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Expression Analysis of Flower MADS-box Genes in Saffron *Crocus* (*Crocus sativus* L.) Supports a Modified ABCDE Model

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ABSTRACT

Crocus sativus L. is a monocot triploid species, member of the family Iridaceae, and is considered to be the highest priced spice in the world. It is cultivated for its flowers and more specifically for its red stigmas. The flower of *Crocus* is bisexual and it is sterile. The dry form of stigmas constitutes saffron. In order to uncover and understand the molecular mechanisms controlling flower development in cultivated *Crocus* and its relative wild progenitor species, and characterize a number of *Crocus* flower mutants we have cloned and characterized different full length cDNA sequences encoding MADS-box transcription factors belonging to the different ABC and E-class MADS box proteins. Herein, we review the isolation of *Crocus* MADS box genes and primarily discuss their expression patterns in leaves and the four flower organs: outer tepals, inner tepals, stamens and carpels. Expression analysis of the isolated MADS box genes support the hypothesis that a modified ABCDE model in the flower of *Crocus* is responsible for the development of the different *Crocus* flower organs and the transformation of the sepals and petals into tepaloid organs, designated outer tepals and inner tepals, respectively.

Keywords: flower, gene expression, MADS-box genes, monocots

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INTRODUCTION

Flowering plants or angiosperms represent one of the most successful and diverse groups of organisms on the planet, with more than 250,000 extant species in the wild and thousands more varieties generated by horticulturists through hybridization and other breeding methods. Although angiosperms such as orchids, roses and snapdragons have very characteristic flowers, most flowers contain four organ types and they have highly conserved developmental molecular mechanisms (Krizek and Fletcher 2005). The majority of flowers have four types of floral organs: two outer whorls of sterile organs, the sepals and petals (also known as the perianth), and two inner whorls of fertile organs, the male stamens and female carpels, with the carpels positioned centrally. Although the main characteristics of angiosperm flowers are generally conserved, the vast morphological diversity suggests a high degree of plasticity in the genetic control of floral development. Variation is observed in every aspect of floral architecture, including phyllotaxy, merosity, floral symmetry and floral organ identity. In-depth analyses of model species such as *Arabidopsis thaliana* and *Antirrhinum majus* have contributed significantly to our understanding of the genetic pathways that control these morphological components. By using this work as a foundation for comparative studies, a picture is gradually coming into focus of how alterations in floral genetic programs have contributed to the evolution of floral architecture.

Forward mutagenesis studies of that two model species uncovered an intriguing series of homeotic floral mutants (Komaki *et al*. 1988; Bowman *et al*. 1989; Coen and Meyerowitz 1991). In both taxa, the mutants appeared to fall into similar classes named A, B and C: mutations that affected sepal and petal identity were placed into what was termed the A-class; those that affected petal and stamen identity, the B-class; and those that affected stamen and carpel identity, the C-class. For instance, B mutants exhibited the transformation of petals into sepals and stamens in carpels (Bowman *et al*. 1989; Carpenter and Coen 1990). Analysis of double and triple mutants (Bowman *et al*. 1991) suggested a simple and elegant model that explained the major aspects of genetic interactions among the loci; this became known as the ABC model (Coen and Meyerowitz 1991). Fundamentally, the ABC model holds that the overlapping domains of three classes of gene activity, referred to as A, B and C, produce a combinatorial code that determines floral organ identity in successive whorls of the developing flower. Another critical component of the ABC program is that A and C functions are mutually exclusive (Bowman *et al*. 1991), such that elimination of C gene activity causes

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Invited Mini-Review

Table 1 Homologues of the major A, B, C, D, E classes of MADs-box genes from *Crocus*, the two dicots model species *Arabidopsis* and *Antirrhinum* and the monocot model *Oryza*. The number of crocus homologues for each class of MADs-box gene obtained from *Crocus* and method followed to obtain each sequence is also indicated.

Gene class	*Arabidopsis*	*Antirrhinum*	*Oryza*	*Crocus*	Number of isolated *Crocus* homologues	Cloning method (*Crocus*)	Reference (*Crocus*)
A	*APETALA1*	*SQUAMOSA*	*nk*	*nk*	-	-	-
B	*APETALA3*	*DEFICIENS*	*SUPERWOMAN1*	*CsatAP3/DEF*	2	5′ 3′ RACE	Tsaftaris *et al.* 2006
	PISTILLATA	*GLOBOSA*	*OsMADS2, OsMADS4*	*CsatPI/GLO*	5	5′ 3′ RACE	Kalivas *et al.* 2007
C	*AGAMOUS*	*FARINELI*	*OsMADS3*	*CsatAG1*	2	5′ 3′ RACE	Tsaftaris *et al.* 2005
D	*SEEDSTICK*	*nk*	*OsMADS13*	*nk*	-	-	-
E	*SEPALLATA1, 2, 3*	*DEFH49, 72*	*LHS1, OsMADS5, 34*	*CsatSEP3*	4	famRCA-RACE	Tsaftaris *et al.*, unpublished
	AGAMOUS-LIKE6	-	-	*CsatAGL6*	2	5′ 3′ RACE	Tsaftaris *et al.*, unpublished
			RAP1	*Csat AP1/FUL*	3	5′ 3′ RACE and RCA-RACE	Tsaftaris *et al.* 2004

nk: not known

the A domain to expand and *vice versa* (Drews *et al*. 1991; Gustafson-Brown *et al*. 1994).

Later, based on protein - protein interactions, the ABC model was extended to the ABCDE model (Theissen and Saedler 2001). Whereas the E-class genes together with the B and C genes control stamen formation, the C- and E-class genes regulates carpel formation and the D-class genes are involved in ovule development. According to the model, two dimers of each tetramer recognize two different DNA sites (termed CArG-boxes) on the same strand of DNA, which are brought into close proximity by DNA bending.

With the exception of APETALA2, all of the organ identity genes identified to date are members of the pan-eukaryotic MADS transcription factor family (Becker *et al*. 2003; Messenguy and Dubois 2003). MADS-box genes are characterized by the highly conserved MADS-box domain and can be divided into the type I and type II main lineages that are present in plants, animals and fungi (Theissen *et al*. 2000; De Bodt *et al*. 2003b). These lineages differ in the amino acid sequence of the MADS-box as well as in the domain structure of the predicted protein. Most of type II proteins exhibit a typical MIKC-structure where the MADS-domain is followed by a short I (intervening) domain, a well conserved K (keratin-like) domain and a variable C-terminal region, while type I proteins lack the K-domain, forming a structure of a MADS-box followed by a rather undefined and length-variable C-domain (De Bodt *et al*. 2003a).

Although monocot flowers contain stamens and carpels, they differ from eudicot flowers in the type of organs that are present in the outer whorls. Liliaceae family members often have two outer whorls of showy petal-like tepal organs, whereas grass flowers have paleas, lemmas and lodicules in place of sepals and petals. A modified ABC model in which B function is present in whorls 1, 2 and 3 has been proposed to explain the presence of tepals in Liliaceae flowers (van Tunen *et al*. 1993b). This is supported by the observation of B-class *AP3/DEF*-like and *PI/GLO*-like gene expression in the outer three whorls of tulip flowers (Kanno *et al*. 2003), and the absence or low expression of *AP3/DEF*-like genes in the outermost whorl of other monocots that produce distinct sepals and petals (Ochiai *et al*. 2004). Studies in *Zea mays* and *Oryza sativa* indicate that B-class genes have similar roles in grass and eudicot flowers (Whipple *et al*. 2004). Loss of the single *AP3/DEF*-like gene in maize *SILKY 1* and *O. sativa SUPERWOMAN 1*, results in replacement of lodicules by paleas or lemmas, or palea-like organs, respectively, and the replacement of stamens by carpels (Ambrose *et al*. 2000; Nagasawa *et al*. 2003). These homeotic transformations are similar to those observed in *Arabidopsis* and *Antirrhinum* B-class mutants, indicating that paleas and lemmas are homologous to sepals, and lodicules are homologous to petals. Maize contains two potential C-class *AG/PLE*-like genes (*ZAG1* and *ZMM2*), but redundancy makes it difficult to determine their exact roles (Mena *et al*. 1996). Mutations in ZAG1 affect floral determinacy but not organ identity. Carpels are still produced in rice plants with reduced expression of the *AG/PLE*-like gene *MADS3* (Kang *et al*. 1998), indicating that other factors are required for carpel specification in grasses. The *YABBY* gene *DROOPING LEAF* (DL) is one such factor (panel b), as mutations in DL result in complete homeotic transformations of carpels into stamens (Nagasawa *et al*. 2003; Yamaguchi *et al*. 2004). Although A-class *AP1/SQUA*-like genes have been identified in maize and rice, their roles in flower development are not well defined.

THE FLOWER OF *CROCUS*

Studying flower development and flower organs formation is not only significant for improving our understanding of basic regulatory mechanisms of flower initiation and organ identity, but could have practical applications in crops cultivated for their flowers. *Crocus*, an autumn flowering geophyte of the Mediterranean region, is an example of such a crop with flowers of economic importance. *Crocus* is a monocot triploid species belonging to the Iridaceae family, whose red stigmatic styles constitute saffron, a popular food additive with delicate aroma and attractive colour. Saffron has also medicinal properties and is used in the colouring industry. The flower of *Crocus* is bisexual and as sterile it has an exclusively vegetative propagation forming only 3-4 cormlets each season. Perianth consists of six petaloid tepals; three tepals in whorl 1 (outer tepals) and three tepals in whorl 2 (inner tepals, **Fig. 1B**). Androecium consists of three distinct stamens and the gynoecium consists of a single compound pistil with: three carpels, a single three-branched style, and an inferior ovary. Several phenotypic flower mutants have been described, such as flowers with larger numbers of styles and stamens as well as flowers without stamens (Grilli Caiola *et al.* 2004). *Crocus* blooms only once a year and is hand harvested. After mechanical separation of tepals, the stigmas are hand separated from carpels and dried. The size and the amount of individual stigmas collected from each flower influence total yield and quality of saffron. Between 70,000 and 200,000 flowers are needed to produce 1 kg of dried saffron, which equates 370–470 h of work. Consequently, the cultivation of this crop for its flowers and specifically its stigmas is very labour-intensive leading to high costs (Tsaftaris *et al.* 2004). Thus, understanding flower development in *Crocus* could reveal ways to increase yield and lower production costs since flower and more specifically isolated stigmas comprise the valuable commercial part of the plant.

Towards this goal we have cloned and characterized representatives of all MADS-box genes from *Crocus*, and very recently we obtained the *A*PETAL*A2* homologue from *Crocus* (unpublished data).

In order to isolate genes involved in flowering and flower development we developed and improved new methods and protocols such as the Rolling Cycle Amplification RACE (RCA-RACE) (Polidoros *et al.* 2006; Tsaf-

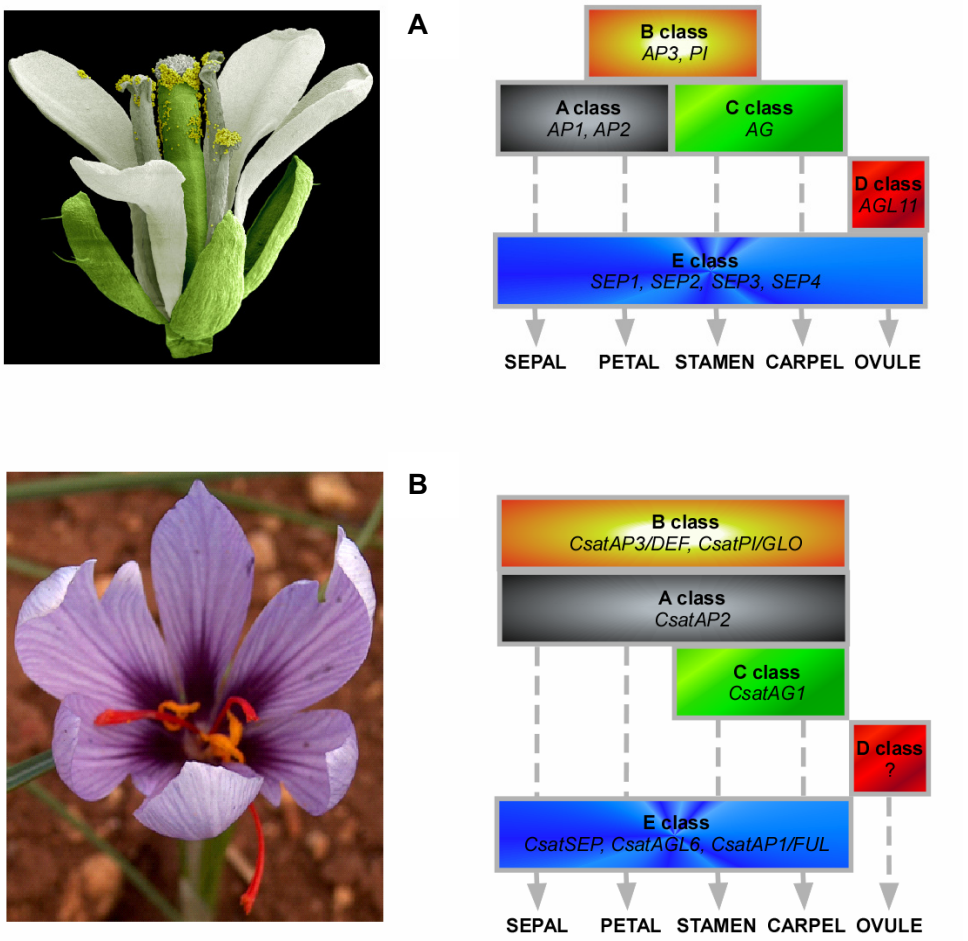

Fig. 1 (**A**) The classic ABC model (Coen and Meyerowitz 1991) for floral organ identity in *Arabidopsis* is shown as orange, grey and green boxes. Based on recent additions to the ABC model, D- and E-class genes are shown as red and blue boxes. (**B**) The modified ABC model for floral organ identity in *Crocus*.

taris *et al.* 2007, 2010) and Family RCA-RACE (Kalivas *et al.* 2010). Applying the above methods allowed us to isolate full length cDNAs and genomic sequences of target genes playing critical roles in flower formation. In a previous review of MADS-box genes involved in the flower of *Crocus* we described their comparative structural and phylogenetic relationships (Tsaftaris *et al.* 2007). **Table 1** displays homologues of the major B, C, and E-classes of MADS-box genes from *Crocus*, the two dicots model species *Arabidopsis* and *Antirrhinum* and the monocot grass model crop rice. The number of *Crocus* homologues for each class of MADS-box sequences obtained from *Crocus* and the method followed to obtain each *Crocus* sequence is also indicated.

EXPRESSION OF *CROCUS* MADS-BOX GENES IN FLOWER

The expression pattern of all the isolated MADS-box genes from *Crocus* in leaves, flowers and in four different flower organs: outer tepals, inner tepals, stamens and carpels was compared by RT-PCR. More specifically the expression pattern of the isolated B, C and E-class MADS-box genes from *Crocus* was examined using cDNA synthesized from leaves, whole flowers and the flower organs sepals, petals, stamens, and carpels.

B-class MADS-box genes

B-class genes have been isolated from several monocot species and their function has been examined in mutants, such as the si1 of maize (Ambrose *et al.* 2000) that exhibit homeotic conversions of stamens into carpel-like and lodicules into palea/lemma-like organs. In rice, the expression patterns and mutant analysis of an *AP3*-like *SPW1* and two *PI*-like *OSMADS2* and *OSMADS4* genes provide supportive evidence for conservation of B-function as predicted from the ABC model in this plant (Kang *et al.* 1998; Moon *et al.* 1999; Nagasawa *et al.* 2003). The above data point to a conserved role for B-class proteins between dicots and monocots of the grasses family. There is also enough evidence to suggest that B-function is conserved in other monocots. The Asparagus AODEF is expressed exclusively in whorls 2 and 3 during the hermaphrodite stages of flower development and its expression is detected in the respective organs of the male but is reduced in the female flowers (Park *et al.* 2003). The lily LMADS1 protein is detected only in petals and stamens although the gene is expressed in all 4 whorls. Additionally, a truncated *LMADS1* lacking the MADS domain, when expressed ectopically in Arabidopsis, can confer a negative dominant phenotype resembling ap3 mutants that have petals transformed into sepal-like, and stamens into carpel-like structures (Tzeng and Yang 2001).

It has been suggested that probably a conserved role of B-class genes in monocots and dicots is the specification of male reproductive organs, while their role in the formation of lodicules in grasses or tepals in Liliales and Asparagales may be not similar to that in formation of petals in eudicots, since homology of these organs remains controversial (Kramer and Irish 1999; Ambrose *et al.* 2000). However, it has also been suggested that formation of petaloid organs in whorl 1 in several eudicots could be due to the transference of the B-function in this whorl (Baum and Whitlock 1999). The same has been proposed as explanation for the formation of tepals in lilies and tulips (Theissen *et al.* 2000). Expression of B-class genes in whorl 1 is not an uncommon phenomenon in monocots, since it can be observed (especially when in addition to northern analysis, sensitive PCR

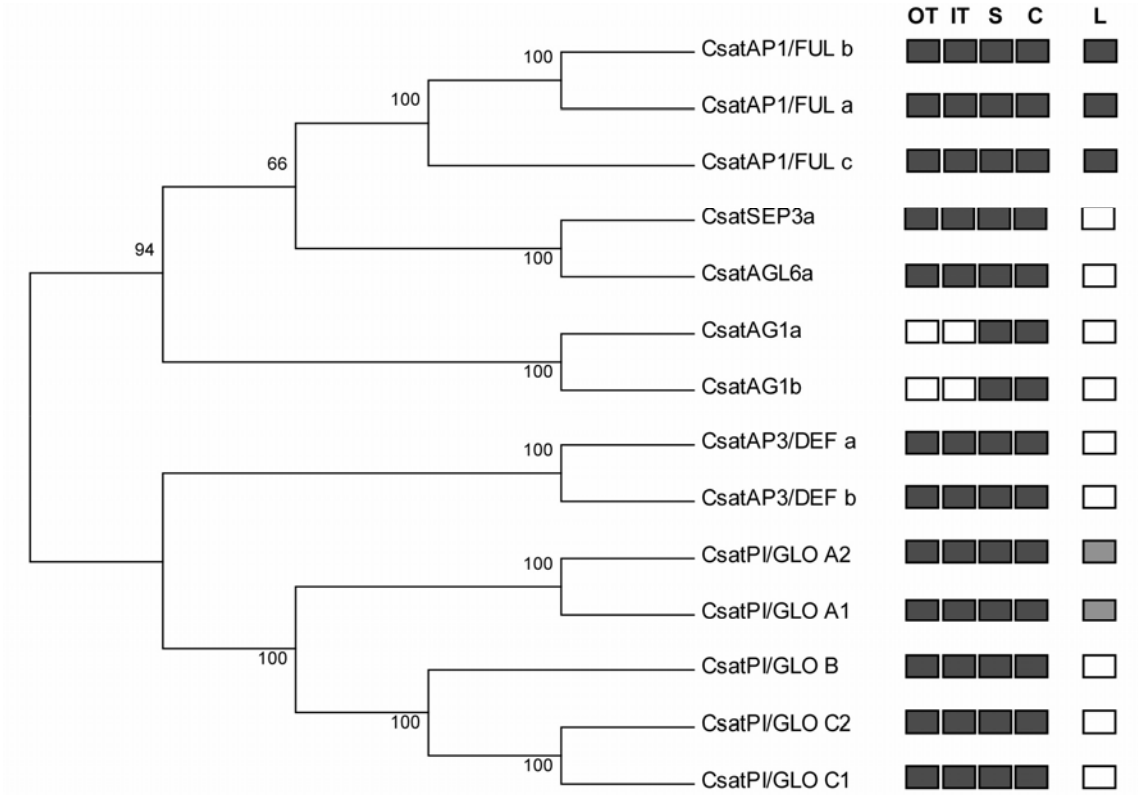

Fig. 2 Phylogeny and expression patterns of the major classes of MADS-box proteins from *Crocus*. The tree was generated by the Neighbor-Joining method using the p-distance correction. Numbers next to the nodes are bootstrap values from 1000 replications. The grey box indicates the identification of the MADS-box transcripts in the flower organs: outer tepals (OT), inner tepals (IT), stamens (S), carpels (C) and in the leaves (L).

techniques are used) in several species (Johansen *et al.* 2002). However, there are examples where expression in whorl 1 was not followed by accumulation of active protein and did not support presence of B-function, as in lily (Tzeng and Yang 2001). In tulip the AP3-like genes *TGDEFA* and *TGDEFB*, as well as, the PI-like *TGGLO*, are expressed in whorls 1, 2 and 3 (Kanno *et al.* 2003). Presence of both, *AP3*-like and *PI*-like proteins in whorl 1 should be a strong indication to explain formation of petaloid organs since ectopic expression of both *AP3* and *PI* in whorl 1 in *Arabidopsis* resulted in the conversion of sepals into petals demonstrating that these genes are sufficient to provide B-function in flowers (Krizek and Meyerowitz 1996). Thus, Kanno *et al.* (2003) provided evidence to support the modified ABC model that was proposed by van Tunen *et al.* (1993b) to explain the flower morphology in tulip. Similar results were obtained in this study for the formation of *Crocus* flower suggesting that the power of the modified ABC model extends in Asparagales. Our data show that the isolated AP3-like *CsatAP3/DEF* sequences and *CsatPI/GLO* are expressed in whorl 1 and may be involved in the homeotic transformation of sepals into tepals. More specifically the experiments revealed the presence of *CsatPI/GLOA* transcripts both in flowers and weakly in leaves while the *CsatPI/GLOB* and *CsatPI/GLOC* transcripts were present only in flowers (**Fig. 2**).

The expression analysis of *CsatAP3/DEF*, the other B-class MADS box gene in *Crocus*, in leaves and flowers revealed the presence of both transcripts only in flowers and not in leaves (**Fig. 2**). The RT-PCR experiment performed with cDNA synthesized from outer tepals, inner tepals, stamens and carpels resulted in the identification of both transcripts in all mature flower parts (Tsaftaris *et al.* 2006). Thus as anticipated from the ABC model and found in other plant species with tepal formation, both B-class genes *CsatAP3* and *CsatPI* extend their expression to whorl1 leading to tepal formation in this whorl instead of sepals.

It is conceivable that even though our results provide supportive evidence for the relevance of a modified ABC model in outer tepal formation in *Crocus*, much has to be done in order to understand flower formation in *Crocus* and other Asparagales species. Further experiments are underway to understand homeotic transformations in *Crocus* flowers and to characterize and possibly exploit the numerous flower mutants (lack of stamens, multiple flower organs, etc.) frequently observed in fields cultivated with this asexually propagated crop.

C-class MADS-box genes

C-class genes, such as Arabidopsis *AGAMOUS* (AG) and *Antirrhinum PLENA* (PLE), play important roles in the specification of stamen and carpel identity, the control of floral meristem determinacy, and the negative regulation of A-class gene activity (Coen and Meyerowitz 1991). The rice genome contains two C-class genes, *OsMADS3* and *OsMADS58*, that arose through gene duplication (Kang *et al.* 1998; Yamaguchi *et al.* 2006). Molecular genetic studies on these two rice C-class genes have revealed further functional diversification of duplicated MADS-box genes (Yamaguchi *et al.* 2006). *OsMADS3* and *OsMADS58* are expressed in the stamen and carpel whorl, like typical eudicot C-class genes, but their temporal patterns of expression differ from each other. *OsMADS3* is expressed only in the presumptive region of stamen and carpel primordia just before the initiation of these organs, whereas *OsMADS58* is expressed in the stamen and carpel whorls before initiation and during the development of stamen and carpel primordia. In an insertional knockout mutant of *OsMADS3*, stamens are homeotically transformed into lodicules, whereas carpels develop almost normally. Thus, *OsMADS3* plays a predominant role in stamen specification. By contrast, RNA-silenced lines of *OsMADS58* develop indeterminate flowers that reiterate a set of floral organs including lodicules, stamens, and abnormal carpel-like organs. In these reiterated flowers, stamens develop almost normally, but carpels are morphologically abnormal. Thus, *OsMADS58* has a critical function both in the establishment of floral meristem determinacy and in normal carpel development. These results indicate that the original functions of C-class genes have

been partitioned into two paralogous genes, *OsMADS3* and *OsMADS58*, during the evolution of rice. The mechanism underlying this functional diversification of C-class genes in rice may be partially explained by variation in the temporal expression of the two C-class genes. For example, *OsMADS3* is down-regulated in the floral meristem before meristem activity is terminated, whereas expression of *OsMADS58* is maintained even after carpel initiation. The predominant function of OsMADS58 in floral meristem determinacy cannot be explained by its expression profile alone, however, because *OsMADS58* is expressed uniformly in both whorl 3 and whorl 4. Another possibility is that differences in the proteins that interact with the two MADS-domain proteins may have led to this functional diversification. That is, OsMADS3 and OsMADS58 may interact specifically and independently with different factors that are preferentially expressed in whorl 3 and whorl 4, respectively. The exact molecular mechanism underlying the functional diversification of OsMADS3 and OsMADS58 during the evolution of rice remains an interesting subject for future study. Another role of the C-class genes in rice, revealed by functional studies, is the control of lodicule positioning (Yamaguchi *et al.* 2006). In both a loss-of-function line of *OsMADS3* and an RNA-silencing line of *OsMADS58*, ectopic lodicules form at the palea side of whorl 2, leading to a radially symmetric arrangement of lodicules, whereas lodicules develop only at the lemma side of whorl 2 in wild type. The spatial expression patterns of *OsMADS3* and *OsMADS58* are also asymmetric in the wild-type flower; that is, the two genes are down-regulated at the lemma side of whorl2 where lodicules develop, but are up-regulated in the region adjacent to the palea. These observations indicate that C-class genes in rice negatively regulate lodicule development at the palea side in the wild-type flower.

The expression analysis for the isolated C-class *CsatAG1* gene in *Crocus* organs revealed the presence of the transcript only in flowers and more specifically restricted in the reproductive parts of the flower: stamens and carpels and not in the perianth. Thus the C-class *CsatAG1*, respects the ABC model where C-class *AGAMOUS* is expressed only in the reproductive parts of the flower (**Fig. 2**; Tsaftaris *et al.* 2005). Furthermore this expression pattern, in agreement with the ABC model of floral organ development, provides the basis for classification of *CsatAG1* in the C-class MADS-box genes.

E-class MADS-box genes

Three groups of genes are included in the E-class MADS-box gene isolated from *Crocus*, namely *CsatA*PETAL*A1/FRUITFUL*, *CsatSEPALATA* and *CsatAGAMOUS LIKE-6*. Originally, many laboratories working with monocots including ours, described the homologues *AP1/FRU* sequences identified and described as *A*PETAL*A1* or *FRUITFUL*. Despite the homologies between *A*PETAL*A1* and *FRUITFUL* all the isolated sequences from monocots so far belong to the FRUITFUL sub-group and no APETALA1 function was described in all monocots so far. Thus we describe as E-class gene the *CsatAP1/FRU*, were its FRU-like sequence belongs.

The results from *Crocus* showed that transcripts of the three isolated genes *CsatAP1/FULa*, *CsatAP1/FULb*, and *CsatAP1/FULc* are present in leaves, as well as, in flowers of *Crocus* (**Fig. 2**). Expression analysis performed in sepals, petals, stamens, and carpels resulted in the identification of the *CsatAP1/FULa*, *CsatAP1/FULb*, and *CsatAP1/FULc* transcripts in all tissues examined (Tsaftaris *et al.* 2004). In *Arabidopsis*, expression of *AP1/FUL* occurs specifically in the tissues and at the developmental stage in which floral fate is assumed. In the flower, expression of *AP1/FUL* is restricted to petals and sepals. In contrast, the three isolated *CsatAP1/FUL* genes from *Crocus*, are *AP1/FUL*-like MADS-box genes expressed in vegetative as well as in all floral tissues of the plant. There are also several examples of MADS-box genes belonging to different homeotic types that are expressed in vegetative tissues and have different functional roles (Zhang and Forde 1998; Alvarez-Buylla *et al.* 2000; Gocal *et al.* 2001; Skipper 2002; van der Linden *et al.* 2002). The similarities in expression pattern of many monocot *AP1/FUL*-like MADS-box genes, including the rice *OsMADS18*, the barley *BM3*) (Schmitz *et al.* 2000) and the three isolated in this study *Crocus CsatAP1/FUL* genes, as well as other such genes with floral and vegetative expression may indicate a novel class of MADS-box genes in monocots, and possibly reflect a novel, yet unidentified role of the corresponding proteins as transcriptional regulators in these species.

The expression analysis of *CsatSEP3a*, a second E-class MADS box gene in *Crocus*, in leaves and flowers revealed the presence of the transcript only in flowers and not in leaves. Furthermore the *CsatSEP3a* transcripts were also detected in sepals, petals, stamens and carpels (**Fig. 2**, Tsaftaris *et al.* submitted).

Unlike the functional-structural conservation, the expression pattern of *SEP*-like transcripts differs between monocots and eudicots. Within monocots, *SEP*-like genes have been most intensively studied in grasses, including the important cereals maize and rice (Tzeng *et al.* 2003). Similarly to other monocots, expression of the isolated *SEP3*-like genes of *Crocus* was detected in all four whorls of flower organs. This pattern of extended expression of E-class genes together with B-class genes reported previously (Tsaftaris *et al.* 2006; Kalivas *et al.* 2007) is compatible with tepal formation in whorl 1. *Muscari armeniacum* is another member of Asparagales that has petaloid organs in the outer two whorls and expression of B-class genes extended to whorl 1, similarly to *Crocus* (Nakada *et al.* 2006). Thus, the extended expression of B- and E-class genes in whorl 1 fits the modified ABCE model proposed to explain tepal formation in tulip and other nongrass monocots (van Tunen *et al.* 1993a; Kanno *et al.* 2003; Kanno *et al.* 2007).

Expression analysis of a third subgroup of E-class MADS-box gene isolated in our laboratory, *CsatAGL6a*, indicated expression in flowers but not in leaves and all four flower organs examined (**Fig. 2**). The Arabidopsis *AGL6* genes named *AGL6* and *AGL13* despite their close relation as a result of their very recent duplication show quite different expression patterns. While *AGL6* is expressed in all four types of floral organs (Mouradov *et al.* 1999), the expression of *AGL13* is restricted to ovules (Rounsley *et al.* 1995). But none of them is expressed in leaves, too. In other monocots such as *Asparagus officinalis* the *AGL6*-like *AOM3* is expressed not only in flower organs, but also in the different meristems present on the apical region of the shoot during the flowering season (Losa *et al.* 2004). *ZAG3* and *ZAG5* are maize AGL6-like genes and their expression was found to be floral-specific and present in both male and female maize inflorescences (Mena *et al.* 1995). *ZAG3* is expressed in carpels, but not in stamens, and in the sterile floral organs but not in glumes (Mena *et al.* 1995). In that respect it is interesting the absence of expression of *AGL6* transcripts in a *Crocus* field isolated mutant with flowers lacking stamen (Tsaftaris *et al.*, in preparation).

Expressing *AGL6*-like genes under the control of the CaMV 35S promoter has dramatic effects on growth of Arabidopsis plants, such as extremely reduced plant size, very early flowering, and the formation of terminal flowers (Hsu *et al.* 2003). Even though such data, probably reflecting gain-of-function effects based on ectopic expression, are difficult to interpret, especially when obtained in a heterologous background they are repeatable after expressing *CsatAGL6a* in *Arabidopsis* under the control of CaMV 35S promoter (Tsaftaris *et al.*, in preparation). *AGL6* genes are basal genes in the MADS-box family and further work is required particularly in well studied flowers from model plants where mutant isolation in combination with transgenic and silencing technologies could better clarify their role.

CONCLUSIONS

Previous studies in dicots species, including *A. thaliana* and *A. majus*, have shown that the B-class genes *AP3/DEF* and *PI/GLO* are expressed in the developing petals and stamens throughout the ontogeny of these organs. The gene products function as heterodimers such that losses of either *AP3/DEF* or their respective partners *PI/GLO* cause homeotic replacement of petals by sepaloid structures and of stamens by carpels. Expression analysis in the different flower organs showed that unlikely to the typical model, the expression of all *CsatPI/GLO* extends into the first whorl of tepals. The extended expression of both B-class genes *paleoAP3/DEF* and *PI/GLO* to the first whorl could be supportive evidence for their heterodimerization not only to form petals or inner tepals in *Crocus*., but also in combination with the extended expression of E-class genes, for the homeotic transformation of sepals into outer whorl 1 tepals in *Crocus* (**Fig. 1B**).

Many monocot flowers have petaloid perianths in whorls 1 and 2, and it is difficult to fully account for this type of floral morphology using the classical ABC model (Coen and Meyerowitz 1991). On the basis of morphological analyses of tulip mutants, van Tunen *et al*. (1993a) hypothesized that the formation of tepals is due to the expanded expression of B-class genes into whorl 1. Moreover, a number of studies in nongrass monocots, such as tulip (Kanno *et al.* 2003), *P. equestris* (Tsai *et al.* 2004; Tsai *et al. 2005*), *A. praecox* (Nakamura *et al.* 2005), *M. armeniacum* (Nakada *et al.* 2006), and *D. crumenatum* (Xu *et al.* 2006), provide support to a simple modification of the ABC model, the so-called modified ABC model (van Tunen *et al.* 1993a). In *Crocus*, *D. crumenatum* and *M. armeniacum*, the B-class genes are expressed in whorls 1, 2, and 3, which fits the modified ABC model, but are also expressed in whorl 4, which does not fit the modified ABC model. However, the protein localization of the B-class gene products is still unclear.

In order to uncover the molecular mechanism of petaloid tepal development in monocots, such as *Crocus*, functional studies with mutant analyses and genetic transformation are needed. MADS-box gene function during the *Crocus* flower development could be revealed during studies on loss-of-function mutants such as stamenless mutant described here, where the *CsatAGL6a* gene is not expressed and moreover, during studies on combinations of such mutants in diploid *Crocus* species like *C. cartwrightianu*s, where genetic crosses between different parents are feasible.

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