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ORIGINAL ARTICLE

Gui-Sheng Li · Zheng Meng · Hong-Zhi Kong ·
Zhi-Duan Chen · Günter Theissen · An-Min Lu

Characterization of candidate class A, B and E floral homeotic genes from the perianthless basal angiosperm *Chloranthus spicatus* (Chloranthaceae)

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Abstract The classic ABC model explains the activities of each class of floral homeotic genes in specifying the identity of floral organs. Thus, changes in these genes may underlay the origin of floral diversity during evolution. In this study, three MADS-box genes were isolated from the perianthless basal angiosperm *Chloranthus spicatus*. Sequence and phylogenetic analyses revealed that they are *AP1*-like, *AP3*-like and *SEP3*-like genes, and hence these genes were termed *CsAP1*, *CsAP3* and *CsSEP3*, respectively. Due to these assignments, they represent candidate class A, class B and class E genes, respectively. Expression patterns suggest that the *CsAP1*, *CsAP3* and *CsSEP3* genes function during flower development of *C. spicatus*. *CsAP1* is expressed broadly in the flower, which may reflect the ancestral function of *SQUA*-like genes in the specification of inflorescence and floral meristems rather than in patterning of the flower. *CsAP3* is exclusively expressed in male floral organs, providing the evidence that *AP3*-like genes have ancestral function in differentiation between male and female reproductive organs. *CsSEP3* expression is not detectable in spike meristems, but its mRNA accumulates throughout the flower, supporting the view that *SEP*-like genes have conserved expression pattern and function throughout angiosperm. Studies of synonymous vs nonsynonymous nucleotide substitutions indicate that these genes have not evolved under changes in evolutionary forces. All the data above suggest that the genes may have maintained at least some ancestral functions despite the lack of perianth in the flowers of *C. spicatus*.

Keywords *Chloranthus spicatus* · MADS box · Basal angiosperms · Perianths

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Nucleotide sequences data from this article have been deposited with the EMBL/GenBank Data Libraries under accession numbers AY316311, AY397762 and AY379963.

G.-S. Li · H.-Z. Kong · Z.-D. Chen · A.-M. Lu (✉)
Laboratory of Systematic and Evolutionary Botany,
Institute of Botany, Chinese Academy of Sciences,
Xiangshan, Beijing, 100093, People's Republic of China
e-mail: anmin@ibcas.ac.cn

G.-S. Li
Graduate School, Chinese Academy of Sciences,
Beijing, 100039, People's Republic of China

Z. Meng (✉)
Laboratory of Photosynthesis and Environmental Molecular
Physiology, Institute of Botany, Chinese Academy of Sciences,
Xiangshan, Beijing, 100093, People's Republic of China
e-mail: zhmeng@ibcas.ac.cn

G. Theissen
Lehrstuhl für Genetik, Friedrich-Schiller-Universität Jena,
Philosophenweg 12,
07743 Jena, Germany

Introduction

The origin and early diversification of flowers during evolution may have significantly contributed to the "abominable mystery," i.e. the sudden occurrence of diverse angiosperms in a relatively short time span during the Early Cretaceous, about 130–90 million years ago (MYA). Quite a number of hypotheses have been developed over the years to explain the origin of the angiosperm flower, such as euanthium/pseudanthium scenarios, Anthophyte and Neopseudanthium hypotheses, but they all have remained very speculative or even have been dismissed (for a review, see Frohlich 1999; Crepet 2000; Stuessy 2004; Theissen and Becker 2004). However, radically new insights into the evolution of flowers came from the study of genes involved in floral development, which led to the widely known ABC model (Coen and Meyerowitz 1991). According to this model, organ identity within each floral whorl is determined by a particular combination of gene expression: expression of class A genes alone specifies the sepals; expression of class A genes and class B genes together specifies the petals; class B genes and class C genes together specify the stamens; and class C genes alone determine the carpels. Recently, the ABCD model (Colombo et al. 1995), the ABCDE model and the floral quartet model, which focuses on the biochemical interac-

tion of the corresponding gene products, all have been proposed to refine the ABC model (Theissen 2001; Theissen and Saedler 2001). Almost all class ABCDE genes are nicknamed as MIKC^c-type MADS-box genes (MADS is the acronym of *MCM1* from yeast, *AGAMOUS* from *Arabidopsis*, *DEFICIENS* from *Antirrhinum*, *SRF* from human (Schwarz-Sommer et al. 1990)), referring to their conserved structure comprising an M (MADS), I (intervening), K (keratin-like) and C (C-terminal) domain (Schwarz-Sommer et al. 1990; Ma et al. 1991; Henschel et al. 2002). The proteins encoded by ABCDE genes act as transcription factors to determine floral organ identity, very likely by activating or repressing specific target genes. Some studies suggested that the specific structure of the genetic network involved in floral development, whether or not conserved throughout angiosperms, may have contributed to the evolution of flowers (Baum and Whitlock 1999; Ma and dePamphilis 2000; Theissen et al. 2000; Johansen et al. 2002; Irish 2003).

Additionally, an improved phylogeny of angiosperms provides a framework to study the evolution of the flower (Qiu et al. 1999; Soltis et al. 1999, 2000; Kuzoff and Gasser 2000). The eudicot model plant *Arabidopsis* and the monocot model plant *Oryza* enable a study of floral evolution at the level of comparative genomics (Bennetzen 2002; Goff et al. 2002; Yu et al. 2002). Most importantly, the relationships among basal angiosperms appear to be resolved, and the ANITA groups (ANITA is the acronym of *Amborella*, Nymphaeaceae, Illiciales, Trimeniaceae and Austrobaileyaceae) are currently considered as the earliest extant angiosperms (Qiu et al. 1999; Zanis et al. 2002). The phylogeny suggests that the early angiosperm flowers had spiral phyllotaxis, with a small but indeterminate number of undifferentiated perianth organs. From this simple flower, a whorled floral arrangement, with a fixed number of sepals and petals, evolved quite early in angiosperm. Reversions to spiral phyllotaxis, changes in merosity and losses of floral parts occurred several times independently in the early evolutionary history of the angiosperms (Soltis et al. 2000; Endress 2001a). Therefore, although it may now appear quite safe to assume that the class C floral homeotic function is conserved throughout extant angiosperms, it is not yet clear to which extent distinct A and B functions have been established in the basal groups of angiosperms (Ma and dePamphilis 2000; Theissen et al. 2000).

The plant family of Chloranthaceae is important for understanding the early diversification of angiosperms (Crane et al. 1995). The family consists of the genera *Chloranthus*, *Sarcandra*, *Ascarina* and *Hedyosmum*, in which the flowers of the derived *Chloranthus*, *Sarcandra* and *Ascarina* possess no perianth, whereas the ancestral *Hedyosmum* possesses a perianth in female flowers (Endress 1987; Doyle et al. 2003). Thus, the family is very interesting for studying perianth evolution. In inflorescences of *Chloranthus spicatus*, flowers of all stages of ontogeny can be readily found throughout the growth period. These inflorescences consist of a terminal spike and seven to eight lateral spikes in decussate pairs in the axils of scale-like bracts; each spike contains 16–18 flowers in decussate pairs, with each flower being in the axil of a bract. The perianthless flowers are bisexual and have an abaxial stamen attached on the back of the single carpel. The stamen is three-lobed and contains four thecae, one on the outer margin of both lateral lobes and one on both margins of the middle lobe (Endress 1987) (Fig. 1).

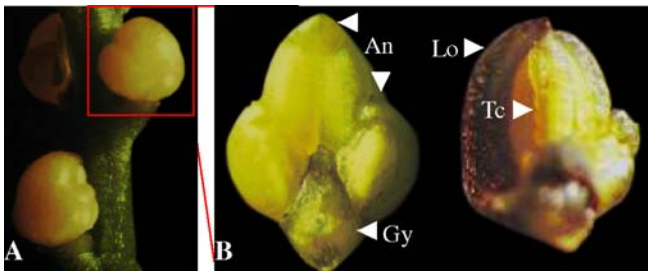

Fig. 1 The flower of *Chloranthus spicatus*. **a** Two mature flowers of one inflorescence. **b** One mature flower containing a gynoecium (*below*) and a three-lobed androecium with four thecae. *An* Androecium, *Gy* gynoecium, *Lo* lobes, *Tc* theca

Our interest is to learn more about the relationship between the phylogeny of MADS-box genes and the dramatic deviations of floral structures in *C. spicatus* from the "standard" flower Bauplan, especially with respect to the reduction in the perianth and changes in the corresponding floral homeotic genes (if any). Partial sequences of *CsAP3* and *CsPI* have already been isolated previously (Kramer and Irish 2000). Here, we report the complete coding sequences of *CsAP1*, *CsAP3* and a partial cDNA sequence of *CsSEP3*. Sequence analyses, phylogeny reconstructions and in situ hybridization expression analyses were performed. Our data demonstrate considerable conservation of gene structure and expression that reflect the general conservation of the angiosperm flower Bauplan. However, a few peculiarities of floral homeotic genes are also obvious, which are probably more of consequences than causes of the loss of the perianth during evolution of the Chloranthaceae.

Materials and methods

Isolation of *CsAP1*, *CsAP3* and *CsSEP3*

Total RNA was prepared using Trizol (Invitrogen) from the spikes of *C. spicatus* cultivated in the Botanical Garden, Institute of Botany, Chinese Academy of Sciences, Beijing. Poly(A) mRNA was extracted using Oligotex mRNA Mini Kit (Qiagen), and single-stranded cDNA was synthesized with Superscript II (Invitrogen). Double-stranded cDNA was isolated by 3′ rapid amplification of cDNA ends (3′ RACE) with the degenerate primers 5′-ATGGG(G/A)AG GGGTAGGGTTC-3′ (for *CsAP1*), 5′-ATGG(C/G)(A/G) AGAGG(G/A)A(A/G)(GA)ITI(C/G)A-3′ (for *CsAP3*) and 5′-CT(C/T)TGTGA(G/T)GCTGAAGTTGCTC-3′ (for *CsSEP3*), using two-cycle polymerase chain reaction (PCR) program (Kramer et al. 1998; Litt and Irish 2003). PCR products of about 1.0 kb (kilobase pair) were cloned into pGEM-T

vector (Promega). Sequencing of cDNA from three independent clones was performed with the ABI PRISM dye terminator kit (PE Applied Biosystems, Foster City, CA).

Sequence analysis of *CsAP1*, *CsAP3* and *CsSEP3*

Firstly, the cDNA sequences were conceptually translated with DAMBE software (Xia and Xie 2001). Then the amino acid sequences representing the maximum open reading frames (ORFs) were used in Blastp against GenBank hosted by NCBI (http://www.ncbi.nlm.nih.gov). Thus, they were identified as MADS-domain-containing proteins, and thereafter, the MADS box and K box of the corresponding genes were defined with reference to Purugganan et al. (1995). The amino acid sequences were further compared with close homologues using CLUSTALX1.81 (Thompson et al. 1997). Thus, their characteristic amino acids and motifs were identified.

Phylogenetic analysis of *CsAP1*, *CsAP3* and *CsSEP3*

Accessible MADS-box genes from *Chloranthus* were used. *Magnolia* and *Houttuynia* are relatively closely related taxa and have putative orthologues; thus, their representative MADS-box genes were also employed in the phylogenetic analysis. Finally, sequences of *Saururus* and *Peperomia*, like *Houttuynia* basal angiosperms without perianths, are also included. *CRM3* from the fern *Ceratopteris* was used to represent the outgroup since it is clearly a homologue, but not an orthologue of the *C. spicatus* genes (Munster et al. 1997; Henschel et al. 2002). The multiple alignment of amino acid sequences was produced with CLUSTALX1.81 under default settings. The MADS, I and K domains were used to construct phylogenetic trees. MEGA3 (Kumar et al. 2004b) was used to construct neighbor-joining (NJ) trees under the Poisson correction and complete deletion. PAUP4b10 (Swofford 1999) was used to construct maximum-parsimony (MP) trees under default settings, and PHYML was used to construct maximum-likelihood (ML) trees under default settings. The bootstrap values were calculated using 100 replicates.

In situ hybridization of *CsAP1*, *CsAP3* and *CsSEP3*

The spikes at various developmental stages were fixed in formalin (10%)/acetic acid (5%)/alcohol (47.5%) (FAA) overnight at 4°C and embedded in Paraplast Plus. The PCR products beginning at the specific 3′ terminus of *CsAP1* (nts 541–809), *CsAP3* (nts 481–854) and *CsSEP3* (nts 322–829) were used as the template to synthesize the sense and antisense probes initiated by SP6 and T7 promoter, respectively. The probes were synthesized with DIG Northern Starter Kit (Roche), and the in vitro transcription reactions and quantification were performed following the manufacturer's protocol. The section (10 μm) pretreatment and hybridization were performed as described by Wilson (2000) with the following modifications: slides were incubated with 7 μg/ml proteinase K (Sigma) for 45 min at 37°C, then acetylated with 0.25% acetic anhydride in 100 mM triethanolamine with pH 8.0 for 5 min at room temperature. Probes were hybridized in 100-μl volume medium overnight at 42°C. After hybridization, the slides were incubated at 37°C in NTE containing 20 mg/ml RNase A for 30 min and followed by several rinses in NTE and a final wash in 0.5×SSC at 52°C. Blocking steps and detection of hybridized transcripts using anti-digoxigenin antisera conjugated to alkaline phosphatase (Roche) were performed as the manufacturer's protocol.

Evolutionary force analysis

While some molecular variations are selectively neutral, some are negative or positive. However, functional evolution may correlate to either relaxed negative selection or positive selection (Bielawski and Yang 2001; Zhang 2003). Under relaxed purifying selection, the rate of amino acid replacement will increase but never exceed that of syn-

Table 1 Summary of parameters in the analysis of evolutionary force

Model	Forebranch	ω_f	lnL	ω_b
M0			−7,723.41	0.164
M1			−7,659.55	
M2	*CsAP1*	0.1483a	−7,709.10	0.193
	CsAP3	0.1241a		
	CsPI	0.0836a		
	CsAG1	0.1407a		
	CsAG2	0.0361c		
	CsSEP3	0.0363c		
	CsAP1	0.1412a	−7,721.04	0.154
	MpMADS15	0.2108a		
	ScMADS669	0.2899a		
	PcFL1	0.1737a		
	PcFL2	0.2743a		
	HcAP1	0.1943a		
	CsAP3	0.1212a	−7,711.41	0.151
	MpMADS7	0.2258a		
	ScMADS651	1.1785c		
	HcAP3	0.9118c		
	PhAP3	0.1988a		
	CsPI	0.0923a	−7,715.44	0.158
	MpMADS8	0.1274a		
	PhPI1	0.2096a		
	ScMADS658	0.3246a		
	HcPI1	1.2928c		
	HcPI2	0.6056c		
	CsSEP3	0.0420c	−7,716.40	0.176
	MpMADS13	0.1172a		
	HcSEP1	0.0669b		
	HcSEP3	0.2804a		

a, Not significant; *b*, significant; *c*, very significant

onymous substitution; only positive selection can produce the nonsynonymous (d_N) to synonymous (d_S) substitution ratio (ω) significantly larger than 1. The tree and codon alignment employed in phylogenetic analysis were used here. PAML3.14beta3 software was used under branch-specific and site-specific model (Yang 1998; Yang and Nielsen 2002). Thus, whether candidate class A, B and E genes in *Chloranthus spicatus* have made their function derived correlating to the lack of perianths is tested (Table 1).

Results

Sequence analysis of *CsAP1*, *CsAP3* and *CsSEP3*

Conceptual translation reveals that CsAP1 has 235 amino acids (aa), CsAP3 219 aa and CsSEP3 241 aa. According to a multiple alignment including APETALA1, APETALA3, AGAMOUS and SEPALLATA3 from *Arabidopsis*, CsAP1 has a 57-aa MADS domain, 33-aa I domain, 68-aa K domain and 77-aa C domain (Fig. 2). CsSEP3 has a 57-aa MADS domain, 32-aa I domain, 68-aa K domain and 84-aa C domain (Fig. 3). A partial cDNA sequence of *CsAP3* has been cloned previously (Kramer and Irish 2000). Here, the complete coding sequence of *CsAP3* is reported. CsAP3 consists of 57-aa MADS domain, 29-aa I domain, 68-aa K domain and 65-aa C domain. CsAP3 shows a PI-derived motif, but, remarkably, only a partial paleoAP3 motif (Kramer et al. 1998), which is truncated by a point mutation from guanine (G) to thymine (T) at the position nts 658 in the cDNA, causing the change from an otherwise highly conserved aspartic acid (D) codon (GAG) to a premature stop codon (TAG) (Fig. 4). Our data corroborate the view (Kramer et al. 1998) that the unusual *CsAP3* cDNA sequence is genuine and does not simply reflect a sequence error.

The most recent common ancestor of mosses and vascular plants contained at least one MIKC^c-type and one

Fig. 2 An alignment of the amino acid sequence of *CsAP1* with close homologues. MADS domain, K domain (consisting of K1, K2, K3 motif in *shade*) and C-terminal motif are showed by *double*, *single* and *wave underlines*, respectively. Gly-110 and other conservative charged amino acids are highlighted in *bold*

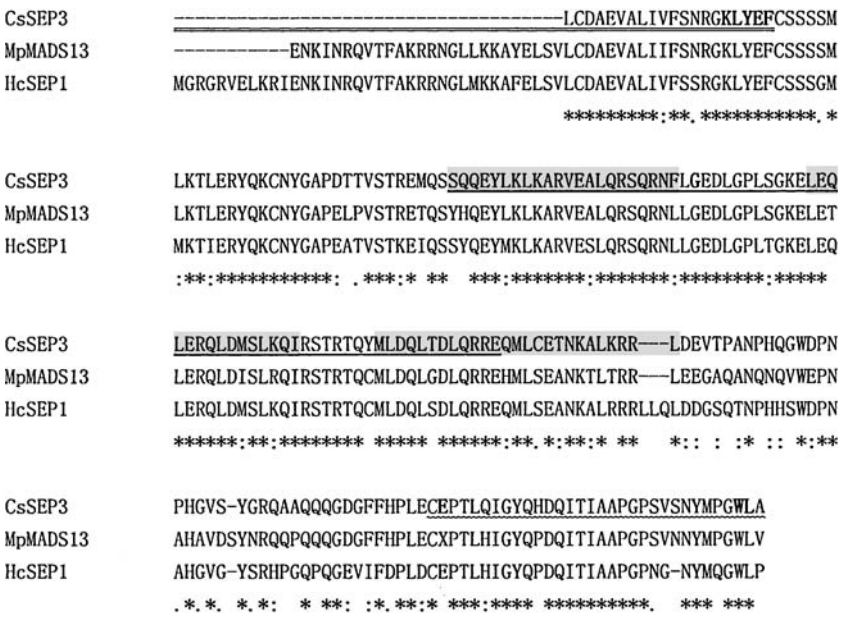

Fig. 3 An alignment of conceptual amino acid sequence of *CsSEP3* with MpMADS13 from *Magnolia* and HcSEP1 from *Houttuynia*. Its MADS domain and K domain (consisting of K1, K2, K3 motif in *shade*) and C-terminus are shown by *double*, *single* and *wave underlines*, respectively. The βII-strand, Gly-110 and the Glu and WL motif in the C-terminal motif are highlighted in *bold*

MIKC*-type gene. MIKC*-type genes have a relatively long I domain, for example, 62 aa in PPM3 and 87 aa in PpMADS2 (Henschel et al. 2002). The I domain can specify and strengthen dimerization. The K domain also contributes to the specific and strong dimerization and can be subdivided into K1, K2 and K3 motifs (Yang et al. 2003). Sequence analysis clearly indicates that *CsAP1*, *CsAP3* and *CsSEP3* are of the MIKC^c-type (Figs. 2, 3). The so-called Gly (G)-110, which may facilitate the formation of a loop between K1 and K2 helices in all MIKC^c-type proteins (Yang et al. 2003), also occurs in CsAP1, CsAP3 and CsSEP3.

CsAP1 possesses many charged residues in the K region and has the conserved hydrophobic C-terminal motif "L/MPPWML" (Litt and Irish 2003). Its C-terminal motif may be responsible for the transcriptional activation and formation of higher complexes (Cho et al. 1999; Honma and Goto 2001; Lamb and Irish 2003). CsSEP3 has the C-terminal motif "MPGWL," which is conserved in SEP3-like proteins but somewhat differs from the "PGWML" in SEP1/2/4-like proteins (Litt and Irish 2003; Ditta et al. 2004). Additionally, the characteristic amino acid Glu (E) flanks the C-terminal motif in SEP3-like proteins, whereas it is N (Asn) in SEP1/2/4-like proteins (unpublished data).

In CsAP3, Thr-97 is uncharged while Arg-102 is basic; on the other hand, in CsPI, Glu-97 is acidic while Gln-102 is uncharged. Thus, CsAP3 and CsPI may function as heterodimers rather than homodimers, resembling the I+5

CsAP3	YG-TH*LCLG	TATGGT---ACCCATTAGCTGCGCCTTGGATAA
TtsAP3	YG-TQDLRLA	TATGGG---ACCCAGGATCTGCGCCTAGCATAA
AreAP3	YG-THDLRLA	TATGGA---ACCCATGATCTGCGCCTCGCATAG
SrhAP3	YG-THDLRLA	TATGGG---ACCCATGATCTGCGCCTTGCTTAA
AeAP3-1	YG-IHDLRLA	TATGGG---ATCCACGATCTGCGCCTTGCATAA
PhAP3	YG-IYDLRLA	TACGGA---ATTTATGATCTTCGCCTTGCATGA
HtcAP3	YG-IPDLRFG	CGTTTT---GGTTAAGACATGTGTCCTTCATAA
HhMADS1	YG-PHDLRLA	TATGGG---CCACATGATCTTCGTCTTGCATGA
TcAP3	YS-SHDLRLA	TACAGT---TCTCATGACCTTCGCCTGGCATGA
SmAP3	YG-FHELRLA	TATGGA---TTCCATGAGTTGCGGCTCGCATGA
ZmSILKY1	YG-FHDLRLG	TACGGC---TTCCACGACCTCCGCCTGGGCTAG
OsMADS16	YGGNHDLRLG	TACGGCGGCAACCACGACCTGCGCCTCGGTTGA
IhAP3-1	YS-THELRLG	TATAGC---ACCCACGAGTTACGTCTTGGCTAA
IhAP3-2A	FD-FHDLRLV	TTCGAT---TTTCATGATTTAAGGCTTGTGTAA
IhAP3-2B	FD-FHDLRLV	TTCGAT---TTTCATGATTTAAGGCTTGTGTAA
IhAP3-3A	YN-FHDLRLG	TACAAC---TTTCATGATTTACGCCTTGGCTAA
IhAP3-3B	YN-FHDLRLG	TACAAC---TTTCATGATTTACGCCTAGGCTAA
NymAP3	YG-CHDLILG	TATGGG---TGCCACGATTTAATTCTAGGATGA

Fig. 4 The truncation occurred in the paleoAP3 motif in CsAP3 and its origin. The homologues from basal angiosperms are compared in terms of the paleoAP3 motif. The point mutation highlighted in underline causes the truncation, whereas subsequent region can be translated into conservative amino acids

ionic interaction observed in leucine zipper dimers (Yang et al. 2003). However, the majority of Magnoliid dicot and ANITA grade AP3 and PI homologues encode acidic residues at position 97 and basic residues at position 102; thus, they may have the capacity to function as homodimers (Stellari et al. 2003). Additionally, CsAP3 and CsPI both possess Asn-98, which may contribute to the strength and specificity of heterodimer formation (Yang et al. 2003).

Phylogenetic analysis of *CsAP1*, *CsAP3* and *CsSEP3*

In the NJ tree, with the designated outgroup CRM3 from fern *Ceratopteris* (Fig. 5), we have identified at least six major clades ("gene subfamilies"). These clades are represented by *PI*, *AP3*, *AG*, *STK*, *AP1* and *SEP1/2/3/4* from *Arabidopsis*, respectively, and correspond well to MADS-box gene clades defined previously in the literature (Becker and Theissen 2003; Litt and Irish 2003; Stellari et al. 2003; Kramer et al. 2004a, and references cited therein). These clades obtained high bootstrap values and hence may quite reliably document the phylogeny of MIKC^c-type genes. The tree reflects the duplication between the *AP3* clade and the *PI* clade, and between the *AG* and the *STK* clade, and the close relationship between the *AP1* and the *SEP* clade also demonstrated in previous studies. The phylogenies in terms of class A (*AP1*), B (*AP3+PI*), C (*AG*), D (*STK*) and E (*SEP*) genes also reoccur. *Chloranthus* has members in all these six clades: *CsPI*, *CsAP3*, *CsAG1*, *CsAG2*, *CsAP1* and *CsSEP3*. Homologues from all clades have also been isolated from *Magnolia* and *Houttuynia* and at least some from *Saururus* and *Peperomia*. Very similar tree topologies were observed in MP and ML trees (unpublished data). Therefore, *Chloranthus* possesses reasonable candidates for all classes (A–E) of floral homeotic genes.

Expression analysis of *CsAP1*, *CsAP3* and *CsSEP3*

In order to get a clue about the function of *CsAP1*, *CsAP3* and *CsSEP3*, the accumulation of their mRNAs was analyzed in detail by in situ hybridization. To facilitate the description of the observed expression patterns, the designation of developmental stages proposed by Endress (1987) was used.

The *CsAP1* transcripts are firstly detected in the dome-shaped spike primordia and the bract primordia, but not in the surrounding leaves (Fig. 6a). Later, the signal is detected weakly in the young bracts and strongly in the floral primordia (Fig. 6b). Subsequently, very strong signal can be detected in the floral primordia, and no signal is produced in the bracts (Fig. 6c, floral primordium morphologically undifferentiated; Fig. 6d, initiation of androecium subdivision). Strong signal can be detected in the androecial primordia and the gynoecial primordia (Fig. 6e). When the thecae and the ovules are differentiated, there are more

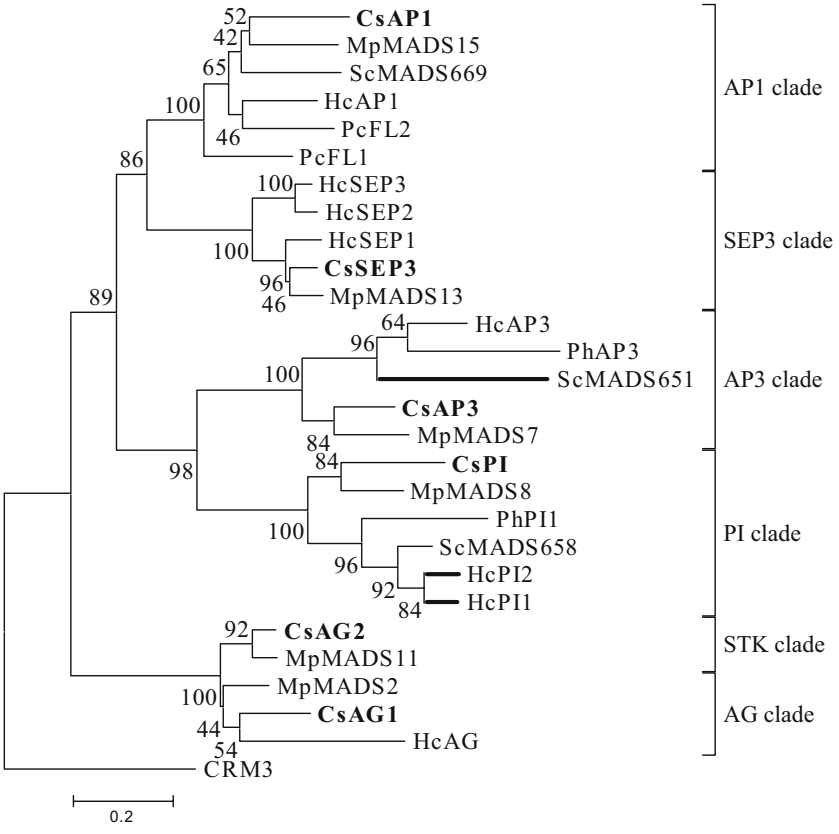

Fig. 5 The NJ tree constructed using the MADS, I and K domains under the Poisson distance and complete deletion. The numbers above the nodes give the bootstrap values from 100 replicates. Clades are labeled by *brackets* at the *right margin*. The *lines* highlighted in *bold* in the tree represents under relaxed or positive selection. CsAP1, CsAP3 and CsSEP3 proteins and others from *Chloranthus* are shown in *bold*

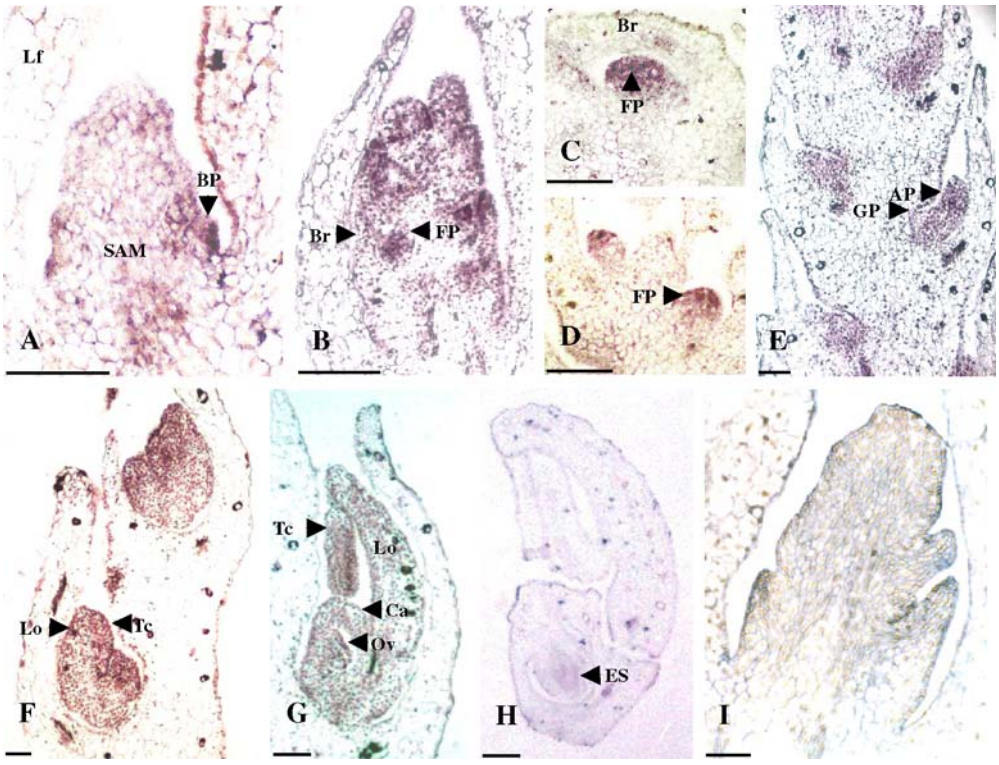

Fig. 6 The accumulation of the *CsAP1* mRNA in the flowers of *C. spicatus*. **a**–**h** Signal produced by antisense probe; **i** sections hybridized by sense probe. **a** Spike primordia with emerging bract primordia. **b** Emerging floral primordia. **c**, **d** Developing floral primordia. **e** Stamen and carpel primordia shaped. **f** Androecial lobes and thecae. **g** Ovules differentiated. **h** Embryo sacs shaped. *Bar*=100 μm. *SP* Spike primordia, *Lf* leaves, *BP* bract primordia, *Br* bracts, *FP* floral primordia, *AP* androecial primordia, *GP* gynoecial primordia, *Lo* lobes, *Tc* thecae, *Ca* carpels, *Ov* ovules, *ES* embryo sacs

transcripts in the thecae and the ovules than in the lobes and the carpels (Fig. 6f,g). Additionally, weak signal can be seen in the embryo sacs (Fig. 6h).

In contrast to *CsAP1*, the *CsAP3* transcripts are not detected in the dome-shaped spike primordia, bract primordia and leaves (Fig. 7a). The expression of *CsAP3* is first detectable in the floral primordia, whereas no transcripts are detectable in the young bracts (Fig. 7b,c). The transcripts in floral primordia are gradually confined to the apical parts where the androecial meristems may initiate (Fig. 7d). Later, it is obvious that the *CsAP3* transcripts are produced in the androecial primordia but not in the gynoecial primordia (Fig. 7e–g). It is noted that no signal occurs in early gynoecial primordia (Fig. 7f) and later chair-like gynoecial primordia (Fig. 7g,h). It is also observed that relatively strong signal occurs at the abaxial to the adaxial part of the androecial primordia (Fig. 7g,h). When the thecae and pollens are differentiated, strong signal can be detected in the thecae and pollens, whereas no transcripts are detected in the lobes and carpels (Fig. 7i–k).

The *CsSEP3* transcripts cannot be detected in the dome-shaped spike primordia and bract primordia (Fig. 8a). The strong signal can be firstly seen in the floral primordia, whereas no signal occurs in the young bracts (Fig. 8b,d). Later, the strong signal can be detected in the nascent androecial and gynoecial primordia (Fig. 8e–g). When thecae and ovule are differentiated, a strong signal is detectable in the thecae and ovule, but no signal can be detected in the lobes and carpels (Fig. 8h,i).

Evolutionary force analysis

Since the *CsAP3* gene does not have an intact paleoAP3 motif, it is reasonable to hypothesize that this gene, and possibly other ABCE genes from the same species, might have lost some of their original function(s) and thus have evolved under relaxed or changed constraint. To test this hypothesis, we conducted a series of likelihood ratio tests (LRTs) by using the codeml program in the PAML 3.13 package. Our first test compares the one-ratio model M0 with the free-ratio model. The one-ratio model, which assumes a single d_N/d_S (nonsynonymous vs synonymous substitutions per site) value, ω, for the entire tree, leads to a log-likelihood value (lnL) −7,723.41, with an estimate ω= 0.164. This low average ratio suggests that purifying selection is dominating the evolution of the MADS-box genes analyzed. The free-ratio model, which assumes different ω values for each branch within the input tree, results in a log-likelihood value −7,659.55. Thus, the free-ratio model fits the data significantly better than the one-ratio model because the LRT statistic is 2×(diff. lnL)=2×63.86=127.72 (P<0.005, df=55−1=54). This result suggests that, although in general these MADS-box genes have evolved under strong purifying selection, the evolution forces are not quite the same for all the branches.

To test whether the genes concerned have evolved under changed constraints, the two-ratio test, which assigns two different ω values for the foreground lineage (taxa concerned) and the background lineages (all other taxa), was

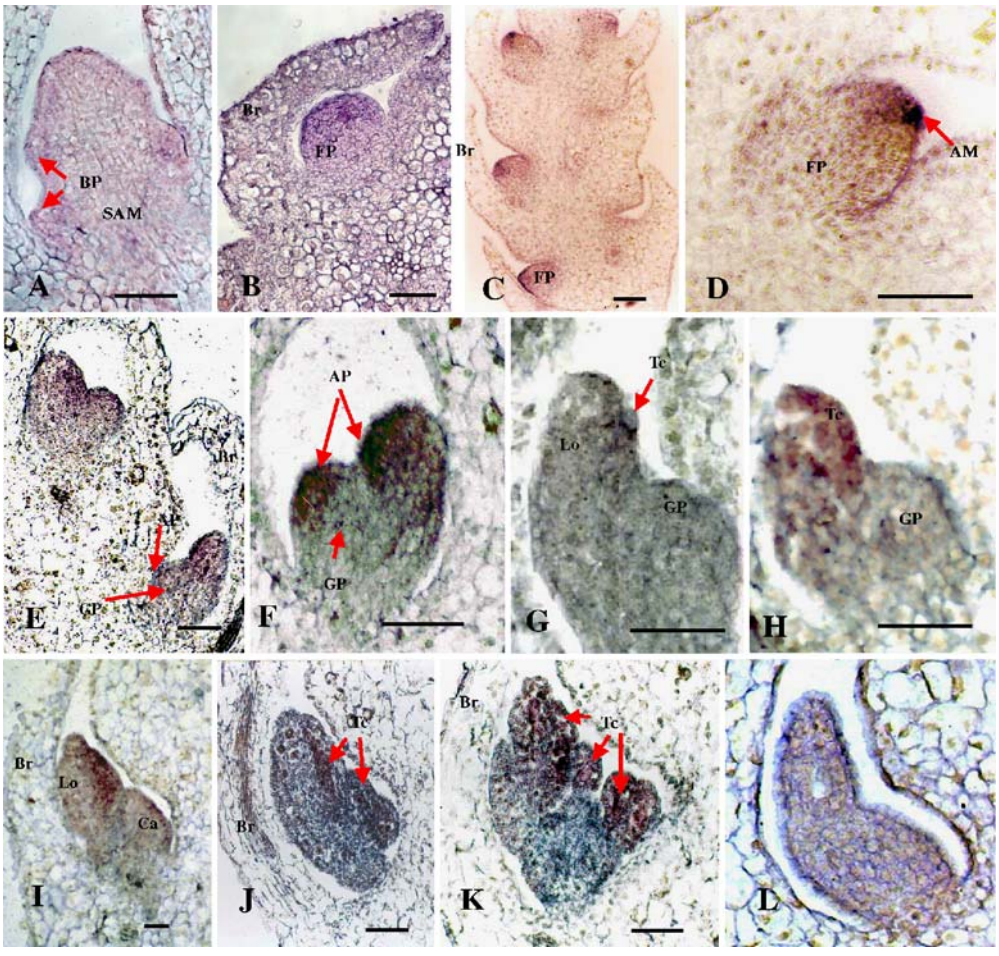

Fig. 7 The accumulation of the *CsAP3* mRNA in the flowers of *C. spicatus*. **a**–**k** Signal produce by antisense probe. **l** Sections hybridized by sense probe. *AM* Androecial meristems, *Po* pollens

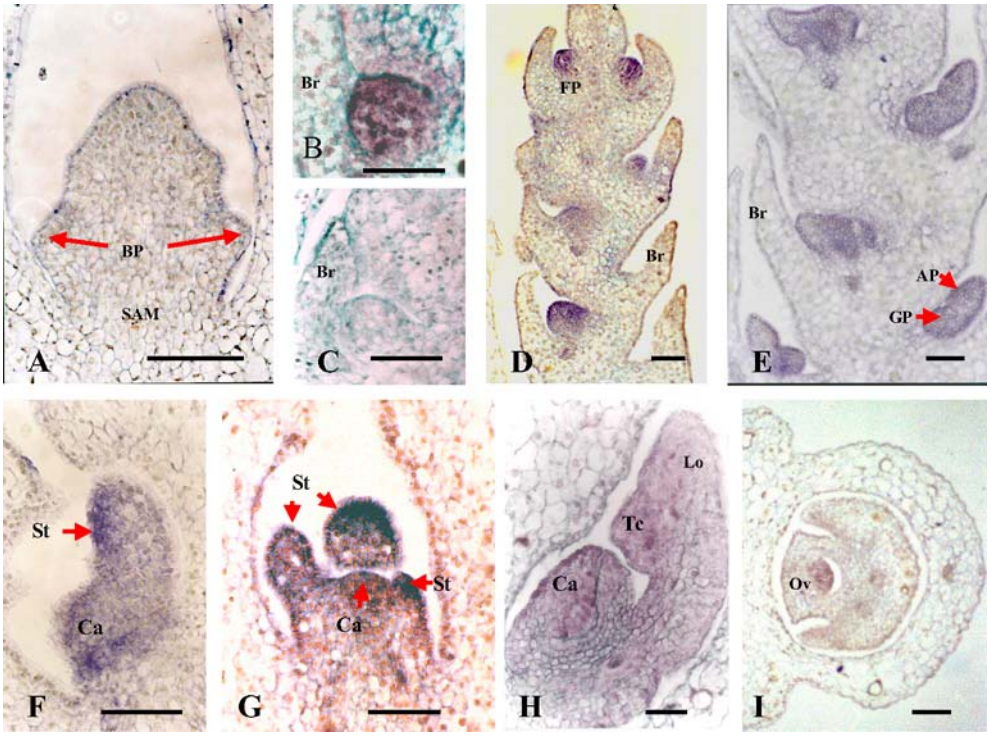

Fig. 8 The accumulation of the *CsSEP3* mRNA in the flowers of *C. spicatus*. **a**, **b**, **d**–**i** Signal produce by antisense probe; **c** sections hybridized by sense probe

conducted and compared with the one-ratio test. When *CsAP1*, *CsAP3*, *CsPI*, *CsAG1*, *CsAG2* and *CsSEP3*, as well as several other genes such as *ScMADS651*, *MpMADS11*, *HcAP3*, *HcPI1*, *HcPI2*, *HcSEP1* and *HcSEP2*, are individually designated as the foreground branch, we found that the two-ratio model fits the data significantly better than the one-ratio model. This suggests that the values of $\omega_{foreground}$ for these genes are indeed different from $\omega_{background}$. However, the ω_f values for the six *Chloranthus* genes are all smaller than ω_b and 1, indicative of more intensified purifying selection for them.

It is worth mentioning, however, that although the overall sequences of the six *Chloranthus* genes have evolved under strong selective constraint, there is no guarantee that every residue(s) in each sequence has evolved under the same constraint. While most residues have evolved under strong purifying selection, some might have evolved under relaxed or altered constraints, or even under positive selection. For this reason, we have conducted a third likelihood ratio test to compare the site-specific model M3 to the branch-site model MB. The site-specific "discrete" model M3, with K=2 or 3, assumes that the ω ratio varies among codon sites, and that there are two or three site classes in the sequence. The first class of sites is highly conserved, with a smaller ω value, whereas the second and the third classes of sites are weakly conserved or even under positive selection, with bigger ω values. The branch-site model is an extension to the M3 model, but adds a new site class for the foreground sequence. In our case, both M3 and MB models detect no sites under positive selection along the branch leading to *CsAP1*, *CsAP3*, *CsPI*, *CsAG1*, *CsAG2* and *CsSEP3* (data not shown), suggesting that all these *Chloranthus* genes have evolved under functional constraints.

Discussion

In this study, *CsAP1*, *CsAP3* and *CsSEP3* were isolated from the perianthless basal angiosperm *C. spicatus*. Sequence analyses and phylogeny reconstructions strongly support the close phylogenetic relationships of *CsAP1*, *CsAP3* and *CsSEP3* to candidate class A, class B and class E genes, respectively. Expression patterns determined by in situ hybridization indicate that they are relatively extensively expressed during flower development. Therefore, it appears quite likely that they all contribute to flower development in *C. spicatus*, and that the most recent common ancestor (MRCA) of *Chloranthus* and eudicots (which was the MRCA of the vast majority of extant angiosperm species) contained already *AP1*-like (or *SQUA*-like), *AP3*-like (or *DEF*-like) as well as *SEP3*-like (or *AGL2*-like) MIKC-type MADS-box genes. Whether these genes were involved in the specification of floral meristem and stamen and carpel identity, respectively, or some (also) already in the formation of floral perianth organs remain to be seen.

CsAP1 function may reflect the ancestral function of *AP1*/*SQUA*

The expression pattern of *AP1*/*SQUA* genes is quite diverse (Huijser et al. 1992; Menzel et al. 1996; Hardenack et al. 1994; Sung et al. 1999; Yu and Goh 2000; Hart and Hannapel 2002) and deserves a more detailed analysis with respect to function and evolution. Based on previous studies, we assume the following:

(1) Class A (*SQUA*/*AP1*) gene expression in vegetative tissue is functionally irrelevant since all transgenic plants ectopically expressing A genes confer no phenotypic change in vegetative organs and class A gene loss of function mutants displays no vegetative phenotype either.
(2) As also noted previously (Theissen et al. 2000; Kramer and Hall 2005), *SQUA*-like gene expression in inflorescence/flower meristems may reflect the ancestral function of these genes in specifying meristem identity since nearly all transgenic plants changing *SQUA*-like gene expression alter the flowering time.
(3) *AP1*/*SQUA*-like gene expression in sepal/petal primordia may represent its redundant function with paralogues or overlapping function with other partners such as *AGL24* (Ferrandiz et al. 2000; Yu et al. 2004) since nearly all *AP1*/*SQUA*-like genes are expressed in sepal/petal primordial, whereas some transgenic plants of *AP1*/*SQUA*-like genes confer homeotic mutations and some do not.

Evolution of *SQUA*-like genes by gene duplication, sequence divergence and fixation in the lineage that led to *Arabidopsis* resulted in *AP1*, *CAULIFLOWER* (*CAL*) and *FRUITFULL* (*FUL*) (Irish 2003; Litt and Irish 2003), which have been recruited for different functions, namely, specifying floral meristems and sepal/petal identity (Mandel et al. 1992), specifying floral meristem identity and specifying aspects of carpel structure as well as inflorescence meristem identity (Kempin et al. 1995; Ferrandiz et al. 2000), respectively. Maybe these genes have functionally diversified in a demand for providing the capability to specify increasingly complex structures during the evolution of flowering plants. For example, the latest study on the overexpression of *AGL24* in the *ap1* mutant background showed that *ap1 agl24* double mutants recover perianth organ development, suggesting that *AP1* function is not essential for the process (Yu et al. 2004).

Within basal angiosperms, very few *AP1*/*SQUA*-like genes have been identified, and their expression studies are almost unknown. In this study, *CsAP1* expression has been shown in inflorescence and floral meristems, young bracts, the developing stamens and carpels and even in ovules. The expression pattern of *CsAP1* may reflect the ancestral function of *SQUA*-like genes in the specification of inflorescence and floral meristems, but a strict functional

analysis of *CsAP1* will require further studies on protein–protein interactions, the identification of target genes and the determination of mutant phenotypes.

CsAP3 functions in the perianthless basal angiosperm flower

DEF from *Antirrhinum* (Sommer et al. 1990), *AP3* from *Arabidopsis* (Jack et al. 1992), *OsMADS4* from rice (*Oryza*) (Kang et al. 1998) and *SILKY1* from maize (*Zea*) (Ambrose et al. 2000) are all expressed in petals or lodicules and stamens, respectively, to specify the identity of these organs (Winter et al. 2002), suggesting conservation of expression patterns and functions of *AP3*-like genes throughout angiosperms.

Class B genes include two closely related lineages, represented by *AP3* and *PI* in *Arabidopsis* and by *DEF* and *GLO* in *Antirrhinum*, respectively. These two lineages, *AP3*/*DEF* and *PI*/*GLO*, were produced by a gene duplication that occurred after the existence of the most recent common ancestor of seed plants but obviously before the radiation of the angiosperms (Winter et al. 2002; Becker and Theissen 2003). As B genes in gnetophytes and conifers (gymnosperms) are exclusively expressed in male reproductive structures (Mouradov et al. 1999; Sundstrom et al. 1999; Winter et al. 1999; Fukui et al. 2001), it was suggested that B gene expression was originally restricted to the progenitor of stamens (Baum and Whitlock 1999). Expression studies of class B genes in several basal eudicot species suggested that their function may vary within the eudicot lineage (Kramer and Irish 1999), which is in line with the origin of novel genes in other gene duplication events just before the radiation of the core eudicots (Kramer et al. 1998, 2004a; Litt and Irish 2003). Similar to the observed history of the *AP1* gene duplication, the ancestral *AP3* lineage, named the paleo*AP3* lineage, underwent also a gene duplication event, followed by a frameshift mutation in one of the copies, producing the eu*AP3* and *TM6* (paleo*AP3*) lineages (Kramer et al. 1998; Stellari et al. 2003; Vandenbussche et al. 2003). The function of genes in the paleo*AP3* lineage is little known, partly due to the fact that *Arabidopsis* has an orthologue of eu*AP3* (*AP3* itself) but lacks a paleo*AP3* representative. Recently, it was shown that *Petunia hybrida DEFICIENS* [*PhDEF*, also known as green petals (*GP*)] can contribute towards both petal and stamen identity, but that *PhTM6* is functional only in the stamens (Vandenbussche et al. 2004). The expression pattern of *CsAP3* in the perianthless flower of *C. spicatus* is very strictly confined to the stamens, strongly supporting the view that *AP3*-like genes have a conserved expression domain in male reproductive organs that traces back to the MRCA of extant seed plants (Theissen et al. 2000) and ancestral function in differentiation between male and female reproductive organs (Winter et al. 1999).

Implications for the lack of perianth in *C. spicatus*

The classic ABC model proposes that class A genes are responsible for the identity of sepals and class A and B genes for petal identity (Coen and Meyerowitz 1991). Recently, class E genes were shown to be also necessary to specify the identity of perianth organs (Pelaz et al. 2000; Theissen 2001; Ditta et al. 2004). Candidates of these genes might have been recruited for the development of perianths in monocots and basal angiosperms (Ma and dePamphilis 2000; Theissen et al. 2000; Whipple et al. 2004). Analyses of phylogeny and expression pattern demonstrate that *CsAP1*, *CsAP3* and *CsSEP3* are candidate class A, B and E genes in *C. spicatus*, and that they function in the development of flowers in this perianthless species. Furthermore, *CsPI*, *CsAG1* and *CsAG2* may be candidate class B, C and D genes necessary to floral development. Thus, some core functions of ABCDE genes may also be conserved in this species.

Taken together, our data are compatible with the view that the perianthless state of *C. spicatus* is derived rather than ancestral, in line with the hypothesis that the most recent common ancestor of all extant angiosperms had a perianth, even though the identity of respective organs is unclear (Theissen et al. 2000).

The question remains as to whether the loss of the perianth in the Chloranthaceae is causally linked to changes in floral homeotic genes or vice versa. According to the ABC model, floral homeotic genes specify the identity of floral organs, but they usually do not determine the presence or absence of these organs in the first place. Only occasionally are complete floral whorls absent in class A, B or C gene mutants (http://www.arabidopsis.org/; Bowman et al. 1989; Schwarz-Sommer et al. 1990; Coen and Meyerowitz 1991), but to the best of our knowledge, never are perianth organs absent in class B gene mutants, and never the complete perianth is absent in any floral homeotic mutant. Even though we cannot exclude such a scenario for *Chloranthus*, we, therefore, currently prefer the "null hypothesis" that there is, for example, no *CsAP3* expression in petals simply because there are no petals, and that genetic changes other than mutations in floral homeotic genes have caused the loss of the perianth within Chloranthaceae. It would be interesting to investigate, therefore, whether there are specific *cis*-regulatory elements controlling *AP3* gene expression in petals, and whether they show hallmarks of degeneration in *CsAP3*. Studies on the *CsAP3* promoter might thus be quite informative. Along similar lines of reasoning, it is interesting to see that the paleoAP3 motif at the C-terminal end of CsAP3 is truncated (Kramer and Irish 2000; this work). This might be due to the fact that integrity of the motif is important for specifying petals, but not for stamens. Recently, Lamb and Irish (2003) presented data to suggest that a paleoAP3 motif is sufficient (and thus a euAP3 motif not required) for stamen development in *Arabidopsis*, whereas a paleoAP3 motif is not sufficient

(and a euAP3 motif required) for petal development. However, Whipple et al. (2004) reported that an *AP3*-like gene from maize with a paleoAP3 rather than an euAP3 motif can substitute *ap3* mutants from *Arabidopsis* even with respect to petal development, suggesting that a paleoAP3 motif suffices for both petal and stamen development in *Arabidopsis*. Maybe in Chloranthaceae, a full paleoAP3 motif is only required for petal, but not for stamen development. 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. Alternatively, even stamen development might require a full paleoAP3 motif, and there is a second *AP3*-like gene in the *C. spicatus* genome with a full paleoAP3 motif that has not been isolated so far.

Obviously, the lack of the perianth does not affect the distinctive expression of *CsAP3* in stamens. Conservation of the ancestral function in the specification of stamen identity might largely explain why we could not detect relaxed purifying selection for *CsAP3* sequence evolution.

Chloranthus is another case in point that in angiosperms the development of perianths is relatively "open" compared to that of fertile organs (Endress 2001b). Thus, the genetic network for petal development might be quite independent from that of fertile organ development, and the interactions among class A, B and E genes might be more flexible than those among class B, C and E genes.

Conclusively, according to the evolutionary force shown by MADS, I and K domains, it appears likely that known candidate classes A, B, C, D and E genes in *Chloranthus* conserved their ancestral function rather than adopted a derived function. According to the "floral quartet" hypothesis, the corresponding proteins are expected to form functional complexes. However, the potential influence conferred by unknown duplicates should be considered.

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