI was even observed for CsAP3 and CsAP3M, so it seems unlikely that the

considerable functional deficits of these sequences in the complementation or gain-of-function analyses described above are caused by impairments in heterodimerization with the endogenous AtPI.

As expected, the MADS-deleted CsAP3 and CsAP3M protein can form heterodimers with CsPI (Fig. 6k,m). In addition, all three proteins can also form homodimers (Fig. 6j,l,n), a feature that has been found also for some other AP3-like and PI-like proteins in noncore eudicots, including monocots such as lily (*Lilium*) and tulip (*Tulipa*), but not in core eudicots (Riechmann *et al.*, 1996; Hsu & Yang, 2002; Winter *et al.*, 2002b; Tzeng *et al.*, 2004; Vandenbussche *et al.*, 2004). However, proteins in which the IK region was from AtAP3 (AtAP3pC and AtAP3pCM) were not able to form homodimers (Fig. 6f,g), corroborating the view that dimerization specificity is largely determined by the region upstream of the C-terminal domain.

Discussion

AP3-like (or, in the case of gymnosperms, *AP3*/*PI*-like) MADS-box genes encoding a C-terminal paleoAP3 motif could only partially substitute for *AtAP3* in some *A. thaliana* complementation or gain-of-function experiments, suggesting that sequence divergence between paleo*AP3*-type and eu*AP3*-type genes during evolution somehow affected gene function (Winter *et al.*, 2002a; Lamb & Irish, 2003). The euAP3 motif can be considered a prime candidate for causing these differences in gene function, because it originated from its precursor, the paleoAP3 motif, by a translational frameshift which resulted in a dramatic sequence change (Vandenbussche *et al.*, 2003; Kramer *et al.*, 2006).

However, the role of the euAP3 motif of *AP3*-like MADS-box genes for full class B floral homeotic gene function in specifying petal and stamen identity has been a matter of controversy. Based on a series of experiments similar to those reported here, Lamb & Irish (2003) reported that, when the PI-derived motif and the euAP3 motif are deleted, the AtAP3 protein is able neither to rescue any aspect of the *ap3-3* mutant phenotype nor to generate a gain-of-function phenotype (transition of carpels to staminoid organs) in the fourth floral whorl of wild-type plants, indicating that a C-terminal domain is required for AtAP3 function *in vivo*. When the C-terminal region of AtAP3 was substituted by that of AtPI, a marginal effect of rescue was seen in the third whorl, but no complementation of second-whorl organs and no ectopic phenotype in gain-of-function analyses was observed (Lamb & Irish, 2003), indicating that not just any C-terminal domain of a class B protein is sufficient for proper AtAP3 function. When the PI-derived motif and the euAP3 motif were substituted by the PI-derived and paleoAP3 motifs of the *DeAP3* gene from the basal eudicot *Dicentra eximia* (fringed bleeding heart; Papaveraceae), weak complementation of stamen formation in the third floral whorl of transgenic *ap3-3* mutants was observed, and a weak ectopic phenotype in the case of gain-of-function analyses involving wild-type plants; again, however, no rescue at all of petal formation in *ap3-3* mutants was obtained (Lamb & Irish, 2003). On the basis of these results, Lamb & Irish (2003) concluded that AP3-like proteins with a paleoAP3 motif might suffice to specify the identity of stamens, but that petal formation in eudicots requires an AP3-like protein with a euAP3 motif. The correlation of a possible independent derivation of petals at the base of core eudicots with a gene duplication and sequence divergence leading to euAP3-type proteins, in addition to paleoAP3-type proteins, was the basis of the hypothesis that the acquisition of the euAP3 motif in addition to the PI-derived motif played a role in the origin of petals in core eudicots (Lamb & Irish, 2003).

Subsequently, however, Whipple *et al.* (2004) showed that the cDNA of the *SILKY1* gene of maize (*Z. mays*), when expressed under control of the *AtAP3* promoter, can rescue *ap3-3* mutants in terms of both stamen and petal development. This is remarkable given that *SILKY1* is a paleo*AP3*-type rather than a eu*AP3*-type gene. Similarly, it has also been shown that overexpression of *TM6*-like genes carrying paleoAP3 motifs can rescue the loss-of-function phenotypes of eu*AP3* lineage genes (de Martino *et al.*, 2006; Rijpkema *et al.*, 2006). A plausible interpretation of these results is that the euAP3 motif or paleoAP3 motifs are not essential for specifying petal or stamen identity. The lack of petal rescue of the *ap3-3* mutant by the expression of *DeAP3* (Lamb & Irish, 2003) may reflect a derived state of the basal eudicot paleo*AP3*-like gene of *D. eximina* resulting from sequence divergence (Whipple *et al.*, 2004). For example, the paleoAP3 motif of DeAP3 might have compromised the dimerization of the chimeric protein with AtPI, or may have prevented the formation of higher order complexes. Alternatively, insufficient expression levels of the respective transgene may have prevented the rescue of the *ap3-3* mutant phenotype in the experiments by Lamb & Irish (2003), especially as Whipple *et al.* (2004) reported relatively high levels of transgene expression in their successful complementation experiments involving the *SILKY1* sequence.

While this manuscript was in preparation, Piwarzyk *et al.* (2007) reported that truncated forms of AtAP3 and AtPI, which lack the conserved C-terminal motifs, are nevertheless capable of specifying floral organ identity in similar experimental set-ups as those reported here and by others (Lamb & Irish, 2003; Whipple *et al.*, 2004; Piwarzyk *et al.*, 2007).

In the experiments reported here, the cDNA sequence of the paleo*AP3*-type gene *CsAP3* from the basal angiosperm *C. spicatus* was an extremely poor substitute for the *AtAP3* gene. We used the *5D3* promoter piece of *AtAP3*, as Lamb & Irish (2003) did, while Whipple *et al.* (2004) employed a slightly larger promoter fragment (positions −1312 to −16 vs −1087 to +1 relative to the translation start codon ATG).

Therefore, and because of potential transgene position effects, differences in expression level cannot be excluded as a

cause of the different degrees of rescue found when different types of paloAP3 motif-containing transgenes were used. However, on the basis of our results we do not believe that it should be concluded that the paleoAP3 motif is generally inferior to the euAP3 motif in specifying petal identity, because, in experiments reported here, full complementation of the *ap3-3* mutant was achieved with constructs containing the paleoAP3 motif of *C. spicatus* at expression levels that were considerably below the expression level of wild-type *AtAP3* (see e.g. Figs 2f and 4a, *5D3*::*AtAP3pC3-16* and Table 1).

Another clue as to why functional substitution in *A. thaliana* of AtAP3 by CsAP3 and CsAP3M sequences was poor is possibly provided by the yeast two-hybrid analyses, which revealed that the CsAP3 and CsAP3M proteins can form homodimers (Fig. 6). Assuming that they do so not only in yeast, but also *in planta*, the behaviour may prevent or at least reduce heterodimerization with AtPI. Class B function, however, may depend on heterodimer formation, while CsAP3 and CsAP3M homodimers might be unable to activate or repress the correct target genes, for example because higher order complex formation or specific DNA recognition is impaired.

The domain swap experiments reported here indicate that neither the presence of a paleoAP3 motif nor its truncation by a precocious stop codon compromises petal formation *per se*, thus corroborating the findings of Whipple *et al.* (2004). Our results are also more compatible with the findings of Piwarzyk *et al.* (2007) than with those of Lamb & Irish (2003). When fused to the MIK region from the *A. thaliana* AtAP3, these C-terminal motifs are closely compatible with both stamen and petal formation in *A. thaliana* (Figs 2–4). However, both CsAP3 and CsAP3M showed only a weak rescue of the mutant phenotype, restricted to stamen development. This is in marked contrast to the ability of the paleo*AP3*-type gene *SILKY1* to rescue the *ap3-3* mutant. Sequence-specific differences or, again, differences in expression level may account for this. However, it is clear from the experiments outlined here that the N-terminal MIK region, and not the C-terminal motifs, is mainly responsible for the functional difference between CsAP3 and AtAP3.

The findings of Whipple *et al.* (2004) and Piwarzyk *et al.* (2007) and those outlined here suggest that the C-terminal domain of AP3-like proteins is of less functional relevance than previously assumed (Lamb & Irish, 2003; Vandenbussche *et al.*, 2003). The 'degeneration' of the paleoAP3 motif in CsAP3 may reflect this. Experiments with other kinds of MIKC-type MADS-box genes have shown that the lineage-specific C-terminal motifs are of minor functional importance (Zachgo *et al.*, 1995; Davies *et al.*, 1996; Krizek & Meyerowitz, 1996; Egea-Cortines *et al.*, 1999; Johansen *et al.*, 2002; Yang *et al.*, 2003; Berbel *et al.*, 2005). For example, the PI homologous protein PsPI from pea (*Pisum sativum*), which lacks the PI motif, is able to complement specifically the floral defects of *pi-1* mutants (Berbel *et al.*, 2005). In addition to CsAP3, paleoAP3-type proteins with truncated or even absent paleoAP3 motifs have also been observed in other cases, such as OMADS3 from the orchid (*Oncidium Gower Ramsey*) and ApDEF from *Agapanthus praecox* (both species are monocots) (Hsu & Yang, 2002; Nakamura *et al.*, 2005). Although the functionality of these proteins has not yet been critically tested, these findings further argue against the functional importance of the paleoAP3 motif.

The extent to which class B floral homeotic gene function has been conserved throughout the angiosperms has been a contentious issue. Expression studies suggested that the function of *AP3*-like genes in specifying petal identity is not conserved outside the core eudicots, possibly reflecting several independent origins of petals (Kramer & Irish, 1999, 2000). A recent study on the lower eudicot *Aquilegia* suggested that *AP3/PI* homologues do not contribute to the identity of some types of petaloid organs (Kramer *et al.*, 2007). In poppy (*Papaver somniferum*), the AP3 lineage has undergone gene duplication, with one gene copy required for petal development and the other responsible for stamen development (Drea *et al.*, 2007). However, work on the paleo*AP3*-type gene *SILKY1* from maize and its orthologues in some other grasses demonstrated that *AP3*-like genes are involved in specifying lodicules and that lodicules are probably homologous to petals (Ambrose *et al.*, 2000; Whipple *et al.*, 2004, 2007). Grasses (Poaceae) belong to the monocots and, like the Chloranthaceae, are hence outside the eudicots. These findings thus suggest that the involvement of *AP3*-like genes in the specification of petal identity represents a rather old developmental programme within the angiosperms. It appears quite likely, therefore, that the precursor of the *CsAP3* gene at the base of the Chloranthaceae was involved in specifying petals, but that this function was lost when petals were abolished in the lineage that led to *Chloranthus*. The fact that the expression of the *CsAP3* gene from *C. spicatus* is now exclusively restricted to stamen primordia (Li *et al.*, 2005) thus also very probably represents a derived rather than an ancestral state.

Previously it was tempting to speculate that the truncation of the paleoAP3 motif of the CsAP3 protein by a precocious stop codon was a consequence of the loss of a perianth in the lineage that led to *Chloranthus*. It has been hypothesized that in Chloranthaceae a full paleoAP3 motif might only be required for petal and not for stamen development, so that the paleoAP3 motif degenerated after the perianth was lost. However, the finding that even the truncated paleoAP3 motif of *CsAP3*, when only fused to a suitable MIK region, is sufficient to specify petals in *A. thaliana* argues against a link between the petal loss (cause) and degeneration of the C-terminal motif as a result of absence of selection against a precocious stop codon (consequence).

While the C-terminal domain appears to be of little importance for dimerization of MIKC-type proteins (for a notable exception, see Tzeng *et al.*, 2004), it might be quite relevant for the formation of multimeric complexes involving these transcription factors (Egea-Cortines *et al.*, 1999; Kaufmann

et al., 2005). The requirement for and sufficiency of a euAP3 motif for class B function in some experiments in *A. thaliana*, even when provided fused to the MIK region of AtPI, were thus plausibly explained by an essential role of this motif in higher order complex formation (Lamb & Irish, 2003). However, in contrast to PI-like proteins from some other species (Winter *et al.*, 2002b), there is no evidence that AtPI can homodimerize *in vivo*, let alone form multimeric complexes. Moreover, the finding that wild-type and chimeric AP3-like proteins with a paleoAP3 motif rather than a euAP3 motif can functionally substitute AtAP3 (Whipple *et al.*, 2004; this work) suggests that these proteins can form the same higher order complexes as AtAP3, thus further invalidating the argument.

Previous ectopic expression studies have shown that the IK region of the MIKC-type proteins AtAP3 and AtPI is required to confer a specific mutant phenotype (Krizek & Meyerowitz, 1996; Krizek, 1999). This is fully compatible with the findings outlined here that specificity in providing the class B function during flower development (specifying petal and stamen identity) resides largely in MIK, the region upstream of the C-terminal domain, of the euAP3-type protein AtAP3.

Our findings, as well as those of others (Whipple *et al.*, 2004; Piwarzyk *et al.*, 2007), concerning the dispensability of the C-terminal motifs raise the question as to why the PI-derived motif and the euAP3 motif have been conserved during evolution for probably > 100 Myr (de Martino *et al.*, 2006). Conservation over such a long period makes it very unlikely that these motifs simply have no function, and Piwarzyk *et al.* (2007) hence suggested that this function is redundantly specified elsewhere in the protein, and is only relevant if this other function is compromised. We would like to suggest a simpler explanation: the function of the C-terminal motifs might just be too subtle to be detected with the usual methods of developmental genetics. If mutation of the C-terminal motifs lowers the fitness of the affected plants by, say, 1% or even less, conservation of the motifs would be extremely likely. However, such effects might easily escape the powerful yet somewhat insensitive analysis of transgenic plants. Determination of the fitness of mutant compared with wild-type *A. thaliana* plants under a set of natural conditions might be required to detect subtle functions of the C-terminal motifs. Obviously, these two explanations are not mutually exclusive.

In conclusion, our data suggest that the MIK region rather than the C-terminal domain is crucial for functional specificity of AP3-like floral homeotic proteins in the development and evolution of petals and stamens. Future domain-swap experiments dissecting the MIK region may help to define which individual domains and subdomains are of critical functional relevance.

Acknowledgements

We thank Guisheng Li, Wei Wang, and Xiaoqiu Du for laboratory assistance. This work was supported by the National Nature Science Foundation of China (grants 30530090, 30121003 and 30770212).

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Supplementary Material

The following supplementary material is available for this article online:

Fig. S1 Quantitative real-time PCR analysis.

Fig. S2 RT-PCR analyses of expression of *AtAP3cCM* in *Arabidopsis thaliana ap3-3* flowers.

Fig. S3 Northern blot analyses of constitutive expression of *AtAP3*, *AtAP3pC*, *AtAP3pCM* (a) and *AtAP3cC* (b) in *Arabidopsis thaliana* leaves.

Table S1 Primers used in this paper

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