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Identification and possible role of a MYB transcription factor from saffron (*Crocus sativus*)

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ABSTRACT

The MYB family is the most abundant group of transcription factors described for plants. Plant *MYB* genes have been shown to be involved in the regulation of many aspects of plant development. No *MYB* genes are described for saffron, the dried stigma of *Crocus sativus*, utilized as a colorant for foodstuffs. In this study, we used RACE-PCR to isolate a full length cDNA of 894 bp with a 591 bp open reading frame, encoding a putative CsMYB1 from *C. sativus*. Comparison between gDNA and cDNA revealed no introns. Homology studies indicated that the deduced amino acid sequence is similar to members of the R2R3 MYB subfamily. Expression analysis showed the presence of high transcript levels in stigma tissue and low levels in tepals, whereas no signal was detected in either anthers or leaves. The RT-PCR analysis revealed that *CsMYB1* expression is developmentally regulated during stigma development. Furthermore, expression analysis in stigmas from different *Crocus* species showed a correlation with stigma morphology. No transcripts were found in stigma tissues of *Crocus* species characterized by branched stigma morphology. Taken together, these results suggest that CsMYB1 may be involved in the regulation of stigma morphology in *Crocus*.

Introduction

Gene expression is regulated by transcription factors. Among these transcription factors, the MYB genes form one of the largest families in plants. They have diverse functions, indicating their importance in the control of different plant processes. The first MYB-like gene characterized in plants was maize C1 (Cone et al., 1986; Paz-Ares et al., 1987), which is involved in the regulation of the anthocyanin pathway. MYB proteins can be classified into three subfamilies depending on the number of adjacent repeats in the MYB DNA domain binding (one, two or three) (Stracke et al., 2001). These refer to MYB-like proteins with one repeat as MYB1R factors, with two as R2R3-type MYB factors, and with three repeats as MYB3R factors. A common characteristic of MYB proteins is the presence of a functional DNA binding domain which is conserved among animals, plants and yeast (Lipsick, 1996). Plant *MYB* genes have been shown to be involved in the regulation of many aspects of plant development, e.g. control of cell morphogenesis (Lee and Schiefelbein, 2001; Higginson et al., 2003) and regulation of floral and seed development (Shin et al., 2002; Steiner-Lange et al., 2003; Albert et al., 2011). It has been reported that *MYB* genes also play a key role in hormone signaling pathways and metabolism (Newman et al., 2004). The subfamily containing the two-repeat R2R3 is the largest in higher plants; members of this subfamily have been described for monocotyledons and dicotyledons. In *Arabidopsis*, rice and grape over 100 R2R3 *MYB* genes have been isolated, although only a few of them have been functionally characterized (Matus et al., 2008). Most of these genes are positive transcription regulators, e.g. *AmMYB* 305 and *AmMYB* 340 isolated from *Antirrhinum majus*, that upregulate the expression of phenylalanine ammonia-lyase (PAL), thus accomplishing the activation of the phenylpropanoid pathway (Moyano et al., 1996). On the other hand, some *MYB* genes, such as *AtMYB4*, can also act as negative regulators in *Arabidopsis thaliana*, repressing the expression of cinnamate 4-hydroxylase (Tamagnone et al., 1998; Jin et al., 2000). In strawberry, the *FaMYB1* gene plays a role, repressing transcription in order to balance the levels of anthocyanin pigments produced at the later stages of strawberry fruit maturation, and/or regulating metabolite levels in various branches of the flavonoid biosynthetic pathway (Aharoni et al., 2001).

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Saffron is a spice derived from the dried stigma of the *Crocus sativus* flower, which is a species belonging to the Iridiaceae family. As a sterile triploid plant, its propagation is vegetative and spreads by means of corms. Saffron only blooms once a year and the planting and harvesting of corms, along with the removal of stigmas, is done manually, making saffron the world's most expensive spice. Mainly used as a colorant for foodstuffs, it is highly appreciated for its aromatic and flavoring properties, while also being used in the confectionary and liquor industries. In recent years, numerous studies have focused on the biological and pharmaceutical properties of saffron, having proven its effectiveness in the reduction of cholesterol and triglyceride levels in blood (Abdullaev and Frankel, 1999), its ability to combat neural disorders (Abe and Saito, 2000), as well as its application in chemotherapy where it is used as an attenuator for the adverse effects of cisplatin (Premkumar et al., 2003).

A large cDNA EST library has recently been published by D'Agostino et al. (2007). 13% of the sequences encode putative transcription factors. One of the most abundantly expressed sequences encodes a MYB-like protein with high similarity to LhMYB (from *Lilium*, GenBank accession BAB40790), which is highly expressed in flowers.

The flower of *C. sativus* has an underground ovary and a long style divided at the top into three red trumpet-like stigmas (Grilli-Caiola, 2003). All plant stigmas perform the same functions: capture pollen, support hydration and germination, and offer entry points and guidance to pollen tubes en route to the ovaries. Stigmas are able to discriminate between different pollen grains and promote outcrossing or self-fertilization by the coordinated timing of their maturation with pollen release. Stigma development is linked to the development of other floral organs. In saffron, shoot growth and flower initiation have started by early July, before this time no change in the size of the bud or that of the naked shoot apex is observed and the bud seems to be dormant. During July the apex becomes dome shaped and the primordial leaf then begins to form. The sheathing leaves then start to grow more quickly than the shoot apex, piercing through the soil and protecting the growth of the young shoot. This is followed by the bract primordial stage, the formation of stamen primordia, the initiation of the perianth and finally the formation of gynoecium. All of the flower parts are already differentiated by the end of August (Molina et al., 2005). At anthesis, the pistil of *C. sativus* has dry-type stigmas with papillae covered with a thick continuous cuticule. Stigmas are erect until anthesis, but as the flower opens, they bend downwards. Stigmas from the *Crocus* genus present different color patterns; varying from pale yellow found in *C. goulimyi* to red dark in *C. sativus*. The quantitative and qualitative changes in the carotenoid and the apocarotenoid profile in *C. sativus* and other *Crocus* stigmas along with the genes involved in the accumulations of these products have been previously studied (Castillo et al., 2005; Ahrazem et al., 2010; Rubio-Moraga et al., 2010). Other phenotypical differences such as the pattern of branching stigmas and the genes responsible for the control of this mechanism have not been studied. R2R3-MYB transcription factors might be important candidates since in *A. thaliana* a group of R2R3 MYB genes has been described as responsible for the control of axillary meristem formation (Keller et al., 2006; Muller et al., 2006).

In this study, we isolated a *MYB* gene from *C. sativus*, with high similarity to *LhMYB*, designated as *CsMYB1*. The results from RT-PCR showed a higher expression in saffron stigmas and a relatively low one in tepals, with no transcripts detected in anthers and leaves. We further analyzed its expression pattern in different developmental stages of stigma maturation, resulting in no transcript detected in the earlier stages (yellow and orange) and high levels in the later stages (red stigmas). In order to provide more information concerning the *CsMYB1*, we analyzed its expression in other crocuses. Our results suggested that CsMYB1 may regulate stigma development together with other genes, since no transcripts were found in *Crocus cancellatus* and *Crocus pulchellus*, which have more branched stigmas than the other crocuses included in this study.

Materials and methods

Plant material

Crocus sativus, *C. cartwrightianus*, *C. hadriaticus*, *C. pallasii*, *C. ocreocreticus*, *C. kotschyanus*, *C. goulimyi*, *C. niveus*, *C. cancellatus* and *C. pulchellus* were obtained from private collections in the UK (Pottertons Nursery). Plant tissues were independently harvested and frozen in liquid nitrogen and stored at −80 °C until required.

DNA extraction

DNA was extracted from 150 to 300 mg of leaf material using a modified Doyle and Doyle method (Doyle and Doyle, 1990). Leaf material was then ground to a fine powder in liquid nitrogen and placed in a microcentrifuge tube with 2 mL of extraction buffer (2% CTAB, 100 mM Tris–HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, and 0.01% proteinase K) plus 40 μL of 2-mercaptoethanol. Following incubation at 65 °C for 30 min, 1.4 mL of chloroform:isoamyl alcohol (24:1) was added, mixed and centrifuged at 8000 rpm for 30 min; the supernatant was transferred to a new tube and then repeated three times. DNA was precipitated with isopropanol (2/3 volume of supernatant), then centrifuged at 8000 rpm for 30 min, the supernatant discarded and the pellet washed in 70% ethanol containing 10 mM ammonium acetate for 20 min. The pellet was dissolved in 100 μL of TE buffer (10 mM Tris–HCl pH 7.4, 1 mM EDTA) and the DNA was reprecipitated with 1/2 volume of ammonium acetate 3 M and 2.5 volumes of ethanol. After centrifuging at 8000 rpm for 30 min, the pellet was redissolved in TE buffer with 10 μg/mL RNase and incubated at 30 °C for 30 min. The extracted DNA was quantified with a spectrophotometer and diluted to 30 ng/μL in TE. The DNA was then stored at −20 °C for further analyses.

Isolation and cloning of the C. sativus MYB gene

mRNA was isolated from developing *C. sativus* stigmas by using Ambion PolyAtrack and following the manufacturer's protocols (Ambion, Inc.). Using 1 μg of poly(A)$^+$ RNA from stigmas was synthesize the first-strand cDNA with a Superscript II reverse transcriptase supplied in the SMART™ RACE cDNA Amplification Kit (Clontech). Primers used for this reaction are described in Table 1.

We used the gene-specific primers described in Table 1 to isolate the cDNA and gDNA with the following cycling program: one cycle at 94 °C for 3 min, 10 cycles at 94 °C for 20 s, 62–0.2 °C/cycle for 20 s, and 72 °C for 1:30 min, and a final extension at 72 °C for 5 min. The amplified PCR product was analyzed by electrophoresis in 1% agarose gel. The PCR product was then cloned into pGEM-T (Promega, Madison, WI, USA). The ligated DNA was transformed into *Escherichia coli* strain JM109. The clones (50 colonies) were picked individually and amplified in 3 mL of LB medium at 37 °C overnight. The plasmid DNA from each clone was extracted using a DNA plasmid Miniprep kit (Promega, Madison, WI, USA) and then sequenced using an automated DNA sequencer (ABI prism 310, Perkin Elmer).

Analysis of mRNA levels in different tissues and from Crocus stigmas

Gene-specific oligos were designed for use in the expression analysis (Table 1). For RT-PCR, total RNA was isolated from *C. sativus*