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Isolation of three homologous *AP1*-like MADS-box genes in crocus (*Crocus sativus* L.) and characterization of their expression

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Abstract

We have cloned and characterized the expression of three homologous Apetala1-like (*AP1*-like) genes from a crocus variety (*Crocus sativus* L) cultivated exclusively in Kozani, at northern Greece. The three different homologous genes were designated *CsAP1* (*C. sativus AP*ETALA*1*) and each one was named *CsAP1a*, *CsAP1b*, and *CsAP1c*. They are the first reported MADS-box genes isolated from this important monocot species cultivated for its flowers.

The deduced amino acid sequence of the three genes indicated high homology with members of the MADS-box family of transcription factors, and particularly with other members of the AP1-like family of MADS-box proteins that control floral-meristem and floral-organ identity. All the isolated sequences lack the typical CaaX-motif that is present in dicot AP1 functional proteins but absent in the monocot homologues thus far examined. CsAP1a and CsAP1b are more similar having 88.2% identical amino acids while CsAP1c is more divergent having 70.9% similarity with CsAP1a, and 64.5% similarity with CsAP1b. Phylogenetic analysis of the isolated genes at the amino acid level indicated that they form a clade with other monocot AP1-like genes from maize (ZmM28), barley (BM3), and rice (OsMADS18). Southern experiments indicated the presence of additional AP1-like homologues in crocus.

Expression analysis indicated the presence of different amount of steady-state mRNAs for all the three homologous genes in leaves, as well as, in the three mature flower parts, namely: tepals, stamen and carpels. Similar expression pattern display many monocot AP1-like MADS-box genes, which comprise a distinct phylogenetic clade of monocot class A MADS-box genes and may reflect a novel, yet unidentified role of their corresponding proteins in these species.

Keywords: MADS-box genes; *Crocus sativus* L.; Floral-organ; AP1-like

1. Introduction

Crocus (*C. Sativus* L.), a monocot triploid species belonging to the Iridaceae family, is cultivated for its red stigmatic lobes that constitute saffron, mainly in southern Europe, Iran, and India. It is popular because of its delicate aroma and attractive color and can be used as a food additive, as well as in medicine and the coloring industry. Saffron has three main chemical components that confer the bright yellow color (crocein), a bitter taste (picrocrocein), and a spicy aroma (saffronal). The flowers of crocus are bisexual. Perianth has no distinct sepals and it only consists of six petaloid tepals in two whorls. Androecium consists of three distinct stamens and the gynoecium consists of a single compound pistil of three carpels, a single three-branched style, and an inferior ovary. The flower is sterile.

Crocus blooms only once a year and its collection period in southern Europe is very short (3–4 weeks in October–November). The method of crocus cultivation contributes greatly to its high price. *C. Sativus* requires a strict agroclimatic condition for growth, which has an influence on its quality. To produce high quality saffron, each flower is harvested by hand, and after mechanical separation of tepals, the stigmas are separated by hand from stamens, sorted, and dried. The size of individual stigmas and the amount of style 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 threads, which equates to

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around 370–470 h of work. Consequently, the cultivation of this crop for its flowers and particularly the separation of its stigmas is very labor intensive leading to very high costs.

Among a number of genes controlling flower development, the floral-organ identity genes, including the so called A, B, and C class homeotic genes, determine the fate of floral-organ primordia. In arabidopsis and other angiosperms, the class A genes lead to the formation of sepals in whorl 1, class A and B genes together lead to the formation of petals in whorl two, the class B and C genes specify the formation of stamens in whorl three, and the class C genes are required for the formation of carpels [1–3]. In this ABC model, A and C genes interact in a mutually antagonistic manner.

Many of the A, B, and C class homeotic genes are members of the MADS-box multigene family encoding putative transcription factors which are characterized by a conserved DNA-binding/dimerization domain. Most of plant MADS proteins initially described, consist of a stereotypic organization of conserved domains. The highly conserved MADS domain is located at the amino-terminal end of the protein spanning 60 amino acids, and is involved in promoter DNA-binding and protein–protein interactions. A second conserved domain of about 70 amino acids, the K-box, is located in the central region of the protein and is able to form coiled-coil structures that may promote dimerization [4]. Two other domains, the I and C-domains are less conserved. Evidence suggests that the C-domain is necessary for the formation of higher order MADS multimers [5]. Recent phylogenetic analysis in *Arabidospis* has led to the proposal of two evolutionary lineages represented by type I and type II MADS-box genes, which differ both in the amino acid sequence of the MADS-box as well as in the domain structure of the predicted protein (for review see [6]). Type I factors lack the K-box, conforming a structure with a MADS-box followed by a rather undefined and length-variable domain, while most of type II factors exhibit the typical MIKC structure [6]. Further fine analysis of phylogenetic relationships among the members of the *Arabidospis* MADS-box transcription factors suggested the presence of five subfamilies one of which is the MIKC (type II) subfamily and the others named Mδ (type II) and Mα, Mβ, Mγ (type I) subfamilies [7].

In angiosperm plants, most of the MADS genes are expressed in reproductive tissue, where they control floral-meristem and floral-organ development, but are also important regulators of flowering time and cell-type specificity in floral organs [8]. Some MADS genes, particularly in monocot species, are expressed in non-floral tissues, suggesting that their role may not only be restricted to the control of flower development [9–13]. Until now MADS genes have been cloned from *Arabidopsis*, which contain 107 genes encoding MADS-box proteins, 84% of which are of unknown function [7], and a variety of mostly dicot plant species, few monocots, as well as, non-flowering plants such as gymnosperms and ferns.

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. Despite the high industrial and medicinal importance and value of crocus flowers, there are no reports on MADS genes and their control in flower development in this plant. Therefore, in order to uncover and understand the molecular mechanisms that control flower development and possible homeotic changes in crocus, we made an effort to clone and characterize crocus MADS-box genes. Here, we describe the cloning and characterization of three different homologous *CsAP1* genes, namely *CsAP1a*, *CsAP1b*, and *CsAP1c*, the first reported MADS-box genes isolated from flowers and leaves of cultivated crocus.

2. Materials and methods

2.1. Plant material

C. sativus var. Kozani field growing plants were collected from Kozani, Greece. Sampling was during the late flowering season in October, and samples were flowers and leaves. As the flower of crocus has not distinct sepals and petals, we treated separately the three tepals of the outer whorl and designated them as sepals, while the three tepals of the inner whorl were designated petals. Tissues were separated and immediately frozen in liquid nitrogen and stored at −80 °C until used.

2.2. DNA Isolation, PCR amplification, and sequencing

Genomic DNA was isolated from leaves using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. For amplification of the MADS-box sequences, two degenerate primers were used. Primer MADS-F, 5′-ATCSAGATMAARAGRATHGARAA-3′, corresponding to the MADS-box conserved amino acid sequence: I(E/Q) IKRIE(N/K) and primer MADS-R, 5′-CATCTTGCCAGWRYTAGMRAARAT-3′ corresponding to the conserved amino acid sequence: IF(A/S)(N/S)(S/T)GKM of the MADS genes [14].

PCR was performed as previously described [15]. A 150 bp PCR product was subcloned into the pGEM T easy Vector (Promega) according to the manufacturer's protocol. Sequencing was performed using a LiCor 4200 sequencer. Using the cloned MADS-box fragment as template a digoxigenin labeled DNA probe was synthesized (MADS-probe) using the PCR DIG Probe Synthesis Kit (Roche). This probe was used to monitor the specificity of the PCR products obtained by different primer combinations and to screen individual clones in Southern blots after hybridization using standard techniques.

2.3. Inverse PCR

We used inverse PCR to amplify the flanking sequences of the isolated MADS-box clone. One microgram genomic DNA was digested with *Eco*RI, *Hind*III, and a combination of *Bam*HI and *Bgl*II in a total volume of 25 μl. The reactions were incubated at 80 °C for 10 min to inactivate the restriction enzymes, diluted to 500 μl with 1 × ligation buffer and supplemented with two Weiss units T4 DNA ligase (NEB). After 2 h incubation at 25 °C the DNA was precipitated with ethanol, resuspended in 10 μl sterile water and used as template in PCR experiments. Two sequence specific primers based on the sequence of the previously isolated MADS clone were designed for PCR. These were: Inv1: 5′-GCGCTTCGAGAAGGTGACCTG-3′ and Inv2: 5′-GGCGGCCTCCTCAAGAAAGC-3′. The PCR was performed using 2 μl of the digested and ligated genomic DNA as template, 0.2 μM of the primers Inv1 and Inv2, 0.2 mM dNTPs and 1.25 U Platinum Taq Polymerase (Invitrogen). The thermocycler program was: 2 min at 94 °C, 35 cycles of 1 min at 94 °C, 30 s at 59 °C, 3.5 min and 10 s per cycle at 72 °C and a final extension step of 10 min at 72 °C.

The PCR products were separated on an agarose gel, but were not visible after EtBr staining. Therefore, the gel was blotted on Nylon membrane and hybridized with the digoxigenin labeled MADS-box probe. After development, one hybridized band of about 500 bp derived from the *Hind* III digest could be identified. In order to obtain sufficient DNA for cloning, a reamplification experiment was performed using 1 μl of the initial PCR as template. The rest of the conditions were the same except that the annealing was at 57 °C and the DyNAzyme II DNA polymerase (Finnzymes) was used. Several strong bands could be observed. A Southern blot was performed as described above and only the 500 bp band of the *Hind* III digest gave a strong hybridization signal with the MADS-probe. This band was cut out from the gel, purified with the Qiaex Purification Kit (Qiagen), cloned in the pGEM T easy vector and sequenced.

2.4. RNA Isolation, cDNA synthesis, and cloning

Total RNA from leaves, closed flowers (3 cm in length), sepals, petals, stamens, and carpels was extracted using the RNeasy Plant Mini Kit (Qiagen). On-column digestion of DNA during RNA purification was performed using the RNase-Free DNase Set (Qiagen). The 3′ end of the cloned fragments was isolated by 3′ RACE. First strand cDNA synthesis was performed using 2 μg total RNA, 1 μg 3′ RACE Adapter Primer 5-GGCCACGCGTCGACTAGTAC$(T)_{17}$-3, (Gibco-BRL), 1 mM dNTPs and 200 U M-MuLV reverse transcriptase (NEB) in 50 μl total volume. Specific forward primers were MADS-A: 5′-CCTTACTGCACACAGCGAATC-3′ (designed from the sequence of the PCR product obtained by the Inv1–Inv2 primer pair) for *CsAP1a*, Inv2 for *CsAP1b*, and MADS-F for *CsAP1c*. Ten percent of the synthesized cDNA from closed flowers (*CsAP1a*) or leaves (*CsAP1b* and *CsAP1c*) was used as template in a PCR reaction with 0.2 pmol forward primer, 0.2 pmol Abridged Universal Amplification primer (AUA) 5′-GGCCACGCGTCGACTAGTAC-3′ (Gibco-BRL), 0.2 mM dNTPs and 1 U DyNAzyme II DNA polymerase (Finnzymes). The thermocycler program was: 1 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 56 °C, 1.5 min at 72 °C and a final extension step of 10 min at 72 °C. PCR products were subcloned into a pGEM T easy vector according to the manufacturer's protocol.

To obtain the cDNA's 5′ end, an RNA ligase-mediated rapid amplification reaction was performed, starting with 3.5 μg total RNA pooled from leaves and closed flowers, using the GeneRacer Kit (Invitrogen) according to the manufacturer's instructions. PCR was then performed using the specific primers *CsAP1a* R (5′-AGTCCGAAATGCCATACAACG-3′) for *CsAP1a*, *CsAP1b* R (5′-CATCAGAAGCTTAGCAGATGTA-3′) for *CsAP1b*, and *CsAP1c* R (5′-GATAGAAATCCCCAAGTACACT-3′) for *CsAP1c*. Amplified fragments were then cloned into pCR 4-TOPO vector using the TOPO TA cloning Kit (Invitrogen).

2.5. Southern hybridization

Ten microgram genomic DNA was digested with *Eco*RI, *Hind* III, and *Bam*HI, (enzymes from NEB) and transferred to a positive charged Nylon membrane. The filter was hybridized with the MADS-box probe described in Section 2.2, stripped with 0.2 M NaOH/0.1% SDS for 10 min at 37 °C (twice), and reprobed with a *CsAP1a* gene-specific 401 bp fragment (708–1109 bp). The digoxigenin labeled *CsAP1a* gene-specific probe was prepared with PCR using the cloned *CsAP1a* cDNA as template with the primers *CsAP1a* F (5′-AATGGCACAGCAGGGACACTG-3′) and *CsAP1a* R, and the PCR DIG Probe Synthesis Kit. Hybridization was performed with DIG Easy Hyb buffer at 42 °C according to the manufacturer and stringent washes at 68 °C in 0.5 × SSC/0.1% SDS (twice). Detection was performed using the DIG Luminescent Detection Kit according the instructions and chemiluminescense was detected using the GeneGnome Bio Imaging System (Syngene).

2.6. Comparison and phylogenetic analysis

The nucleotide and deduced amino acid sequence of the *CsAP1a*, *CsAP1b*, and *CsAP1c* cDNAs were used for BLAST searches on the GenBank/EMBL/DDBJ databases, and among the best BLAST hits, 19 class A floral identity genes for which there are published reports, were selected for comparison. Sequence names were changed to include initials of the species where needed. The sequences were: *Betula pendula* BpMADS5 (X99655) and BpMADS3 (X99653) [16]; *Eucalyptus globulus* EgAP1 (AF305076) [17]; *Capsicum annuum* CanMADS6 (AF130118) [18]; *Pisum sativum* PsPEAM4 (AJ279089) [19]; *Arabidopsis*

lyrata AlAP1 (AF143379) [20]; *Arabidopsis thaliana* AtAP1 (Z16421) [21]; *Sinapis alba* SaAP1 (X81480) [22]; *Brassica oleracea* BoAP1 (Z37968) [23]; *Lolium temulentum* LtMADS1 (AF035378) and LtMADS2 (AF035379) [13]; *Oryza sativa* OsAP1-L (AB041020) [24], OsMADS14 (AF058697) [25], OsMADS15 (AF058698) [25], OsMADS28 (CAB56800) and OsMADS18 (AAF04972) [25]; *Hordeum vulgare* HvMADS3 (originally BM3, CAB97351) [26]; *Zea mays* ZmM28 (AJ430695) [27] and ZmMADS3 (AF112150) [27,28]. The deduced amino acid sequences of the genes together with CsAP1 were aligned using the multiple sequence alignment program Clustal W [29]. Phylogenetic relationships of the sequences were performed using the Neighbor-Joining Method with p-distance correction [30]. The tree was constructed using the MEGA 2.1 software [31].

2.7. Expression analysis

Total RNA (1 μg) extracted from sepals, petals, stamens, and carpels was used in a reverse transcription reaction as previously described. PCR was performed in 1 × PCR buffer, 0.2 mM dNTPs, 0.4 pmol forward primer, 0.4 pmol reverse primer, and 1 U of the DyNAzyme II DNA polymerase (Finnzymes) having as template 1/10 of the synthesized cDNA. Primers were *CsAP1a* F and *CsAP1a* R for *CsAP1a*, *CsAP1b* F (5′-GAATTATTTGAGCAGCATCATAT-3′) and *CsAP1b* R for *CsAP1b*, *CsAP1c* F (5′-CGCTCTGTTAATGGATAGTATCC-3′) and *CsAP1c* R for *CsAP1c*, and: actin2-F (5′-CCGGTGTCATGGTTGGTAT-3′) and actin2-R (5′-GCAGGCACATTGAAGGTCT-3′) amplifying a fragment of the actin-beta gene as a control. The cycling parameters were incubation at 94 °C for 1 min, followed by 35 cycles of incubation at 94 °C for 30 s, 50 °C (*CsAP1b* and *CsAP1c*) or 54 °C (*CsAP1a* and actin-beta) 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 a 1.8% agarose gel and amplification products of the expected size could be observed.

Quantitative expression analysis of the isolated *CsAP1a* gene was performed with real-time RT-PCR using an Opticon (MJ Research) real-time PCR system. PCR was performed in 1 × PCR buffer, 0.2 mM dNTPs, 0.4 pmol *CsAP1a* F primer, 0.4 pmol *CsAP1a* R primer, 0.2 × Sybr-Green (Sigma) and 1 U of the DyNAzyme II DNA polymerase (Finnzymes) having as template 1/10 of cDNA synthesized from 2.5 μg of total RNA extracted from leaves and closed flowers. Actin-beta was used as a control for relative gene expression quantification. The cycling parameters were incubation at 95 °C for 1 min, followed by 33 cycles of incubation at 95 °C for 15 s, 52 °C for 30 s, 72 °C for 30 s, plate read at 80 °C and a final extension step of 5 min at 72 °C. For identification of the PCR products a melting curve was performed from 65 to 95 °C with read every 0.2 °C and 10 s hold between reads. 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 reactions were performed in triplicate. Relative quantification was performed using the Q-Gene software for estimation of the normalized gene expression [32].

3. Results

3.1. Cloning of three MADS-box Apetala1-like cDNAs from C. sativus

Three homologous MADS-box Apetala1-like cDNAs designated *CsAP1a*, *CsAP1b*, and *CsAP1c*, were isolated from *C. sativus* using a combination of PCR techniques. *CsAP1a* (1281 bp, GenBank accession number AY337928) was isolated from flowers and contain a 750 bp ORF, which encodes for a 250 amino acid predicted polypeptide. *CsAP1b* (1164 bp, GenBank accession number AY337929) and *CsAP1c* (1257 bp, GenBank accession number AY337930) were isolated from leaves and contain a 735 bp ORF, which encodes for a 244 amino acid predicted polypeptide, and a 741 bp ORF, which encodes for a 246 amino acid predicted polypeptide, respectively. The sequences of the three *CsAP1* cDNAs were more homologous in the coding region spanning the MADS, I and K domains while were more divergent at the C terminal domain and the 5′ and 3′ untranslated regions. A microsatellite locus was revealed at the 5′ untranslated region of *CsAP1a* and *CsAP1b* consisting of a $(GA)_5$ and a $(GA)_{19}$ repeat, respectively.

3.2. Amino acid sequence comparisons, phylogenetic analysis, and copy number of AP1-like genes in crocus

Based on the amino acid sequence similarity of the entire coding region, the three CsAP1 homologous deduced protein sequences can be grouped into Apetala1-like family of proteins [33]. Published genes that belong to the Apetala1 family of proteins and showed high degree of homology to the three CsAP1 were selected for the multiple alignment process (Fig. 1). The MADS domain of the aligned proteins is highly conserved and conserved regions are also observed in the I and K domains, while the C terminal domain is more divergent. In this comparison the three CsAP1 homologues -a, -b, and -c present 87, 82, and 84% identity, 92, 92, and 91% similarity to the consensus AP1-like proteins MADS-box domain and 64, 59, 51% identity, 79, 77, 76% similarity to the consensus K-box of the AP1-like proteins, respectively. The three CsAP1 proteins lack at the C-terminus the CFAA motif, a typical CaaX box recognition motif for farnesyltransferase (FTase). CaaX-boxes (where C is Cys, a is an aliphatic amino acid, and X is Cys, Met, Ser, Ala or Glu) are present in many AP1-like dicot proteins (Fig. 1).

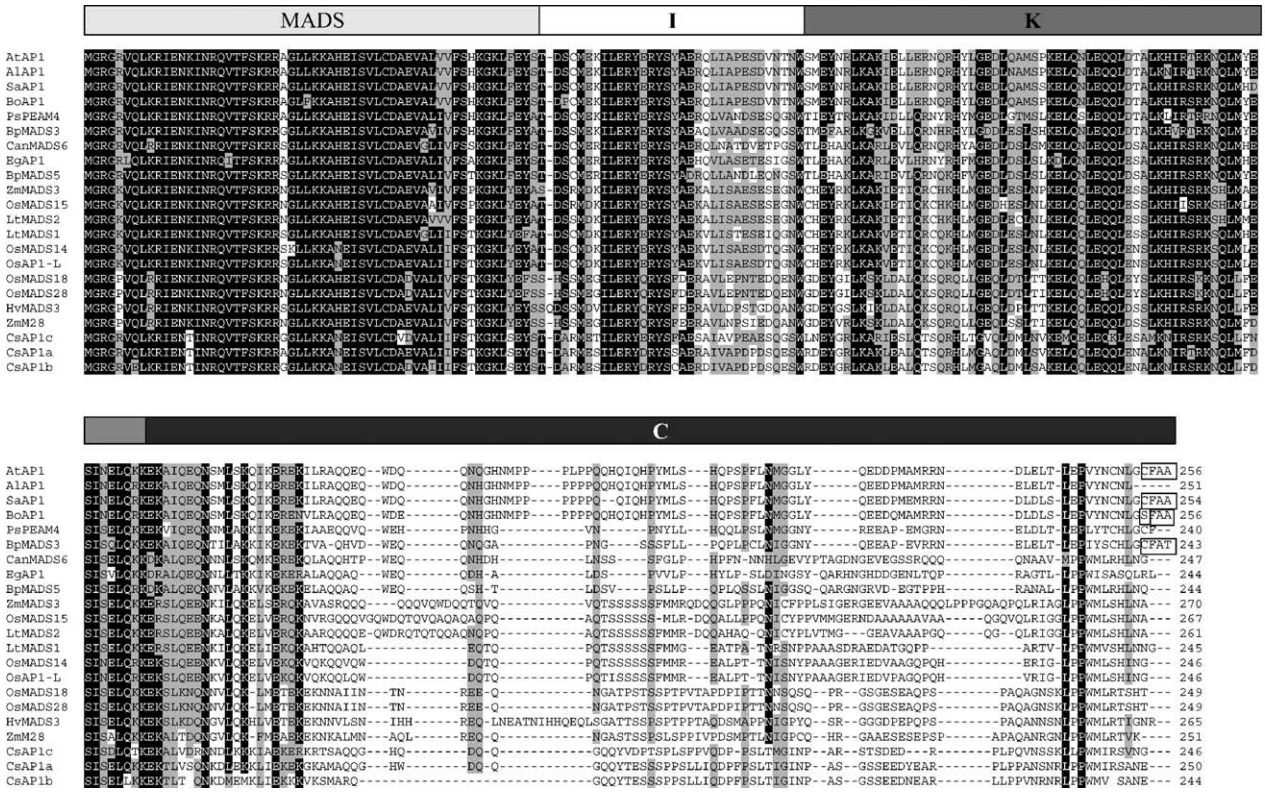

Fig. 1. Alignment of the predicted amino acid sequences of the three CsAP1 proteins and members of the AP1 family in other plants. The CaaX box motif at the C-terminus of BpMADS3, AtAP1, and SaAP1 that is conserved between AP1 and functional homologues from distantly related plant species is boxed, and the substituted cysteine of this box in BoAP1 is boxed separately. Identical amino acids in all proteins are in white letters highlighted by black background, while identical amino acids in more than 75% of the sequences are highlighted by grey background. Dashes indicate gaps to maximize alignment. The MADS-box, I , K, and C-domains are shown by boxes on top of the alignment. The alignments were generated using ClustalW.

The higher BLAST similarity scores between each of the three homologues CsAP1 crocus proteins and sequences in the GenBank were recorded for MADS-box proteins of plants belonging to the class of Liliopsida (monocots). The highest score was for a monocot orchid hybrid (*Dendrobium grex* Madame Thong-In) MADS-box protein DOMADS2 (AAF13261.1) [34] with 61% identity and 74% similarity for CsAP1a, 57% identity and 71% similarity for CsAP1b, and 58% identity and 72% similarity for CsAP1c. Next higher scores for CsAP1a and CsAP1b were for the maize ZmM28 (CAD23441) and the rice OsMADS28 (CAB56800) and OsMADS18 (AAF04972) proteins. For CsAP1c next higher scores were for the chrysanthemum MADS-box transcription factor CDM8 (AAO22981), the *B. pendula* BpMADS3 (CAA67967), and a carrot MADS-box transcription factor (CAC81068).

A phylogenetic tree was calculated for the full length amino acid sequence of the three CsAP1 proteins and the selected Apetala1-like proteins using the Neighbor-Joining Method (Fig. 2). The three CsAP1 proteins fall in a group with the maize ZmM28 MADS-box protein, the barley BM3 (HvMADS3), and the rice OsMADS18 and OsMADS28.

Southern blot experiments revealed the presence of additional putative *AP1*-like orthologs in crocus (Fig. 3). Multiple bands were observed with the MADS-box probe indicating the large number of MADS-box genes in crocus many of which may be *AP1*-like homologues. The *CsAP1a* gene-specific probe revealed three high molecular weight bands after *Bam*HI digestion (there is no *Bam*HI restriction site within the three *CsAP1* mRNA sequences), six after *Hind* III digestion (in *CsAP1a* and *CsAP1b* there is a *Hind* III internal site) and six bands after *Eco*RI digestion (in *CsAP1a-c* no *Eco*RI site exist within the mRNA of the genes, however, there is an *Eco*RI site within an intron of the *CsAP1a* gene (data not shown)). Since no further genomic information is currently available we concluded, based on the results from the *Bam*HI digest, that three homologous genes were hybridized to our probe. With *CsAP1a,b,c* being homologous to each other it is reasonable to predict that these three genes were detected on the membrane.

3.3. Expression analysis

Initial experiments on cDNAs derived from leaves and closed flowers revealed the presence of the *CsAP1a* transcript in both tissues. A real-time PCR experiment was performed to provide quantitative estimation of *CsAP1a* expression. The results indicated that the normalized expression level of the *CsAP1a* transcript is almost double in leaves than that in flowers (Fig. 4).

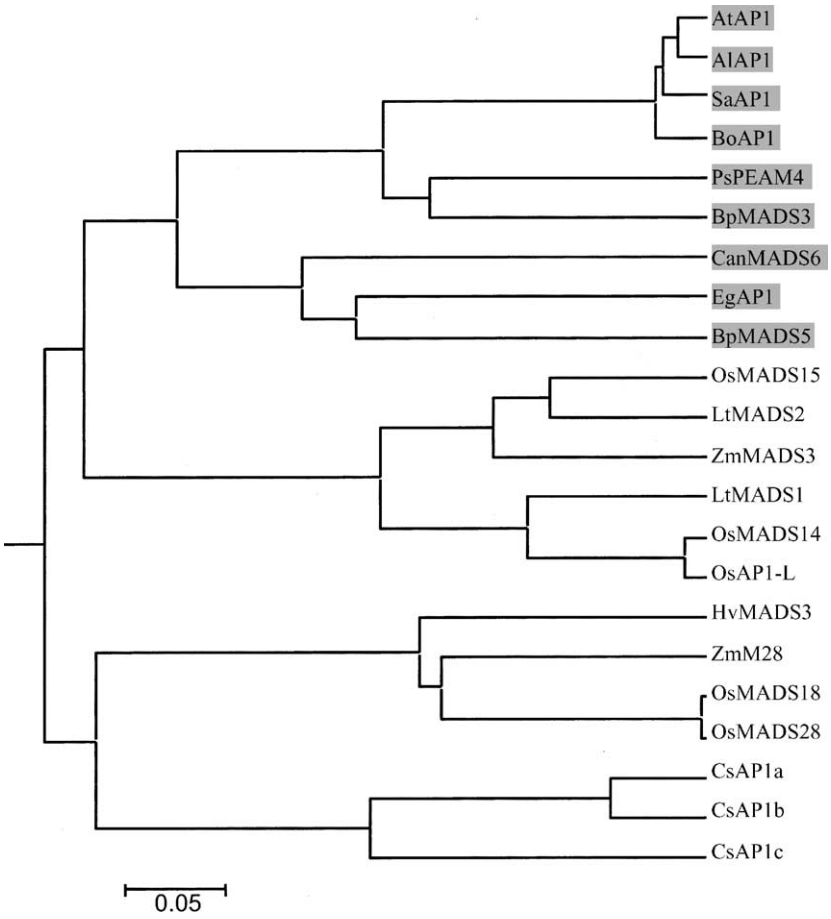

Fig. 2. Phylogenetic relationships of the protein sequences aligned in figure 1. The tree was generated by the Neighbor-Joining method using the p-distance correction. Dicot genes are highlighted in gray. The three CsAP1 sequences are grouped with the maize ZmM28, the barley HvMADS3, and the rice OsMADS18, and OsMADS28.

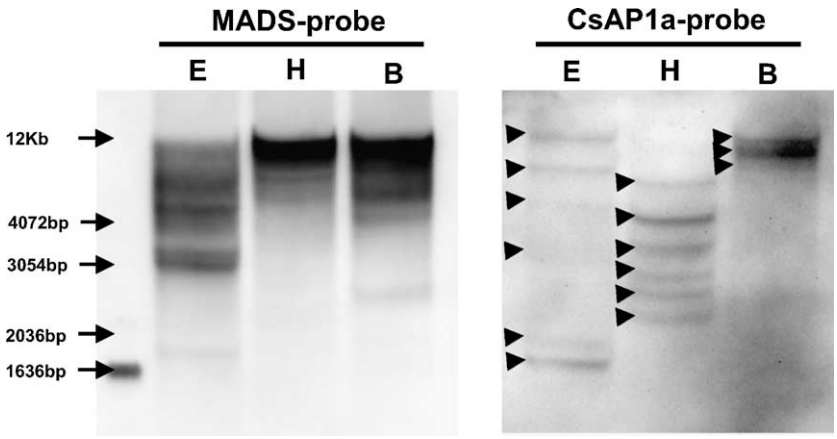

Fig. 3. Southern blot revealing the presence of additional putative AP1 orthologs in crocus. Ten microgram crocus genomic DNA digested with *EcoR*I (E), *Hind* III (H) and *Bam*HI (B), was hybridized with the MADS-box probe described in Section 2.2, stripped and reprobed with a CsAP1a gene-specific 401 bp fragment (708–1109 bp). Multiple bands were observed with the MADS-box probe indicating the large number of MADS-box genes in crocus many of which may be AP1-like homologues. The CsAP1a gene-specific probe hybridized with three high molecular weight bands after BamHI digestion, six after Hind III digestion and six bands after *Eco*RI digestion. Since no further genomic information is currently available the *Bam*HI digestion hybridization pattern suggests that three homologous genes were hybridized to our probe. With *CsAP1a,b,c* being homologous to each other it is reasonable to predict that these three genes were detected on the membrane.

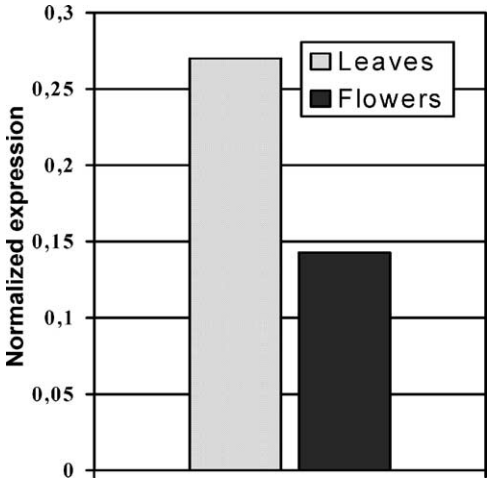

Fig. 4. The mean normalized CsAP1a expression in crocus leaves and flowers as determined by real-time PCR and analyzed using the Q-Gene software.

The expression pattern of all the three homologous genes in leaves and flowers was compared by RT-PCR (Fig. 5). Results show that transcripts of each gene are present in leaves, as well as, in flowers of crocus. The expression pattern of the three genes was also examined in different flower tissues (Fig. 5A). The RT-PCR experiment performed with cDNA synthesized from sepals, petals, stamens, and carpels resulted in the identification of the *CsAP1a*, *CsAP1b*, and *CsAP1c* transcripts in all tissues examined (Fig. 5B). In all the expression experiments the actin-beta gene (Fig. 5C) was used as an RT-PCR positive control. 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).

4. Discussion

In our effort to isolate MADS-box transcription factor genes and assess their involvement in flower development in *C. sativus*, we followed a PCR-based approach and we were able to isolate and examine the expression of three homologous MIKC-type (type II) Apetala1-like MADS-box cDNAs.

AP1 of *Arabidopsis* and its homologue *SQUAMOSA* (*SQUA*) in *Antirrhinum* are MADS-box genes belonging to the SQUA subfamily (also termed AP1/AGL9 clade) which includes MADS-box genes from many other plant species [35]. *AP1* and *SQUA* are key regulatory genes specifying floral-meristem identity in both *Arabidopsis* and *Antirrhinum*. Despite many similarities in their sequence, expression, and functions, only *AP1* appears to have the additional role of class A floral identity gene specifying sepal and petal identity in *Arabidopsis*. There is scarce evidence regarding how the different functions of *AP1*-like genes are conserved between different species. Analysis of the *PEAM4* pea *AP1* functional homologue, from a plant with different floral morphology and inflorescence architecture than that of *Arabidopsis* and *Antirrhinum*, suggested that the *AP1*-like gene dual role in the control of both

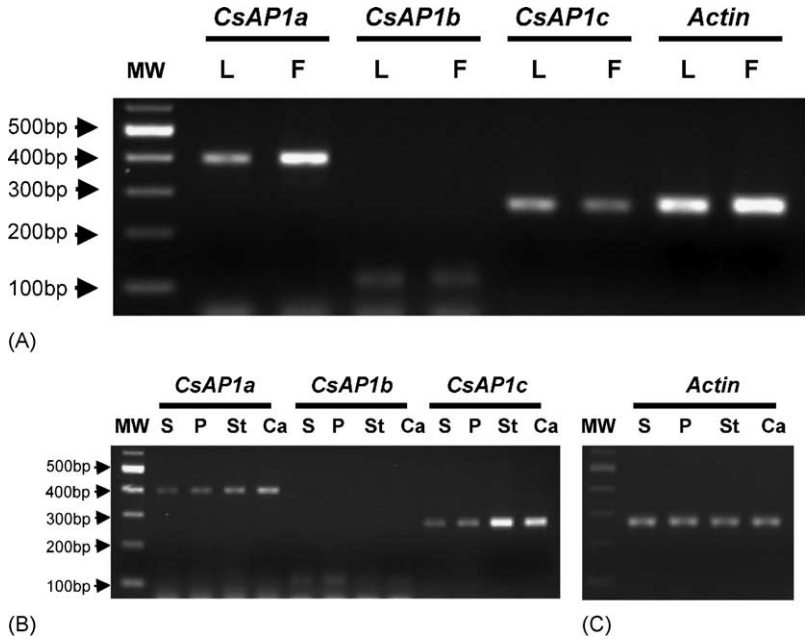

Fig. 5. (A) RT-PCR analysis of the three homologous genes *CsAP1a*, *CsAP1b*, *CsAP1c*, expression in crocus leaves and flowers, (B) different crocus flower tissues and (C) the actin control. Lanes are: MW, 100 bp size marker; L, Leaves; F, Flower; S, Sepals (the three tepals of the outer whorl); P, Petals (the three tepals of the inner whorl); St, Stamens; Ca, Carpels. Amplicons of the expected size are observed. The PCR bands for *CsAP1b* are faint due to the small size of the product and possibly to low target amount or efficiency of amplification.

floral-meristem and floral-organ identity is not restricted to *Arabidopsis*, but can be conserved in species with diverse floral morphologies, such as pea [36]. The *CsAP1* sequence similarity with *AP1*-like genes is not adequate to infer a functional role of these genes in crocus. There are several differences between *AP1* and the three *CsAP1* genes that have to be considered. In *Arabidopsis*, expression of *AP1* occurs specifically in the tissues and at the developmental stage in which floral fate is assumed. In the flower, expression of *AP1* is restricted to petals and sepals. In contrast, RT-PCR experiments revealed that the three *CsAP1* genes are expressed in leaves, as well as in all the flower organs examined. The amino acid sequence of *AP1* and many of its dicot homologues terminates in CFAA (Fig. 1), a typical CaaX box recognition motif for farnesyltransferase (FTase). AP1 is a target of FTase and farnesylation alters the function and perhaps specificity of the transcription factor [37]. The three *CsAP1* genes lack a CaaX-box at the C-terminus, and this, additional to the above mentioned differences, probably suggest divergence from the typical arabidopsis *AP1*-function. Other genes with structural similarity to *AP1* have different expression patterns and function, as for example the *FRUITFULL* (*FUL*) gene (previously called *AGL*8) that is weakly expressed in rosette leaves during vegetative development and is subsequently strongly upregulated in the shoot apex upon the transition to flowering. Experimental evidence suggests that *FUL* regulates the transcription of genes required for cellular differentiation during fruit and leaf development [38].

The three isolated *CsAP1* genes from crocus, are *AP1*-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 classes that are expressed in vegetative tissues and have different functional roles [9–13]. It is conceivable that despite the high level of sequence similarity between *AP1*-like MADS-box genes their expression profile can be quite different. Functional analysis will have to show whether these genes have AP1 function in inflorescence development.

Sequence similarities between the three *CsAP1* genes were higher in the MADS, I and K domains, while the C-domain was more divergent. This is consistent with other studies reporting similar results. Experimental data based on southern hybridization after digestion with different restriction enzymes indicated that multiple MADS-box genes (and possibly more *AP1*-homologues) were present in this species. However, the three isolated homologous *AP1*-like crocus genes were the only with enough similarities at the C-terminus domain to be detected after hybridization with a probe specific for one of them (Fig. 3). Phylogenetic analyses along with 19 class A floral identity genes showed that the three CsAP1 proteins fell in a group with ZmM28, a typical apetala1-like MADS-box representative of maize. This clade also comprise OSMADS18 from rice and BM3 from barley [27]. There are no published data on the expression pattern of ZMM28. OsMADS18 (previously known as FDRMADS 7) is expressed in all rice plant tissues with higher level in the inflorescence. Initially, it is detected in the spikelet apical meristem, and at a later stage, when flower organ primordia differentiate, it is abundantly detectable throughout the organ primordia. When the spikelet reaches maturity its expression is not detected in the lodicules (which are considered as a reduced perianth in rice) or the sterile glumes. OsMADS18 shares considerable sequence similarity with OsMADS14 (previously known as FDRMADS 6) but its expression pattern is quite distinct. OsMADS14 expression is detectable only in flowers and when all organ primordia have developed, its expression appear to be specifically localized in developing stamens and pistil [39]. BM3 is abundantly expressed in all organ primordia and the vascular tissue of the barley floret throughout inflorescence development. Its expression can also be detected in vegetative tissues (nodes and leaves) [26]. The similarities in expression pattern of many monocot *AP1*-like MADS-box genes, including the rice *OsMADS*18, the barley *BM3*, the three isolated in this study crocus *CsAP1* 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 three isolated class A crocus MADS-box genes constitute a start point for our studies of flower development in crocus. We have recently isolated several class B and class C MADS-box genes of crocus and characterized their expression (manuscripts in preparation). Experiments are underway to further characterize the possible specific role(s) of each gene in crocus that will enable us to infer a flower development model of this plant, to understand the formation of a number of mutant flowers observed in the field, and define ways to enhance its productivity exploiting molecular genetic techniques.

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