

From model plants to crops: the MADS – box family of gene controlling flower development in Crocus (*Crocus sativus* L.)

A. Tsaftaris^{1,2}, K. Pasentsis¹, A. Kalivas², A. Polidoros¹

¹Institute of Agrobiotechnology (IN.A.) - Center for Research and Technology Hellas (CERTH), Thessaloniki, GREECE.

²Department of Genetics and Plant Breeding, AUTH, Thessaloniki
Email address: tsaft@certh.gr

Abstract

We have cloned and characterized the structures and expression, of the family of flower specific MADS-Box genes of cultivated Crocus (*Crocus sativus* L). The deduced amino acid sequence of the gene indicated high homology with members of the MADS-box family of transcription factors. In contrast to PISTILLATA (PI) and APETALLA3 (AP3) studies of other plants monocots and dicots and the predictions of the ABC-model for floral organ identity genes, expression studies indicated the presence of the transcripts are not restricted only in the second and third whorl of the flowers but also in the petaloid sepals and stigmata of the mature crocus flower parts, explaining the homeotic transformation of sepals to petals in this species.

Introduction

Arabidopsis plants produce a number of closely spaced rosette leaves during the vegetative phase. Upon the transition to flowering, the internode length increases, and this event causes a significant increase in the distance that separates the last few vegetative (cauline) leaves. The flowers are composed of four whorls of organs, with sepals in the first, or outermost, whorl, petals in the second whorl, stamens in the third whorl, and carpels occupying the fourth whorl, in the center. The simple and elegant ABC model of flower development was proposed to explain the activity of the floral organ identity genes (Coen and Meyerowitz, 2001). According to the model, the combined actions of three different functions, each one active in two adjacent whorls, are responsible for the development of the four types of organs of a typical eudicot flower. The model suggests that A alone specifies sepals, C alone specifies carpels, and the combined activities of AB and BC specify petals and stamens, respectively.

In *Arabidopsis*, *APETALA1* (*API*) and *APETALA2* (*AP2*) are the A function genes, *APETALA3* and *PISTILLATA* are the B function genes, and *AGAMOUS* (*AG*) is the only C function gene. With the exception of *AP2*, all of these genes are members of the MADS-box family of transcription factors, whose expression patterns correspond to their domains of action

Today, MADS box research is revealing a much more detailed and complex picture from the initial ABC model. Whereas the original ABC model was based largely on the analysis of flower developmental mutants using classic forward-genetics approaches, recent progress using reverse-genetics strategies has revealed redundant functions that were missed before. Likewise, recent studies using protein-protein interactions to elucidate complex interaction scheme than the originally proposed heterodimer formation between the B-function genes. Combining these results has led to extensions of the ABC model toward models with a higher complexity, including a novel class of genes called identity-mediating or E-function genes required for B and C floral organ identity functions (Homma and Goto, 2001).

Crocus sativus, a monocot triploid species belonging to the Iridaceae family, is cultivated for its red stigmatic lobes of the carpel that constitute saffron. It is cultivated in Southern Europe (mainly in Greece), 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. The flowers of crocus are bisexual. Perianth consists of 6 petaloid tepals in two whorls. Androecium consists of 3 distinct stamens and the gynoecium consists of a single compound pistil of 3 carpels, a single 3-branched style, and an inferior ovary. The flower is sterile, thus the crop is propagated asexual.

The method of saffron 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 carpels, 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 around 370-470 hours of work. Consequently, the cultivation of this crop for its flowers and specifically its stigmas is very labor intensive leading to high costs.

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 and many homeotic mutants with variable number of flower parts have been identified in farmer's fields (Tsaftaris *et al.* unpublished data).

Towards this goal we report the cloning and characterization and studies on the expression of three homologous Apetala like (A-like) genes designated *CsAP1a,b,c* (Tsaftaris *et al.*, 2004a) two Agamous like (C-like) differentially spliced genes designated *CsAGa,b* (Tsaftaris *et al.*, 2004b) and describe here our efforts in cloning and characterization of the B-like (AP3 and PI) genes as well as the E-like gene Sepallata.

Materials and methods

Crocus sativus field growing plants were collected from Kozani, 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.

Total RNA from leaves, closed flowers (3 cm in length), sepals, petals, stamens and carpels was extracted using the RNeasy plant mini kit (Qiagen). For amplification of MADS Box sequences, two degenerate primers, MADSF (Tsaftaris *et al.* 2004) and MADS2F (van der Linden *et al.* 2002), which corresponding to conserved amino acid sequences of the MADS domain of MADS Box genes from other plant species and was 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-GGCCACGCGTCTAGTA(T)₁₇₋₃, Gibco-BRL), 1mM dNTPs and 200u M-MuLV reverse transcriptase (NEB) in 50µl total volume. 1/25 of the synthesized cDNA was used as template in a touch down PCR reaction with 1pmole MADSF primer, 0.2pmoles Abridged Universal Amplification primer, 0.2mM dNTPs and 1u DyNAzyme II DNA polymerase (Finnzymes). The thermocycler program was 2 min at 94°C, 34 cycles of 45sec at 94°C, 45sec at 54°C, 1 min 45sec at 72°C followed by a final extension step of 15min at 72°C. 1/25 of the PCR product was used as template in a touch down Re-PCR reaction with 1pmole and MADS2F primer, 0.2pmoles Abridged Universal Amplification primer, 0.2mM dNTPs and 1u DyNAzyme II DNA polymerase (Finnzymes).

The thermocycler program was: 1 min at 94⁰C, 30 cycles of 30sec at 94⁰C, 45sec at 50⁰C, 1.5min at 72⁰C and a final extension step of 10min at 72⁰C. Several products between 300 and 1100 bp were excised, re-amplified and cloned into the pGEM T easy vector (Promega) 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 cDNA's 5'end, an RNA ligase-mediated rapid amplification reaction was performed on a pool from total RNA from leaves and flowers using the GeneRacer Kit (Invitrogen) according to the manufacturer's protocol as described in (Tsaftaris *et al.* 2004). Based on the sequence information obtained by the 3' RACE experiments, gene specific primers and degenerate, for the five family of MADS box genes, were designed from the 3-UTR and used to isolate the cDNA's 5'ends following the recommendations of the manufacturer. .

The expression analysis of the isolated MADS- box gene was performed with RT-PCR. One µg of total RNA extracted from leaves, flowers, sepals, petals, stamens and carpels were used in a reverse transcription reaction as described above in the RNA isolation and cDNA. PCR was performed in 1x PCR buffer, 0.2mM dNTPs, 0.4pmoles of two specific primers for each family gene and 1u of the DyNAzyme II DNA polymerase (Finnzymes) using as template 1/50 of the synthesized cDNA. Primers actin2-F and actin2-R, amplifying a fragment of the actin-beta gene, were used as control. The cycling parameters were incubation at 94⁰C for 2min, followed by 30 cycles of incubation at 94⁰C for 30 sec, 54⁰C for 30 sec, 72⁰C for 30 sec and a final extension step of 5 min at 72⁰C. 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.

Results

The 3' RACE experiments on flower cDNA from *Crocus sativus* yielded in the isolation of several clones with an uninterrupted ORF and with homology to AP1, AP3, PI, AG and SEP genes from other plant species. The clones were different so in their coding region as in the size of the 3 UTR. Based on the sequence information obtained, gene specific primers for the five gene families designed to anneal downstream from the putative stop codon were used in the 5' RACE experiments. Several PCR fragments between 700bp and 1100 bp was purified from the gel and cloned into the pGEM T easy vector (Promega) according to the manufacturer's protocol. Eighteen clones were sequenced.

The analysis of the sequencing results using DNA Star revealed that the 5' RACE clones and the 3' RACE clones could be grouped into five populations for the gene CsPI (*CsPIA1*, *CsPIA2*, *CsPIB*, *CsPIC1* and *CsPI2*), into two populations for the gene CsAP3 (*Csap3a* and *Csap3b*), into two population for gene *CsAG* (*CsAG1a* and *CsAG1b*), into two populations for the gene *CsSEP* (*CsSEP1* and *CsSEP2*) and into three population for *CsAPI* (*CsAPIa*, *CsAPIb* and *CsAPI*).

Phylogeny analysis showed that *CsAPI*, *CsAP3*, *CsPI*, *CsAG* and *CsSEP* genes belong to AP1, AP3/DEF, PI/GLO, AG and SEP subfamilies respectively and these genes are closely related to other monocot AP1-, AP3/DEF-, PI/GLO-, AG- and SEP-like genes.

The expression pattern of the five MADS Box genes 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. Experiments revealed the presence of the transcripts for *CsAP3*, *CsSEP* and *CsAG* only in

flowers, instead for *CsAPI* and *CsPI* revealed the presence of the transcript both in flowers and in leaves. The expression pattern of these five families genes was also examined in different flower tissues. The RT-PCR experiment performed with cDNA synthesized from sepals, petals, stamens and carpels resulted in the identification of the transcript in all mature flower parts for *CsAPI*, *CsAP3*, *CsPI* and *CsSEP* and the presence of the transcript, for *CsAG*, restricted in stamens and carpels.

Discussion

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.

Three *API*-, Five *PI*-, two *AP3*-, two *AG*- and two *SEP*-like MADS Box genes were isolated from *Crocus sativus* L. The sequence alignment revealed that the above proteins contain the typical domain structure of plant MADS box proteins consisted of the conserved N-terminal MADS-box, the I domain, the central K domain and the C terminal domain.

In Arabidopsis, expression of *API* occurs specifically in the tissues and at the developmental stage in which floral fate is assumed. In the flower, expression of *API* is restricted to petals and sepals. In contrast, RT-PCR experiments revealed that the three *CsAPI* genes are expressed in leaves, as well as in all the flower organs examined. The three isolated *CsAPI* genes from crocus, are *API*-like MADS-box genes expressed in vegetative as well as in all floral tissues of the plant. Similar expression pattern display many monocot *API*-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.

In contrast to Pistillata and *AP3* studies of other plants monocots and dicots, which follow the ABC model, and the expression of *AP3*- and *PI*-like MADS Box genes is restricted in 2 and 3 whorl, the expression pattern of *CsPI* *CsAP3* revealed that these genes are expressed in floral organs, especially in whorls 1, 2, 3, 4 (outer and inner tepal, stamen and carpel respectively). The expression pattern of *CsPI* in whorl 1 can be explained with the modified ABC model, which proposed to explain the Liliaceae flower morphology by Van Tunen *et al.* (1993). According to this model, class B genes are not only expressed in whorls 2 and 3, but also in whorl 1. Thus the floral organs of whorls 1 and 2 express A and class B genes and, for this reason, get the same petaloid identity.

The function of *CsAG*, like C-class MADS-box gene, is essential for both stamen and carpel formation and in agreement with studies in other plants and the predictions of the ABC-model for floral organ identity genes, its expression studies indicated that the presence of both differentially spliced transcripts is restricted only to flowers and particularly in stamens and carpels of the mature crocus flower parts.

The expression studies for *CsSEP* reveals that *SEP*-like gene is also expressed in the first whorl leading to sepaloid petals in this crop and confirming its requirement for tetramer formation for the establishment of flower organ identity meristem.

References

- Coen E.S., Meyerowitz, E.M. (1991) The war of the whorls: genetic interactions controlling flower development. *Nature*. **353**. 31–37.
- Chung, Y.Y., Kim, S.R., Kang, H.G., Noh, Y.S., Park, M.C., Finkel, D., An, G. (1995) Characterization of two rice MADS box genes homologous to GLOBOSA. *Plant Sci*. **109**. 45–56.
- Honma, T., Goto, K. (2001) Complexes of MADS-box proteins are sufficient to convert leaves into floral-organs, *Nature*. **409**. 525–529.
- Kanno, A., Saeki, H., Kameya, T., Saedler, H., Theissen, G. (2003) Heterotopic expression of class B floral homeotic genes supports a modified ABC model for tulip (*Tulipa gesneriana*). *Plant Molecular Biology*. **5**. 831–841.
- Münster, T., Wingen, L.U., Faigl, W., Werth, S., Saedler, H., Theissen, G. (2001). Characterization of three GLOBOSA-like MADS-box genes from maize: evidence for ancient paralogy in one class of floral homeotic B-function genes of grasses. *Gene*. **262**. 1–13.
- Tsaftaris, A. S., Pasentsis, K., Iliopoulos, I., Polidoros, A. N. (2004a) Isolation of three homologous AP1-like MADS-box genes in crocus (*Crocus Sativus* L.) and characterization of their expression. *Plant Science*. **166**. 1235-1243.
- Tsaftaris, A. S., Pasentsis, K., Polidoros, A. N. (2004b), Isolation of a differentially spliced C-type flower specific AG-like MADS-box gene from Crocus (*Crocus sativus*) and characterization of its expression. *Biol. Plant*. in press.
- van der Linden, C.G., Vosman, B., Smulders, M.J. (2002) Cloning and characterization of four apple MADS box genes isolated from vegetative tissue. *J. Exp. Bot*. **53**. 1025-1036.
- van Tunen, A.J., Eikelboom, W., Angenent, G.C. (1993) Floral organogenesis in *Tulipa*. *Flow. Newsl*. **16**. 33–38.
- Yang, Y., Fanning, L., Jack, T. (2003) The K domain mediates heterodimerization of the Arabidopsis floral organ identity proteins, APETALA3 and PISTILLATA. *The Plant Journal*. **33**. 47-59.