Isolation of a differentially spliced C-type flower specific *AG*-like MADS-box gene from *Crocus sativus* and characterization of its expression

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Abstract

We have cloned and characterized the expression of *Crocus sativus AGAMOUS1* (*CsAG1*), a putative C-type MADS-box gene homologous to *AGAMOUS* (*AG*) from a triploid monocot species crocus (*Crocus sativus* L.). The typical domain structure of MIKC-type plant MADS proteins was identified. Phylogenetic analysis of the deduced amino acid sequence indicated that the isolated gene forms a clade with the *AGAMOUS* homologs from the monocots *Hyacinthus orientalis* and *Phalaenopsis equestris*. A differential splicing event altering the amino acid sequence at the C terminus was identified, leading to the formation of two mRNAs differing ten nucleotides in size. The presence of both differentially spliced transcripts was restricted only to mature crocus flowers and particularly to stamens and carpels.

Additional key words: *AGAMOUS*, monocot, saffron, phylogeny.

Introduction

MADS-box genes encode transcription factors present in several eukaryotic organisms and contain a highly conserved sequence encoding the MADS-domain that is responsible for nuclear localization, DNA binding, dimerization and accessory factor binding (Theissen *et al.* 2000, Immink *et al.* 2002). The first isolated plant MADS-box genes were *DEFICIENS* (*DEF*) from *Antirrhinum* and *AGAMOUS* (*AG*) from *Arabidopsis.* Although initially found in floral tissues where they regulate floral organ identity, it was later established that MADS-box genes also act as regulators of various other aspects of plant development (Rounsley *et al.* 1995, Kim *et al.* 2002). It has been proposed that there are at least two lineages (type I and type II) of MADS-box genes in plants, animals and fungi (Alvarez-Buylla *et al.* 2000). Most of the plant MADS-box genes belong to type II and encode proteins with a stereotypic organization of four domains: the conserved ~55 amino acid (aa) MADS-domain (M), followed by the intervening (I) domain (~30 aa), the keratin-like coiled-coil (K) domain (~70 aa) and the variable C-terminal (C) domain that provides functional specificity. Therefore genes displaying this organization are called MIKC-type and are plant-specific transcriptional regulators. Subsequent work revealed in plants the existence of a large family with at least nine classes of MADS-box genes based on function and expression patterns (Nam *et al.* 2003). In angiosperms, at least five classes of MADS-box genes are involved in control of flower development. Particularly, the ABC model of flower development (Weigel and Meyerowitz 1994) predicts that three classes of floral MADS-box genes, encoding the A, B and C functions, act alone or in combination to specify floral organ identity. Since its initial proposal, the ABC model has been refined to include D- and E-class genes (Ferrario *et al.* 2004) and has been followed by the 'quartet model', which predicts that the identity of the different floral organs – sepals, petals, stamens and carpels – is determined by four combinations of floral homeotic MADS-box proteins (Theissen 2001). Regardless the model, AG-homologous genes are required for stamen, and carpel development and are known as the C-function genes. The class C function of floral organ development has been conserved during the 300 million years of evolution after the divergence of gymnosperms and angiosperms (Zhang *et al.* 2004).

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Abbreviations: BLAST - basic local alignment search tool; MADS - minichromosomal maintenance *1 Agamous deficiens* serum response factor; MEGA - molecular evolutionary genetics analysis; MIKC - MADS-box intermediate keratin-like C-terminal domain structure; PAGE - polyacrylamide gel electrophoresis.
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We are interested in characterizing the expression of genes involved in flower development in crocus, a monocot triploid species belonging to the *Iridaceae* family, since its stigmas constitute the expensive food additive saffron. The flowers of crocus are bisexual. The outer two whorls (the perianth) consists of 6 tepals. The next two whorls form sequentially stamens and carpels. Removal of stamens and separation of stigmas by hand is very labour intensive leading to high costs (see also Tsaftaris *et al.* 2004). It would be desirable to have mutants without stamen formation in crocus flowers or even to transform stamens to carpels, which should double saffron production in a single flower while lowering the cost of production and improving saffron quality. In order to understand and exploit the molecular mechanisms that control flower development in crocus, we started to clone and characterize the expression of MADS-box genes in crocus flowers (Tsaftaris *et al.* 2004). As C-class MADS-box gene function is essential for both stamen and carpel formation we report here the isolation and characterization of *CsAG1*, a C-class *AG*-like gene that is differentially spliced in two products and its expression is restricted to flowers and particularly in stamens and carpels.

Materials and methods

Plants: *Crocus sativus* L. cv. Kozani field growing plants were collected during the late flowering season in October from Kozani, Greece. As the crocus perianth has not distinct sepals and petals we treated separately the three tepals of the outer whorl and the three tepals of the inner whorl. Tissues were separated and immediately frozen in liquid nitrogen and stored at -80 °C until used.

RNA isolation, cDNA synthesis and gene expression analysis: Total RNA from flowers 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*). Isolation of *CsAG1* was accomplished by RT-PCR using standard protocols, essentially as described previously (Tsaftaris *et al.* 2004). First strand cDNA synthesis was performed using 0.5 μg total RNA from closed flowers, 0.25 μg 3′RACE Adapter Primer 5′-GGCCACGCGTCGACTAGTAC$(T)_{17}$-3′ (*Gibco-BRL*, Paisley, UK), 1 mM dNTPs and 200 units M-MuLV reverse transcriptase (*NEB*, Beverly, USA) in 0.05 cm^3 total volume. MADS-box cDNAs were amplified in PCR using the degenerate MADS-F primer 5′-ATCSAGATMAARAGRATHGARAA-3′ and the Abridged Universal Amplification primer (AUA) 5′-GGCCACGCGTCGACTAGTAC-3′ (*Gibco-BRL*). Nested PCR on the amplification products was performed using the primer MADS-2F, 5′-GTKCTYTGYGAYGCYGAGGT-3′ (Van der Linden *et al.* 2002) and the primer AUA. PCR reactions were performed using 0.2 pmoles of each primer, 0.2 mM dNTPs and 1 unit DyNAzyme II DNA polymerase (*Finnzymes*, Espoo, Finland). The thermocycler's program was: 1 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 50 °C, 90 s at 72 °C and a final extension step of 10 min at 72 °C. Several products between 300 and 1100 bp were cloned into the pGEM T easy vector (*Promega*, Madison, USA). Sequencing was performed using a *LiCor 4200* sequencer. BLAST searches identified one clone of 806 bp with homology to *AGAMOUS* genes from other plant species. Based on this sequence, the gene specific primer AGAM-R (5′-ATAGCAATAAGGTACCCAGTCAC-3′) was designed from the 3-UTR and used to isolate the cDNA's 5′end, using the GeneRacer kit (*Invitrogen*, Paisley, UK) according the instructions. A PCR fragment of 1100 bp was purified from the gel and cloned into the pGEM T easy vector (*Promega*) according to the manufacturer's protocol.

Gene expression analysis was performed with RT-PCR. Total RNA (1 μg) extracted from leaves, flowers, tepals, stamens and carpels was used in a reverse transcription reaction as previously described. PCR was performed using primer AGAM-R and primer AG1F (5′-AAGCTTCCTTCAAGCGAACTTG-3′) on 1/50 of the synthesized cDNA as template. The primers were designed to amplify a 263 bp fragment containing the deletion observed in the isolated genes. Primers actin 2-F (5′-CCGGTGTCATGGTTGGTAT-3′) and actin 2-R (5′-GCAGGCACATTGAAGGTCT-3′), amplifying a fragment of the actin-beta gene, were used as control for successful cDNA syntesis. 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.

DNA isolation and Southern hybridization: Genomic DNA was isolated from leaves using the DNeasy plant mini kit (*Qiagen*) according to the manufacturer's protocol. 10 μg genomic DNA was digested with *Eco*RI, *Hind*III and *Bam*HI, restriction enzymes (*NEB*), transferred onto a nylon membrane (*Roche*, Mannheim, Germany) and hybridized with an AG-specific digoxigenin labeled probe prepared with the PCR DIG Probe Synthesis Kit (*Roche*) using the primers AG1F and AGAM-R. Hybridization was performed in DIG Easy Hyb buffer (*Roche*) at 42 °C according to the manufacturer and stringent washes were at 68 °C in 0.5 X SSC / 0.1 % SDS (twice). Detection was performed using the DIG Luminescent Detection Kit (*Roche*) according the instructions and chemiluminescense was detected using the Gene-Gnome Bio Imaging System (*Syngene*, Cambridge, UK).

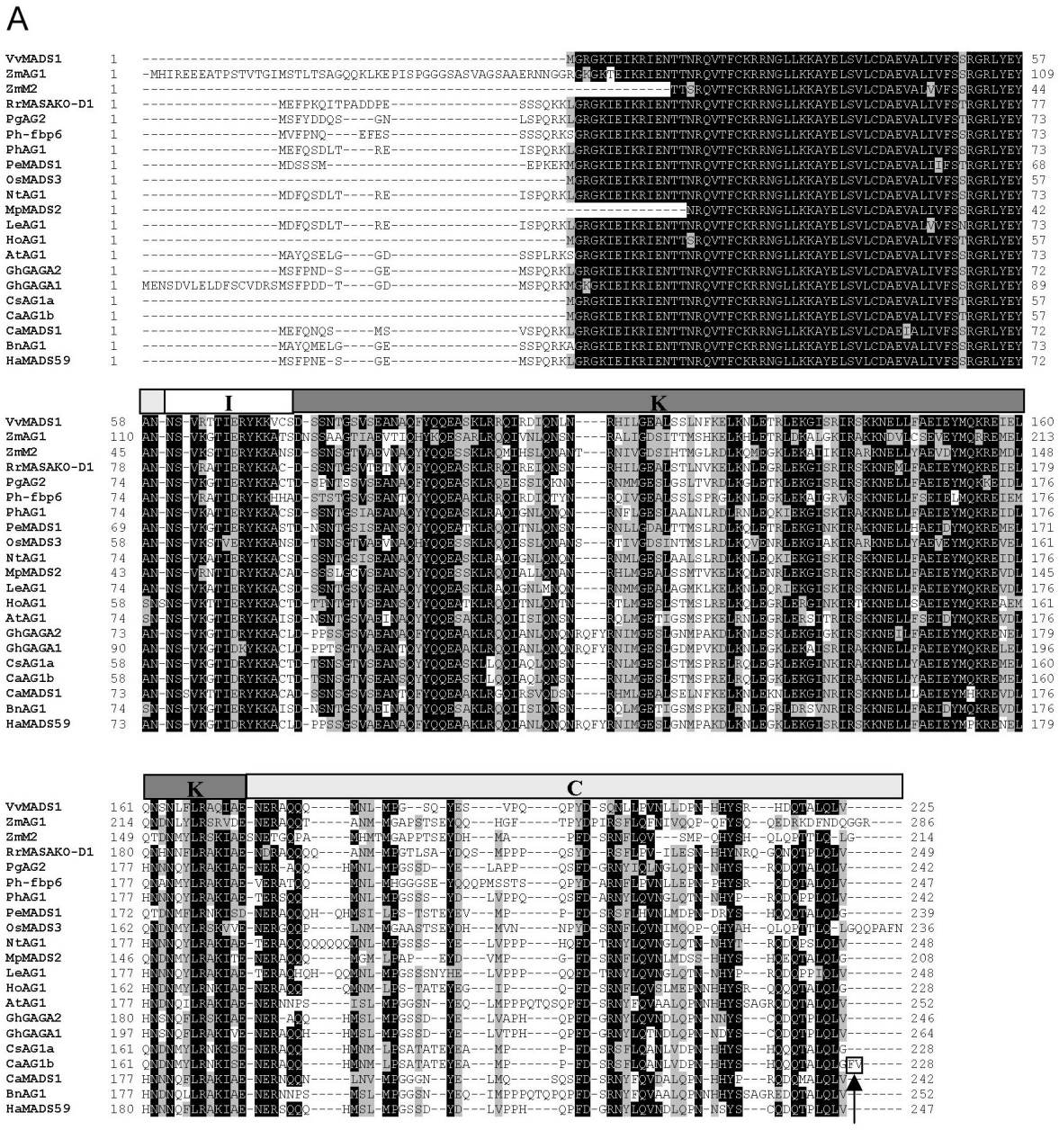

Fig. 1. *A* - Alignment of the deduced amino acid (aa) sequences of *CsAG1a* and *CsAG1b* and other homologous plant AG-like proteins using *Clustal W* (Thompson *et al.* 1994). Identical aa in all the sequences are highlighted in black and in 70 % of the sequences in gray. The 2 aa difference of the alternatively spliced products of *CsAG1*is boxed and indicated by an arrow. Highlighted on top of the alignment are the four domains of the MIKC-type proteins. *B* - Partial alignment of cDNA fragments from *CsAG1a*, *CsAG1b*, and the corresponding genomic fragment, showing the position of the intron. In bold and highlighted are the 5-splice donor element GT and the two alternative 3 splice acceptor elements AG of the intron. Underlined are the stop codons TAA for CsAG1a and TAG for CsAG1b.

Comparison and phylogenetic analysis: The deduced CsAG1 amino acid sequences were used in *BLAST* searches and the best hits were for AG-like genes from *Hyacinthus orientalis* (HoAG1, AAD19360), *Phalaenopsis equestris* (PeMADS1, AAL76415), and *Magnolia praecocissima* (MpMADS2, BAB70737). These, and 16 additional *AGAMOUS* genes were selected for phylogenetic analysis. The sequences were from: *Nicotiana tabacum* (NtAG1, Q43585) (Kempin *et al.* 1993); *Lycopersicon esculentum* (LeAG1, Q40168) (Pnueli *et al.* 1994); *Petunia hybrida* (PhMADS3, Q40885) (Tsuchimoto *et al.* 1993) and (Ph-fbp6, CAA48635) (Angenent *et al.* 1993); *Panax ginseng* (PgAG2, Q40872); *Gerbera hybrida* (GhGAGA1, CAA08800 and GhGAGA2, CAA08801) (Yu *et al.* 1999), *Corylus avellana* (CaMADS1, AAD03486) (Rigola *et al.* 1998); *Arabidopsis thaliana* (AtAG1, P17839) (Yanofsky *et al.* 1990); *Brassica napus* (BnAG1, Q01540) (Mandel *et al.* 1992); *Rosa rugosa* (RrMASAKO-D1, BAA90743) (Kitahara and Matsumoto 2000); *Zea mays* (ZmAG1, JQ2289) (Schmidt *et al.* 1993) and (ZmM2, CAA57074) (Theissen *et al.* 1995); *Helianthus annuus* (HaMADS59, AAO18229); *Oryza sativa* (OsMADS3, S59480) (Kang *et al.* 1995) and *Vitis vinifera* (VvMADS1, AAK58564) (Boss *et al.* 2001). The deduced amino acid sequences of the above genes together with CsAG1a and CsAG1b were aligned using the multiple sequence alignment program *Clustal W* (Thompson *et al.* 1994). Phylogenetic relationships of the sequences were examined using the Neighbor-Joining method with p-distance correction (Saitou and Nei 1987). The tree was constructed using the *MEGA 2.1* software (Kumar *et al.* 2001).

Results and discussion

Isolation and characterization of *CsAG1*: In this study we describe the isolation of *CsAG1* a putative C-type MADS-box gene homologous to *AGAMOUS* (AG) from crocus. Sequencing revealed the presence of two transcripts designated *CsAG1a* (GenBank accession number AY555579) and *CsAG1b* (GenBank accession number AY555580), which are different since *CsAG1a* is missing 10 bp from 890 to 899 in comparison with *CsAG1b*. This deletion alters the coding ORF of *CsAG1* in such a way that the deduced protein sequence of *CsAG1a* is two aa shorter at the C terminus from the deduced protein sequence of *CsAG1b* (Fig. 1*A*). To investigate if the phenomenon is due to differential splicing, we amplified the corresponding genomic DNA fragment by PCR using the primers AGAM-R and AG1F. PCR yielded only one distinct 360 bp band as determined by 5 % polyacrylamide electrophoresis (data not shown). Further, this genomic DNA fragment was sequenced (GenBank accession number AY555581) and revealed the presence of an 100-110 bp intron beginning with the typical plant intron 5' splice donor GT and ending at two nearby 3' splice acceptor AG signals (Lorkovic *et al.* 2000) being 10 bp apart and matching the observed deletion in the two isolated transcripts (Fig. 1*B*). This intron in crocus is located exactly at the conserved position of the 8th intron in plant *AGAMOUS* genes (Zhang *et al.* 2004). Southern hybridization analysis using a probe prepared by PCR with the primers AG1F and AGAM-R revealed that three copies of the *AGAMOUS* gene are present in the genome of crocus (Fig. 2) that is in agreement with the triploid nature of the crop.

Phylogenetic analysis of *CsAG1*: The deduced amino acid sequences were used in *BLAST* searches and the best hits were for AG-like genes from *Hyacinthus orientalis* (HoAG1, AAD19360), *Phalaenopsis equestris* (PeMADS1, AAL76415), and *Magnolia praecocissima* (MpMADS2, BAB70737). These, and several additional *AGAMOUS* genes for which there are published reports were aligned using *Clustal W* (Fig. 1*A*), and their phylogenetic relationships were examined using the Neighbor-Joining method with p-distance correction (Saitou and Nei 1987). The tree was constructed using the *MEGA 2.1* software (Kumar *et al.* 2001). The crocus sequences fall in a group with the *AGAMOUS* genes from a hyacinth (*Hyacinthus orientalis)* and an orchid plant (*Phalaenopsis equestris*), which both similarly to crocus have tepals in the perianth (Fig. 3).

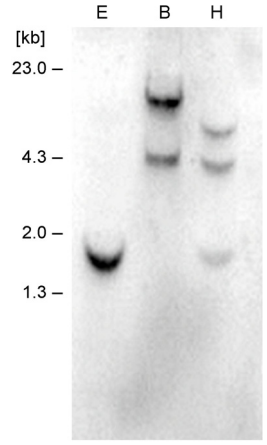

Fig. 2. Southern blot of genomic DNA from *Crocus* digested with: *Eco*RI (E), *Bam*HI (B) and *Hind*III (H) using as probe a PCR fragment of the cloned *CsAG1* genomic DNA. The profile of the *Hind*III digestion indicates that 3 copies of the gene are present in the crocus genome.

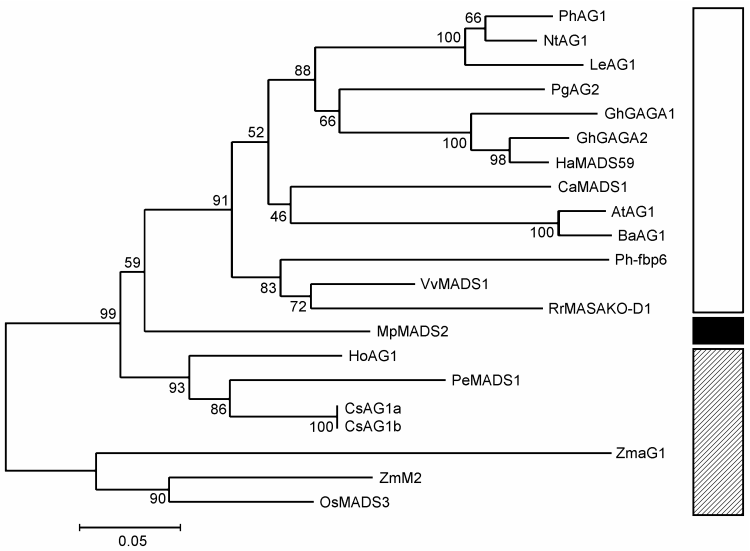

Fig. 3. Phylogenetic relationships of amino acid *AGAMOUS* sequences (alignment in Fig. 1*A*) estimated using the Neighbor-Joining method with p-distance correction. The tree was constructed using the *MEGA 2.1* software. Bootstrap values (1000 replications) are shown at the cross of clades and sequence distances are shown at the bottom of the tree. The crocus sequences are grouped with *AGAMOUS* genes from a hyacinth (*Hyacinthus orientalis)* and an orchid plant (*Phalaenopsis equestris*). *White bar* - eudicot plants, *black bar* - Magnoliids, *stripped bar* - monocot plants.

Expression analysis of *CsAG1* in crocus tissues: Expression analysis of *CsAG1* was performed with RT-PCR using the primer pair AGAM-R and AG1F. Primers actin2-F and actin2-R (Tsaftaris *et al.* 2004) amplifying a fragment of the actin-beta gene were used as a cDNA syntesis control, and all the experiments included a negative RT-PCR control. No amplification could be observed in the negative controls (data not shown).

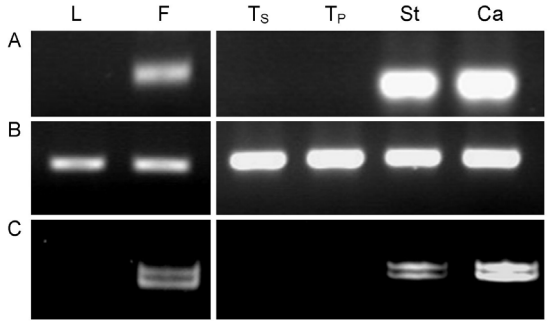

Fig. 4. *CsAG1* expression in leaves and flowers of crocus detected with RT-PCR. cDNA from leaves (L) , flowers (F), tepals of the outer whorl that forms sepals in other plants (T_S), tepals of the inner whorl that forms petals in other plants (T_P), stamens (St) and carpels (Ca) was amplified using: *A* - *CsAG1* gene specific primers, *B* - actin primers, *C* - fine resolution of PCR products from Fig. 3*A* on 5 % native PAGE.

Experiments revealed the presence of the transcript only in flowers and more specifically restricted in stamens and carpels (Fig. 4*A*). This expression pattern is in agreement with the expected according the ABC model of floral organ development and provides the basis for classification of *CsAG1* in the C-class MADS-box genes. A 5 % nondenaturing polyacrylamide gel was loaded with the same PCR products to investigate whether both isolated transcripts were expressed. The fine resolution through native PAGE revealed that both transcripts were present in stamens as well as in carpels (Fig. 4*C*).

Experiments are underway to isolate the complete set of flower-specific MADS-box genes in crocus. Towards this goal we have identified the *APETALA 3* (*AP3*) and *SEPALLATA* (*SEP*) homologs, which we are currently characterizing. Expression of the B-class *AP3* gene normally restricted to the second and third whorl is extended to the first whorl in crocus (data not shown). This could explain the homeotic transformation of sepals to petals in crocus flowers. Isolation and functional analysis of all the classes of MADS-box flower-specific genes will enable us not only to understand homeotic transformations in crocus flowers, but will also help to characterize and possibly exploit the numerous flower mutants (multiple flower organs, lack of stamens, *etc*.) frequently observed in fields cultivated with this asexually propagated crop.

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