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# Tepal formation and expression pattern of B-class paleo*AP3*-like MADS-box genes in crocus (*Crocus sativus* L.)

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**Abstract**

We have cloned and characterized the expression of two flower specific B-class *APETALA3* -like (*AP3*-like) MADS-box sequences of cultivated crocus (*Crocus sativus* L). Based on sequencing data two sequences designated *CsatAP3a* and *CsatAP3b* could be distinguished. These were different in the 5′and 3′ untranslated regions and had five single nucleotide differences in the coding region that led to a single amino acid difference in the coded protein. The deduced amino acid sequences of the genes indicated high similarity with members of the MADS-box family of transcription factors, and particularly with other members of the paleo-AP3 lineage of B-class MADS-box proteins that control floral organ identity. Phylogenetic analysis at the amino acid level confirmed that the isolated sequences belong to the monocot-specific paleoAP3 clade. In the sequence the typical domain structure of plant MADS box proteins was observed. The conserved N-terminal MADS-box, the I domain, the central K domain and a C terminal domain harboring a paleoAP3 motif were identified. Expression analysis indicated that transcripts of *CsatAP3* and also an isolated *PISTILLATA*-like *CsatPIc* sequence are not restricted to organs of the second and third whorls of the flowers but are present also in the tepals of the first whorl and stigmata of the mature crocus flower of the fourth whorl. Extension of *CsatAP3* and *CsatPIc* expression in organs of the first whorl could be a supportive evidence to explain the homeotic transformation of sepals into tepals in crocus.


*Keywords:* *Crocus sativus* L.; Tepals; MADS-box genes; PaleoAP3-like; *APETALA3*; *PISTILLATA*

## 1. Introduction

*Crocus sativus* 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 color. Saffron has also medicinal properties and is used in the coloring industry. Crocus has been cultivated for almost four millennia and its origin has been assigned to Iran, Asia Minor, and most probably Greece as supported by recent data [1]. The flower of crocus is bisexual and it is sterile. Perianth consists of six petaloid three tepals in whorl 1 (outer tepals) and three tepals in whorl 2 (inner tepals). 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 ([1] and references therein), as well as a flower without stamens described in this study. 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 to around 370–470 h of work. Consequently, the cultivation of this crop for its flowers and specifically its stigmas is very labor-intensive leading to high costs [2]. 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.

In higher plants, flower morphology is established by the concerted action of a series of MADS-box transcription factors responsible for space-time regulation of flower organ development. Genetic and molecular analyses of flower-specific

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MADS-box genes and floral homeotic mutants enabled an understanding of their role and led to the proposal of the classic ABC model of flower development [3]. According to this model flower organs that are arranged in four homocentric whorls on the flower originate as follows: sepals in the outermost whorl 1 from the action of A-class MADS-box genes alone; petals in whorl 2 from the combined action of A-class and B-class MADS-box genes; anthers in whorl 3 from the combined action of B-class and C-class MADS-box genes; and finally carpels in the inner whorl 4 from the action of C-class MADS-box genes alone. The initial model as devised from studies in *Arabidopsis* and *Antirrhinum* was originally though to be a simple universal model for floral development, but further research revealed a much more detailed and complex picture [4]. Recent results revealed novel classes of MADS-box genes, namely D-class extending the involvement of MADS-box genes in ovule development and E-class that is required for B and C floral organ identity functions, and consequently leading to revisions of the ABC model toward models with a higher complexity that also envisage the formation of quartets of MADS-box transcription factors for flower organ formation [5–7].

Studying flower development 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 flower. Crocus is an example of such a crop with flowers of economic importance and we have set up a study to understand and possibly improve crocus flower. Towards this goal, we have cloned characterized and studied the expression of three homologous A-class *APETALA*-like genes designated *CsAP1a*,*b*,*c* [2] and two C-class *AGAMOUS*-like differentially spliced genes designated *CsAGa*,*b* [8]. In these reports we used the abbreviation *Cs* as prefix to characterize the crocus genes but since this may cause some confusion because MADS-box genes from *Chloranthus spicatus* have been reported using the *Cs* prefix [9], we decided to use from now on the *Csat* prefix for the crocus genes and will update the GenBank records, accordingly. Expression pattern of the A-class crocus genes is not complying with the classic ABC model as their transcripts are present in all mature flower parts, whereas the two C-class genes are expressed only in stamens and carpels as expected by the model.

Isolation and characterization of B-class genes in crocus could be important in order to understand the formation of tepals in this and other species. Studies on petal development and evolution have focused on the B-class genes that are represented by the two *Arabidopsis* lineages of *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) and their *Antirrhinum* counterparts *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*), respectively [10]. Previous studies in these two species have shown that B-class genes are expressed in the developing petals and stamens throughout the ontogeny of these organs. The gene products function as a heterodimer such that loss of either AP3 or PI causes homeotic replacement of petals by sepaloid structures and of stamens by carpels (see [4] and [10] for reviews). The *AP3*-like linage is further divided into three lineages, namely eu*AP3*, paleo*AP3*, and *TM6* based on sequence diversity [11]. This supports the hypothesis that B-class originated from a common ancestral gene through successive gene duplication events [4]. Several monocot species have flowers that are not typical regarding what is observed in eudicots. In grass family for example lodicules are considered to be homologous to petals and play a role in opening the florets, while palea and lemma structures are thought to be similar to a prophyll and a bract, respectively [12]. A prophyll is a leaf formed at the base of a shoot, while a bract is a leaf-like structure associated with an inflorescence or flower. Thus, according to the definitions of flower organ initiation regions in *Arabidopsis* the origin of lodicules, stamens and pistil can be assigned to whorl 2–4, respectively. Defining whorl 1 is often avoided since the homology of palea and lemma to whorl 1 organs of other plants is controversial at present [13]. Experimental evidence has shown that the function of class B genes is conserved in grasses. In *silky1* (*si1*) mutants of maize (*SI1* is the *AP3* homolog in maize), stamens are replaced by carpels, and lodicules are replaced by bracts that resemble palea/lemma [14]. Similarly, in the *superwoman1* (*spw1*) mutant of rice (*SPW1* is the *AP3* homolog in rice) stamens and lodicules are transformed into carpels and palea-like organs, respectively [15].

Another morphological difference between monocot of other families and eudicot flowers is that instead of sepals in whorl 1 and petals in whorl 2, flowers of species like crocus, tulip and many lilies (among others) have three whorl 1 (outer) and three whorl 2 (inner) petaloid organs called tepals. For the formation of tepals in tulip it was proposed that B-class gene expression in liliaceae is not restricted to whorls 2 and 3 but is extended to whorl 1, which could explain the natural homeotic transformation of sepals to petals [16]. This model was experimentally supported in tulip by recent findings [17], but experimental evidence to indicate wide application of the model for tepal formation in other monocot families is lacking. In this report, we describe the cloning and characterization of two *AP3*-like sequences designated *CsatAP3a* and *CsatAP3b*. We also report the partial isolation of a *PISTILLATA*-like sequence designated *CsatPIc*, and our results on the expression of these genes that are relevant to tepal formation in crocus.

## 2. Materials and methods

### 2.1. Plant material

*Crocus sativus* from the cultivated variety ‘‘Kozani’’ field growing plants were collected from Greece. Sampling was during the late flowering season in October. Tissues were separated and immediately frozen in liquid nitrogen and stored at −80 °C until used.

### 2.2. RNA Isolation, cDNA synthesis and cloning

Total RNA from leaves, closed flowers (3 cm in length), tepals, stamens and carpels was extracted using the RNeasy plant mini kit (Qiagen, Hilden, Germany). On-column digestion of DNA during RNA purification was performed using the RNase-Free DNase Set (Qiagen, Hilden, Germany). For amplification of MADS-box sequences, a degenerate

primer, MADS-2F, 5′-GTKCTYTGYGAYGCYGAGGT-3′ corresponding to the conserved amino acid sequence VLCDAEV of the MADS-box genes [18] was used in 3′ RACE experiments. For amplification of *PISTILLATA*-like sequences, two degenerate primers, PIF1, 5′-AAGCTSTGGGAYGMNAARCA-3′ and PIF2, 5′-RSGARAAYGAYAAYATGCA-3′ were designed corresponding to the conserved amino acid sequences KLWDAKH and KENDNMQ respectively of the K domain of PI genes from other monocots species and were used in 3′ RACE experiments.

First strand cDNA synthesis was performed using 1.5 μg total RNA from closed flowers, 0.75 μg 3′ RACE Adapter Primer 5′-GGCCACGCGTCGACTAGTAC$(T)_{17}$-3′ (Invitrogen, Paisley, UK), 1 mM dNTPs and 200 μM-MuLV reverse transcriptase (New England Biolabs, Beverly, USA) in 50 μl total volume. 1/25 of the synthesized cDNA was used as template in a touch down PCR reaction with 1 pmol MADS-2F primer, 0.2 pmol Abridged Universal Amplification primer, 5′-GGCCACGCGTCGACTAGTAC-3′ (Invitrogen), 0.2 mM dNTPs and 1 u DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland). The thermocycler program was: 1 min at 94 °C; 15 cycles of 30 s at 94 °C; 30 s at 60 °C—0.5 °C/cycle; 1.5 min at 72 °C followed by 20 cycles of 30 s at 94 °C; 30 s at 52 °C; 1.5 min at 72 °C and a final extension step of 15 min at 72 °C. Several products between 500 and 900 bp were cloned into the pGEM T easy vector (Promega, Madison, USA) according to the manufacturer's protocol. DNA sequencing and BLAST similarity searches with EMBL and Genbank nucleotide databases identified a number of clones.

To obtain the *AP3* cDNA's 5′end, an RNA ligase-mediated rapid amplification reaction was performed on a pool of total RNA from leaves and flowers using the GeneRacer Kit (Invitrogen, Paisley, UK) according to the manufactures protocol as previously described [2]. Based on the sequence information obtained by the 3′ RACE experiments, two gene specific primers AP3-R1 (5′-AGCACAACATATTTGTAGGTAG-3′) and AP3-R2 (5′-AGGTAGCAAATTAAGTAGGAAAG-3′) were designed from the 3-UTR and used to isolate the cDNA's 5′ends following the recommendations of the manufacturer.

### 2.3. Phylogenetic analysis

The deduced CsatAP3 amino acid sequences were used in BLAST searches against amino acid sequences in the GenBank, and the best hits identified B-class MADS-box genes belonging to the monocot-specific paleoAP3 lineage. The highest similarity (with 152 identical amino acids out of 214) was for the *Agapanthus praecox* AP3-like protein ApDEF (BAD95987). For phylogenetic analysis 84 B-type MADS-box amino acid sequences (AP3-, PI, and GGM2-like) representing all angiosperm linages and gymnosperms were selected. The B-sister clade of B proteins ($B_S$ proteins) was used as outgroup [19,20]. The AP3-like amino acid sequences from monocots were the *Agapanthus praecox* ApDEF, *Zea mays* ZmSILKY1 (AAF59838), *Oryza sativa* SW1 (AAL18851), *Triticum aestivum* TaMADS51 (BAA33459), *Tulipa gesneriana* TGDEFA (BAC75970) and TGDEFB (BAC75971), *Lilium regale* LRDEF (BAB91550), *Hemerocallis hybrid* HhMADS1 (AAG35773), *Asparagus officinalis* AODEF (BAC75969), *Oncidium sp.* OMADS3 (AAO45824), *Phalaenopsis hybrid* PhMADS17 (AAV28492), *Phalaenopsis equestris* PeMADS4 (AAR26626), PeMADS2 (AAR26628), PeMADS3 (AAR26629), PeMADS5 (AAR26630), *Tacca chantieri* TcAP3 (AAF73935), *Tradescantia reflexa* TRDEF (BAD80745), *Commelina communis* CCDEF (BAD80747), and *Hordeum vulgare* HvAP3 (AAS 48126). Several alignment and phylogeny reconstruction methods were examined. The alignment presented here was performed by the ClustalW method [21] and phylogenetic relationships of the sequences were examined using the neighbor-joining method with p-distance correction [22]. Bootstrap values were derived from 1000 replicate runs. A phylogenetic tree was constructed using the *MEGA 3* software [23].

### 2.4. Southern blot

Ten micrograms genomic DNA was digested with *Eco*RI, *Bam*HI and *Hind*III (enzymes from New England Biolabs, Beverly, USA). The digested DNA was separated on 1.2% agarose gel and transferred to positively charged nylon membranes (Roche, Mannheim, Germany) by capillary transfer. Hybridization was performed in DIG Easy Hyb solution (Roche, Mannheim, Germany) at 42 °C. The probe was prepared using the PCR DIG probe synthesis kit (Roche, Mannheim, Germany) with template the previous cloned cDNA and primers AP3-F (5′-TTGGATGAGTCGTTGAGGCTTGT-3′)/AP3-R2. Chemiluminescence was detected using the DIG Luminescent detection kit (Roche, Mannheim, Germany) according to the manufacturer in a Genegnome (Syngene, Cambridge, USA) bioimager.

### 2.5. Expression analysis

The expression analysis of the isolated MADS-box genes was performed with RT-PCR. One microgram of total RNA extracted from leaves, flower buds, mature flower, whorl 1 tepals, whorl 2 tepals, stamens, and carpels of the wild type flowers, and additionally carpels of a mutated flower lacking stamens were used in a reverse transcription reaction as described in the RNA isolation and cDNA synthesis of Section 2. PCR was performed in 1 × PCR buffer, 0.2 mM dNTPs, 0.4 pmol of the primers AP3-R2 and AP3-F for the AP3 genes, or the primers PICF1 (5-TATAGAAGAAGCCTTGCAGAAC-3) and PICR2 (5′-CTGTTGGTACCCAAGATCCATG-3′) for the *PISTILLATA CsatPIc*, and 1 u of the DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland) using as template 1/25 of the synthesized cDNA. Primers actin2-F (5-CCGGTGTCATGGTTGGTAT-3) and actin2-R (5-GCAGGCACATTGAAGGTCT-3), amplifying a fragment of the actin-beta gene, were used as control for successful cDNA syntesis under the same conditions as above. The cycling parameters were incubation at 94 °C for 2 min, followed by 30 cycles of incubation at 94 °C for 30 s, 54 °C for 30 s, 72 °C for 30 s and a final extension step of 5 min at 72 °C. A control RT-PCR was included for each sample using as template total RNA without reverse transcriptase at the same dilution as the cDNA template. The PCR products were separated on 1.8% agarose gels where amplification products of the expected size could be observed.

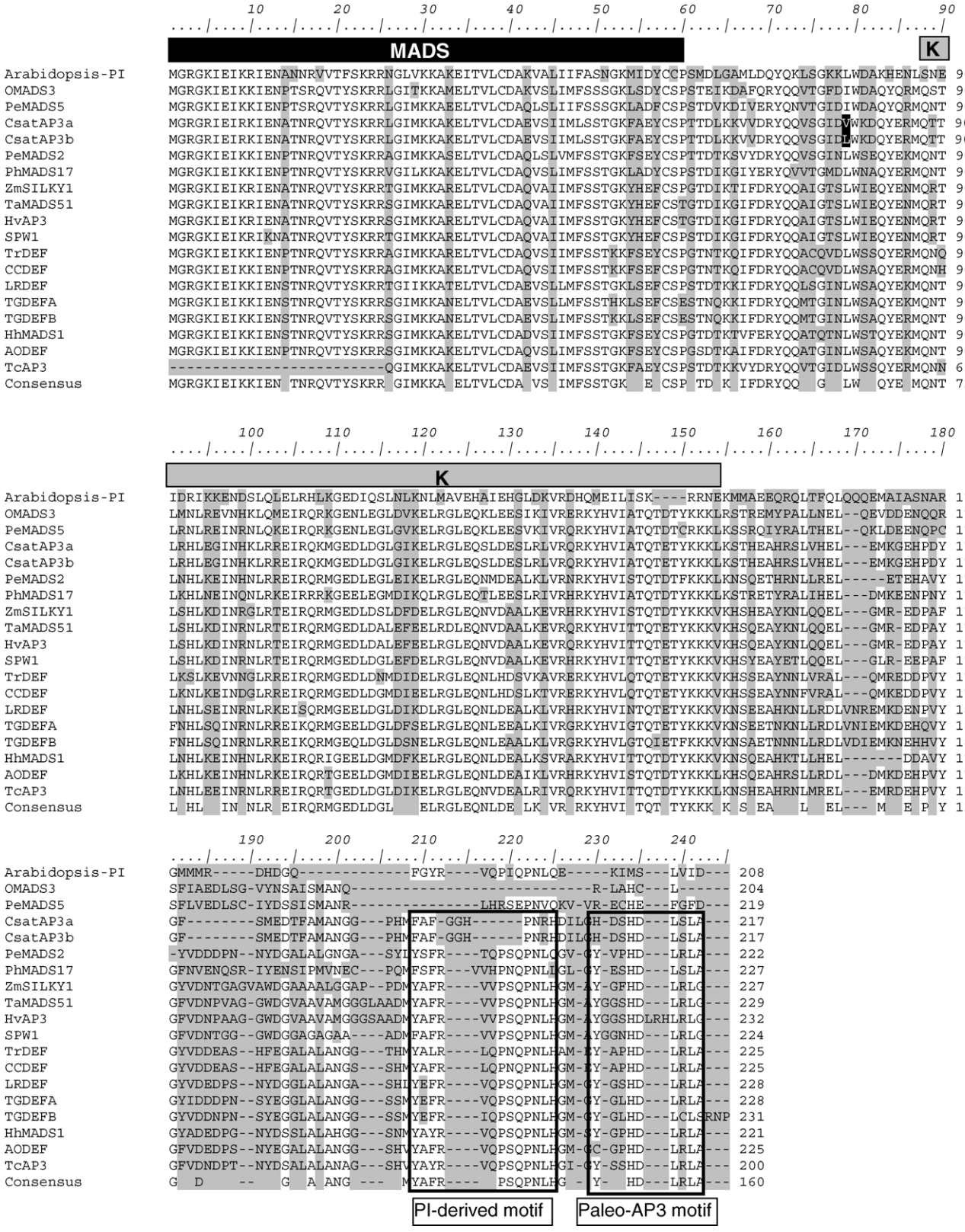


Fig. 1. Alignment of the predicted amino acid sequences CsatAP3a and CsatAP3b with members of the B-class MADS-box proteins in other plants. The MADS and K domains are indicated above the sequences. Identical amino acids in more than 75% of the sequences are in white background while different amino acids are in grey background. The single amino acid difference of the two crocus sequences is shown by white letters in black background. Dashes indicate gaps to maximize alignment. The alignments were generated using ClustalW. The PI-derived motif and the paleo-AP3 motif in the sequences are boxed.

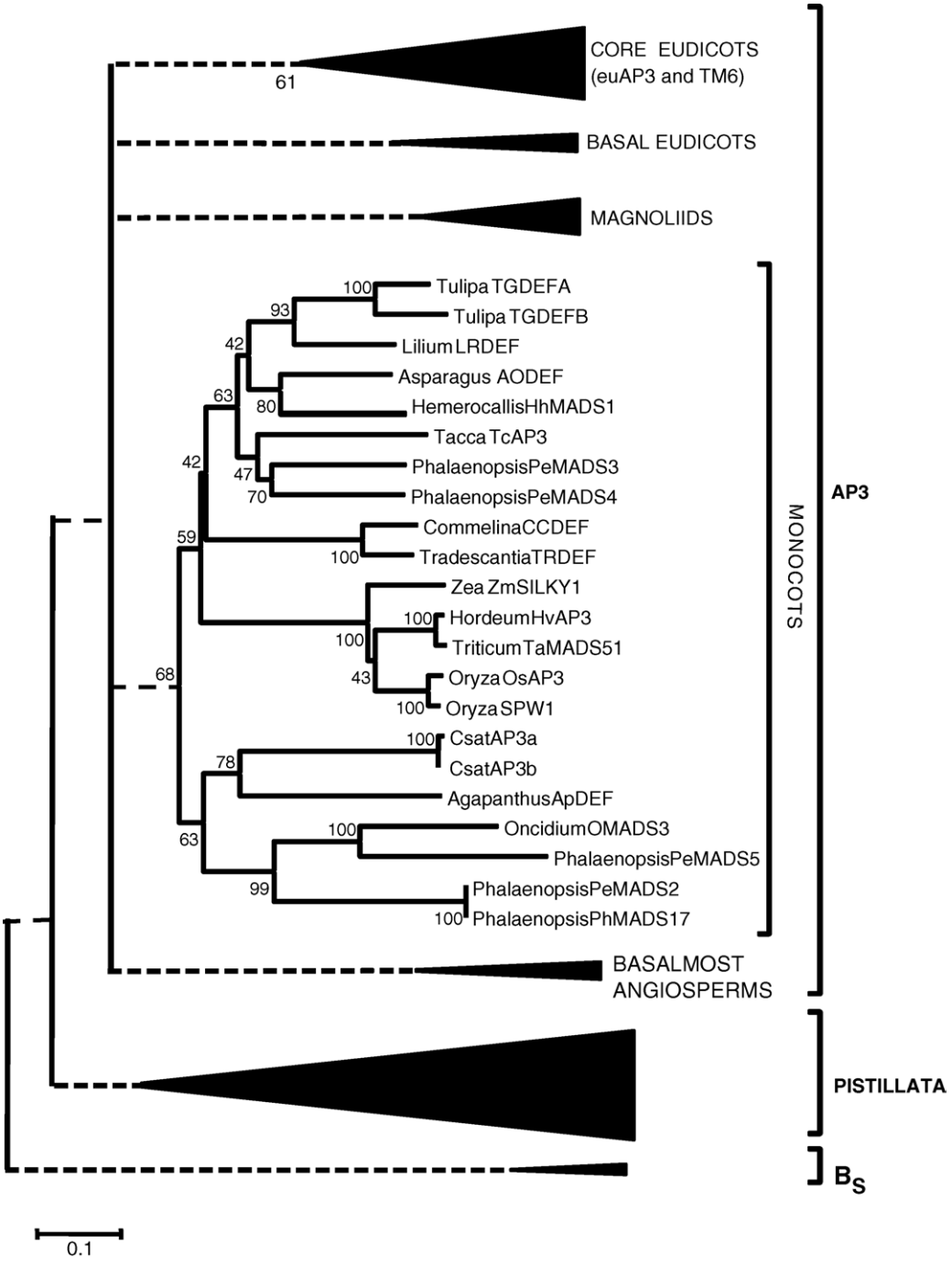


Fig. 2. Phylogenetic relationships of the monocot AP3-like MADS-box proteins. Analysis was performed in the context of the plant B-class MADS-box proteins. Phylogeny was reconstructed from 84 B-type MADS-box amino acid sequences (AP3-, PI, and GGM2-like) representing all angiosperm linages and gymnosperms as well. The B-sister clade of B proteins ($B_S$ proteins) was used as outgroup. Phylogeny of angiosperm linages in the AP3-like branch is not well resolved and an indicative order according to published data (Zahn et al. [30]) is presented. Monocot AP3-like sequences form a separate clade representing the monocot-specific paleoAP3 lineage of B-class proteins. The crocus CsatAP3a and CsatAP3b sequences form a separate branch that includes also *Agapanthus* ApDEF and the orchid sequences OMADS3, PeMADS2, PeMADS5 and PhMADS17. The tree was generated by the neighbor-joining method using p-distance correction. Numbers next to the nodes are bootstrap values from 1000 replications. Dashed lines indicate branches out of scale.

## 3. Results

### *3.1. Cloning of APETALA3 and PISTILLATA-like MADS-box cDNAs from crocus*

3′ RACE experiments on flower cDNA from crocus aiming to the isolation of *AP3*-like sequences resulted in the isolation of eight clones with an uninterrupted ORF and with homology to *AP3* genes from other plant species. The clones were similar in their coding region but different in the size of the 3′ UTR, being 163 bp in five clones and 69 bp shorter in three clones before the polyA tail. Based on the obtained sequence information, the gene specific primers AP3-R1 and AP3-R2, designed to anneal 45 and 29 bp, respectively, downstream

from the putative stop codon were used in 5′ RACE experiments. A PCR fragment of 750 bp was purified from the gel and cloned into the pGEM T easy vector (Promega, Madison, USA) according to the manufacturer's protocol. Seven clones were sequenced.

Analysis of the sequencing results using the SeqMan software package (DNA Star, Madison, WI) revealed that the seven 5′ RACE clones and the eight 3′ RACE clones could be grouped into two contigs. The first contig consisted of the consensus sequence from two 5′ RACE clones and four 3′ RACE clones was designated *CsatAP3a* (GenBank accession number AY948339). The second contig consisted of the consensus sequence of five 5′ RACE clones and four 3′ RACE clones was designated *CsatAP3b* (GenBank accession number AY948340). The two transcripts share 98% similarity and the encoded proteins differ in a single amino acid. Two trinucleotide repeats were present at the 5′ UTR of *CsatAP3a*. The one is a CCT trinucleotide, repeated three times, that is missing from the shorter *CsatAP3b* sequence. The second is a CTT microsatellite, repeated eight times in *CsatAP3a*, which is also present but repeated six times in *CsatAP3b*, providing a means to distinguish between the genes.

Similar 3′ RACE experiments for isolation of *PI*-like sequences resulted in isolation of several clones and characterization of these sequences is underway. We have characterized one partially isolated sequence, which revealed high similarity with reported monocot *PI*-like sequences. The isolated 523 bp long nucleotide sequence designated *CsatPIc*, contains part of an ORF with a stop codon and a 176 bp long 3′ UTR including a 17 bp polyA tail. The partial putative translation product of the gene is 116 amino acid long spanning part of the K and the C-terminus domain of the protein, and in BLAST comparison with GenBank sequences the best scores were obtained for *Tacca chantieri* PI (AAF73942) with 93 identical out of 116 amino acids compared at the C-terminus of the proteins followed by *Phalaenopsis equestris* MADS box protein 6 (AAV83997) with 92 identical amino acids and *Agapanthus praecox* MADS-box transcription factor PI (BAC66962) with 91 identical amino acids of the 116 used for comparisons. Based on the partial *CsatPIc* cDNA sequence we prepared gene-specific PCR primers for expression analysis.

Alignment of the predicted amino acid sequences CsatAP3a and CsatAP3b with the members of B-class MADS-box proteins used in phylogenetic analysis (see Section 2) revealed that the crocus sequences share high similarity with B-class genes in the conserved MIK region while are more divergent at the variable C region (Fig. 1). Within the C region the presence of the paleoAP3 motif in the crocus AP3-like sequences (five out of eight identical amino acids with the consensus paleoAP3 sequence described in Tzeng and Yang [24]) could be identified. The PI-derived motif, which is defined as a region bearing similarity with the conserved PI motif in the PI lineage of B-class proteins [25] was less conserved (five out of 12 identical aminoacids of the consensus PI-derived motif described in Tzeng and Yang [24]). Phylogenetic analysis depicted in Fig. 2, confirmed that CsatAP3 fall into the monocot-specific paleoAP3 lineage of AP3-like proteins.

### 3.2. Southern blot analysis

As shown in Fig. 3, one band of about 9.5 kb from the *BamH*I and one band of about 4.3 kb from the *Hind*III digest could be observed in the southern blot. The *Eco*RI digest revealed the presence of two bands with 0.7 and 1.3 kb in size. Two possible explanations of these results could be proposed. First, since the restriction enzymes used for southern blot do not cut within the isolated cDNAs, a possible explanation is that the cloned sequences represent two different alleles of a single genomic locus. This scenario would assume that no *BamH*I and *Hind*III sites exist in the genomic region recognized by the probe while a putative intron interrupts the probe sequence and harbors an *Eco*RI site. Second, an alternative hypothesis could be that the two sequences represent two different genomic loci that can be distinguished by the different *Eco*RI digestion pattern.

### 3.3. Expression analysis

The expression pattern of the *CsatAP3* and a *PISTILLATA*-like *CsatPIc* gene in leaves and flowers was compared by RT-PCR. In addition, all the experiments included a negative RT-PCR control using a template that was prepared in a similar manner except that reverse transcriptase was omitted in cDNA synthesis. No amplification could be observed in the negative controls (data not shown). Results shown in Fig. 4A revealed the presence of both transcripts only in flowers and not in leaves. The expression pattern of the *CsatAP3* and *CsatPIc* was also examined in different flower organs. The RT-PCR experiment performed with cDNA synthesized from outer tepals, inner tepals, stamens and carpels resulted in the identification of both transcript in all mature flower parts. The *CsatAP3* transcript was also present in a crocus mutant isolated in the field with flowers lacking stamens (Fig. 4B). This mutant was furthermore examined for expression of the crocus *AP1*-

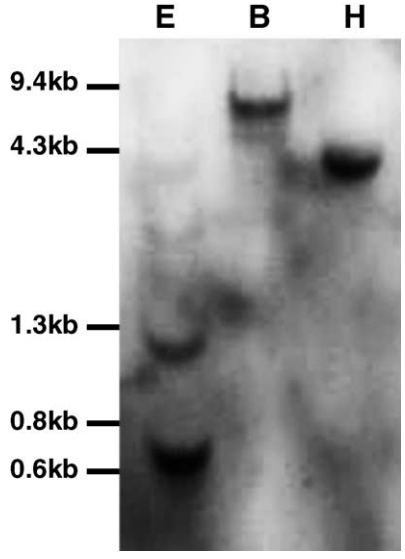


Fig. 3. Southern blot of genomic DNA from crocus digested with: *Eco*RI (E), *Bam*HI (B) and *Hind*III (H) probed by a *CsatAP3*-specific probe.

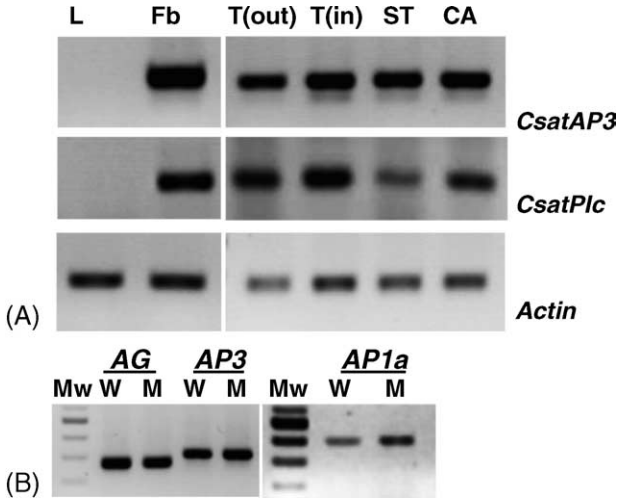


Fig. 4. (A) RT-PCR analysis of *CsatAP3* and *CsatPIc* transcript accumulation in different crocus tissues and flower organs. Transcripts of both class B genes are present in flower buds and all the mature flower organs while are missing from leaves. Control RT-PCR was performed with actin primers. (B) RT-PCR analysis of transcript accumulation in wild type and mutated flowers lacking stamens for the crocus genes *CsAG1* (*AG*), *CsatAP3* (*AP3*), *CsAP1a* (*AP1a*). All genes are expressed in wild type as well as in mutant flowers. Tissues and genotypes are: leaves (L), flower buds (Fb), outer tepals (T(out)), inner tepals (T(in)), stamens (ST), carpels (CA), carpels of a mutated flower lacking stamens (M), carpels of wild type flower (W), molecular weight ladder (Mw).

like [2] and *AG*-like genes [8]. Results in Fig. 4B shows the examined genes were expressed in wild type, as well as, in mutated flowers.

## 4. Discussion

We have isolated two putative B-class paleo*AP3*-like MADS-box sequences from crocus. Sequencing results and Southern hybridization (Fig. 3) do not provide conclusive evidence as to whether the two sequences designated *CsatAP3a* and *CsatAP3b* present in the crocus Greek cultivar ''Kozani'' represent different alleles or different genes. *AP3*-like genes in higher plants have conserved C-terminal motifs, which in higher eudicots is the eu*AP3* motif, while in lower eudicots and magnoliids is the paleo*AP3* motif [11]. Detailed comparison between the paleo*AP3* and the eu*AP3* motif revealed that the later could have derived by an 8-bp insertion at the C-terminus of the former causing a frameshift mutation beyond the insertion site in eu*AP3* genes [26]. The paleoAP3 motif is also conserved in many B-class AP3-like proteins in monocots so far examined including lily, asparagus and tulip [17,27,28]. This led to the suggestion that all monocot AP3-like proteins have paleoAP3 motif [17], which is in agreement with recent hypotheses on the phylogenetic position of monocots in basal angiosperms [29]. This hypothesis is supported by the presence of the paleoAP3 motif in the crocus AP3-like sequences (five out of eight identical amino acids with the consensus paleoAP3 sequence [24]). Another conserved motif in AP3-like proteins is the PI-derived motif which is defined as a region bearing similarity with the conserved PI motif in the PI lineage of B-class proteins [11]. This region is less conserved in all AP3-like lineages including the paleoAP3 lineage of monocots and the crocus sequence has only five out of 12 identical aminoacids of the consensus PI-derived motif described previously [24]. Phylogenetic analysis of all the available monocot AP3-like sequences revealed that CsatAP3a and CsatAP3b, fall together with the other monocot sequences in the paleoAP3 linage clade of the phylogenetic tree. As observed in Fig. 2, the crocus sequences form a separate branch that includes also Agapanthus ApDEF and the orchid sequences OMADS3, PeMADS2, PeMADS5 and PhMADS17. This branch is supported by bootstrap statistics (63%).

Although reconstruction of the monocot, as well as the eudicot AP3-like clades using distance-based methods are well supported by bootstrap analysis (Fig. 2), this does not hold true for the phylogeny of all the entire family of B-class plant proteins. Employing such methods we systematically observed clustering of basal eudicods and magloliids together with monocots in a clade that branched separate from eudicots, which has also been reported by others [20]. This does not conform to current views of angiosperm linage evolution. Recently, a phylogenetic tree of the B-class proteins compatible with evolution of angiosperm linages was reconstructed using manual alignment of the sequences and maximum likelihood statistics [30]. However, since such thorough analysis is beyond the scope of this study, we presented the monocot specific AP3-like clade and indicated the position of other clades according the analysis of Zahn et al.

B-class genes have been isolated from several monocot species and their function has been examined in mutants, such as the *si1* of maize [14] that exhibit homeotic conversions of stamens into carpel-like and lodicules into palea/lemma-like organs. In rice, an AP3-like *SPW1* and two PI-like *OSMADS2* and *OSMADS4* genes have been isolated and their expression patterns and mutant analysis provide supportive evidence for conservation of B-function as predicted from the ABC model in this plant [15,31,32]. 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 [28]. The lily LMADS1 protein is detected only in petals and stamens although the gene is expressed in all four 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 [24]. The crocus mutant examined in this study, which has no stamens has identical expression pattern with the wild type flowers for the *AP3*, *AG*, and *AP1* crocus genes thus far tested, suggesting that lack of stamens in this mutant is probably due to mutation(s) of other gene(s).

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 [14,33]. 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 [34]. The same has been proposed as explanation for the formation of tepals in lilies and tulips [4]. 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 techniques are used) in several species [35]. 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 [24]. In tulip the *AP3*-like genes *TGDEFA* and *TGDEFB*, as well as, the *PI*-like *TGGLO*, are expressed in whorls 1–3 [17]. 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 [36]. Thus, Kanno et al. provided evidence to support the modified ABC model that was proposed by van Tunen et al. 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* sequences are expressed in whorl 1 and may be involved in the homeotic transformation of sepals into tepals. Supportive to this view is evidence revealing that expression of B-class genes in whorl 1 is not restricted to *AP3*-like genes but includes *PI*-like genes (Fig. 2), which is a prerequisite for transference of B-function to whorl 1 organ formation. A thorough analysis of the *PI*-like gene family in crocus is currently underway (Kalivas and Tsaftaris, unpublished results).

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.

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