

# Comparative expression analysis of senescence gene CsNAP and B-class floral development gene CsAP3 during different stages of flower development in Saffron (*Crocus sativus* L.)

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**Abstract** *Crocus sativus*, a monocot triploid species belonging to the Iridaceae family, is cultivated for its red stigmatic lobes of the carpel that constitute saffron. Flower development has been extensively studied in different plants. Different floral developmental pathways have been deciphered in many plants. In *Crocus sativus*, flower is the most important part and understanding the pathway underlying the flower development can pave the way for new avenues to improve its productivity and quality. The combination of class A genes (including APETALA1; CsAP1 and APETALA2; CsAP2), class B genes (including APETALA3; CsAP3 and PISTILLATA; CsPI) and class C genes (including AGAMOUS; CsAG) that are active in each whorl, determines the identity of the organs that will later develop in that whorl. CsAP3 is a class B homeotic gene which promotes petal and stamen formation and has a very important role in flower development. It also activates other genes playing pivotal role in flower development. It has been earlier reported that CsAP3 gene has direct role in activation of CsNAP gene which promotes senescence in plants. Present work was focused on study of relative gene expression changes of CsAP3 and CsNAP gene during different stages of flower development.

CsAP3 gene expression was found maximum during late-preanthesis stages of stigma development. Expression increases from stage 5 to stage 6 of flower development and then reduces again from stage 6 to stage 7. CsNAP gene had moderate expression during stage 3 to stage 4 transition and its expression increased abruptly from stage 6 to stage 7 of flower development. There is no direct concordance in the expression of CsAP3 and CsNAP gene expression in saffron. We may conclude that some other factor(s) may be responsible for initiation of CsNAP expression and CsAP3 gene may directly/indirectly be involved in regulating the factors responsible for CsNAP activation.

**Keywords** Saffron · CsAP3 · CsNAP gene · Homeotic gene · Real Time PCR · Stigma development

## Introduction

*Crocus sativus*, a member of family Iridaceae is believed to have originated in Greece, Iran and Asia (Grilli Caiola et al. 2004) and is commonly used in medicines, food dyes and flavoring agents. The plant produces sterile flowers due triploidy of the chromosomes and thus reproduces asexually through the vegetative multiplication of corms. Flower is the most valuable part comprising of 6 tepals, 3 stamens and 3 stigmas whose development is differentially regulated by Class A-, B- and C- genes as proposed by ABC model. While development of tepals is governed by class A/B genes, development of stamens is governed by class B/C genes and development of carpels is governed by class C genes alone (Theissen and Saedler 2001). In *Arabidopsis thaliana*, MADS-box transcription factors, APETALA3 (AP3) and PISTILLATA (PI) regulate petal and stamen development (Zhang et al. 2011). Being homeotic genes, AP3 and PI have

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highly conserved sequences. AP3 and PI form heterodimers and appears to act together with other MADS box proteins *viz.* AP1 and SEPALLATA (SEP) to regulate development of floral organs (Mara et al. 2010; Yi and Jack 1998). AP3-PI heterodimer bind to the promoter of AP3 and drives its expression through an auto-regulatory positive feedback loop (Mara et al. 2010; Yi and Jack 1998). Two transcripts of CsAP3 gene have been reported in saffron, namely, CsAP3a & CsAP3b, which differ in CCT trinucleotide repeats in 5' and 3' UTRs (Tsiftaris et al. 2006). In this study, we have considered CsAP3a (Gene Bank accession number AY948339) transcript for gene expression analysis. Another target for AP3-PI heterodimer, NAP (NAC-like, activated by AP3/PI) has been found to play important role in promoting senescence in flowers and leaves (Guo and Gan 2006) besides establishment of meristem and separation of floral organs during development (Sablowski and Meyerowitz 1998). In saffron, CsAP3-CsPI heterodimer has also been reported to show positive association with the CsNAP gene expression (Kalivas et al. 2010). The CsNAP gene has been reported to be involved in senescence in saffron (Kalivas et al. 2010). In this study we compare the expression of CsAP3 gene and CsNAP gene at three different stages of saffron flower on the basis of stigma development so as to explore the pattern in which these genes express during flower development.

## Methodology

### Sample collection and storage

The samples were collected at seven different stages of saffron flower defined on the basis of morphological changes during the process of flower development. The seven stages were 1) Corm at dormant stage (June); 2) Appearance of initial dome shaped shoot apex showing formation of first leaves (July–Aug); 3) Transformation of shoot meristem into floral meristem. (Aug); 4) Floral initiation with three carpel primordia visible. (Aug–Sep); 5) Bud with stigma at yellow stage. (Sep–Oct); 6) Bud with stigma at orange stage. (Sep–Oct); 7) Bud with stigma at scarlet stage. (Oct–Nov) (Fig. 1). The samples were collected from the field and immediately dipped in liquid nitrogen. For long term storage these samples were stored at  $-80^{\circ}\text{C}$ .

### RNA extraction

Samples from the seven stages of flower development were ground into fine powder in liquid nitrogen in sterilized mortar and pestle and total RNA was extracted using Trizol Reagent (Sigma Aldrich, USA) following the manufacturer's protocol. RNA quality was checked by measuring the ratio of

absorbance at 260 nm and 280 nm. Only the samples having ratio between 2.1 and 2.3 were taken for cDNA synthesis.

### cDNA preparation

3  $\mu\text{g}$  total RNA was taken as template and first strand cDNA synthesis kit (Bangalore Genei) was used for the synthesis of first-strand cDNA as per manufacturer's instructions. The synthesized cDNA was stored at  $-20^{\circ}\text{C}$  for future use.

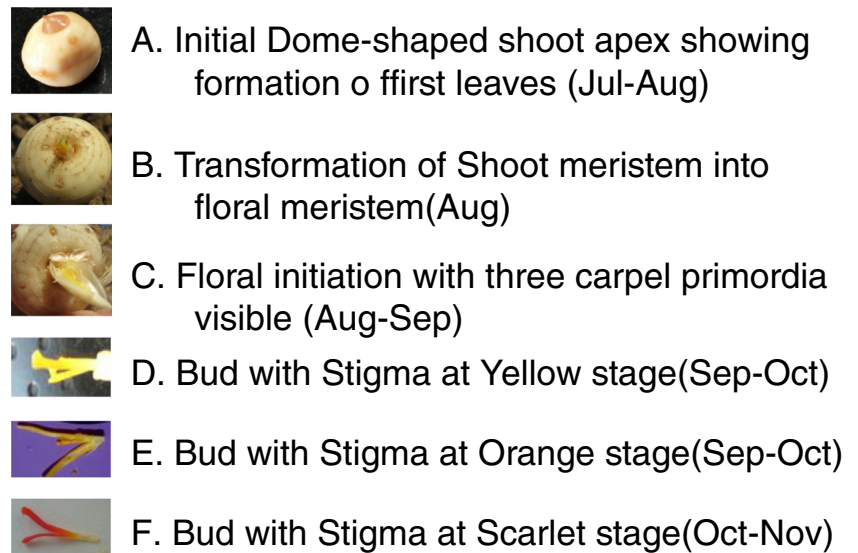
### Reverse transcription PCR and quantitative real time PCR

The real time PCR was performed with LightCycler real time PCR instrument (Applied Biosystems) in 96 well plates in triplicates using SYBR Green master mix (Fermentas). Each reaction contain 5  $\mu\text{l}$  SYBR Green Master Mix, 0.5  $\mu\text{l}$  forward and reverse primers, 3  $\mu\text{l}$  PCR grade water, 1  $\mu\text{l}$  cDNA totaling a final volume of 10  $\mu\text{l}$ . Each reaction was taken in triplicates so as to minimize the deviation in results due to handling errors. The primers used for amplification were CsAP3-F (5'-TTGGATGAGTCGTTGAGGCTTGT-3') and CsAP3-R (5'-AGGTAGCAAATTAAGTAGGAAAG-3') for CsAP3 gene; CsNAP-F (5'-GAGATCGGGGTATTGGAAGG-3') and CsNAP-R (5'-ATCGAATTCCAGCAAACCAG-3') for CsNAP gene and TUB-F (5'-TGATTTCCTCAACTCGACCA GTGTC-3') and TUB-R (5'-ATACTCATCACCTCGTC ACCATC-3') for tubulin gene. Tubulin gene expression was used as reference and scarlet stage was used as positive control. The amplification was carried at  $94^{\circ}\text{C}$  for 2 min incubation and 40 cycles of  $94^{\circ}\text{C}$  for 15 s,  $56^{\circ}\text{C}$  for 15 s,  $72^{\circ}\text{C}$  for 20 s, followed by final extension of  $72^{\circ}\text{C}$  for 2 min. Semi-quantitative gene expression study was carried by reverse transcription PCR using the same primers as in real time PCR with initial denaturation at  $94^{\circ}\text{C}$  for 5 min followed by 25 cycles of  $94^{\circ}\text{C}$  for 1 min,  $56^{\circ}\text{C}$  for 1 min and  $72^{\circ}\text{C}$  for 45 s, final extension of  $72^{\circ}\text{C}$  for 2 min. Product size was verified by agarose gel electrophoresis using 1.5 % agarose gel (Sigma Aldrich, USA). PCR product was visualized by ethidium bromide staining. Advanced relative quantification was done by  $2^{-\Delta\Delta\text{Cp}}$  method (Livak and Schmittgen 2001).

## Result and discussion

Expression study of CsAP3 and CsNAP genes during 7 stages of flower development was done through reverse transcription PCR and quantitative real-time PCR analysis to find out the relative change in expression of these genes. Although CsAP3 gene is involved in flower development but it may have some indirect role in senescence of flower and stigma development, which is an economic part of saffron. It was found that there is drastic increase in expression of CsAP3 gene from orange to

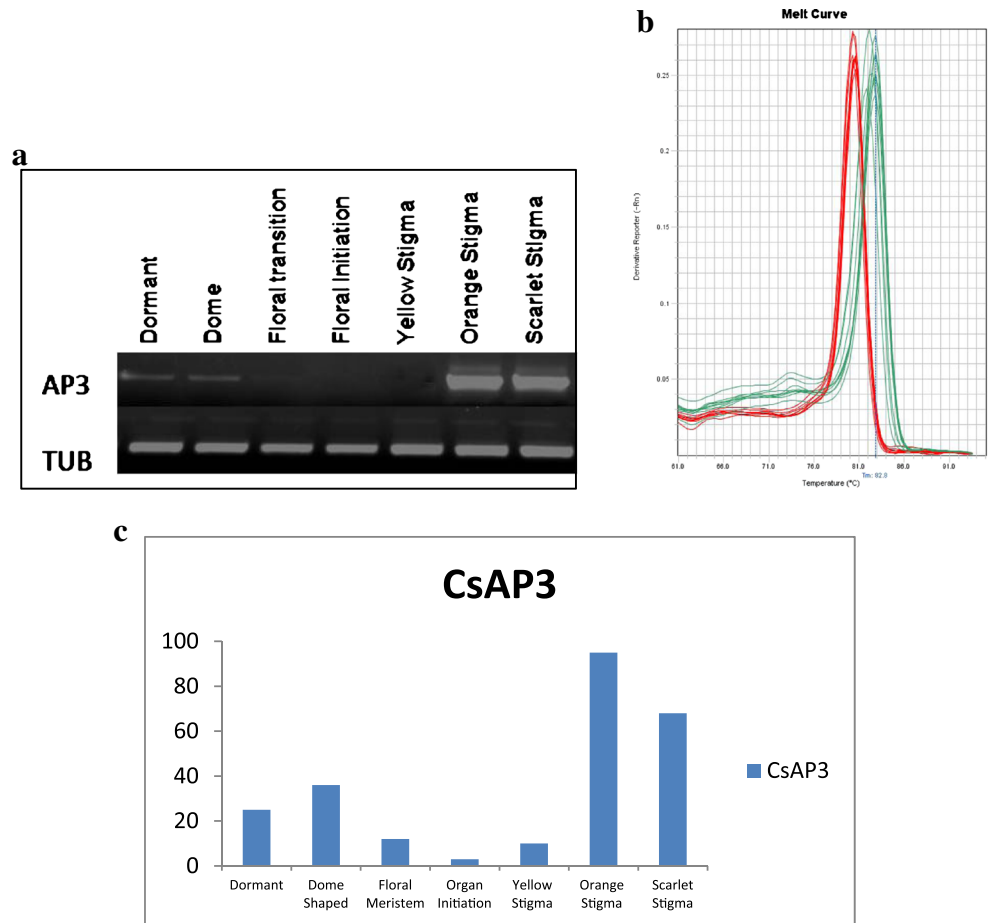
**Fig. 1** Stages of flower development from breaking of dormancy to scarlet stage



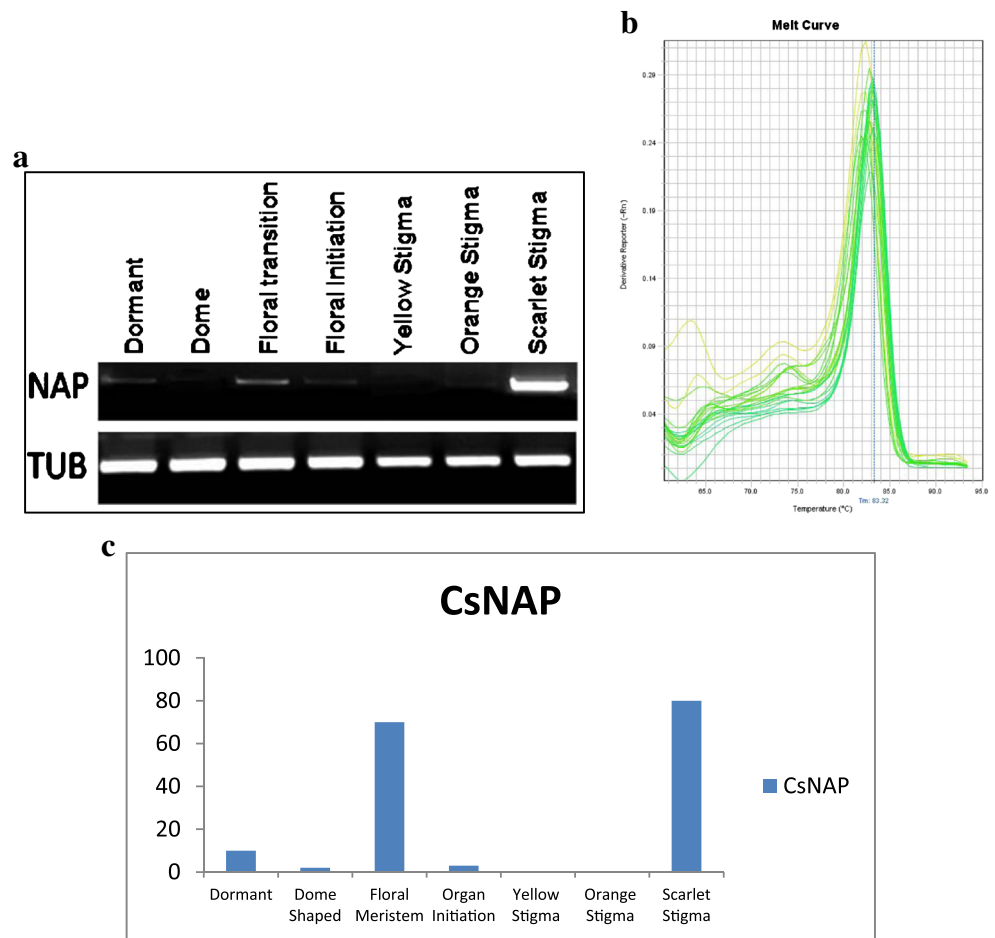
scarlet stages of flower development (Fig. 2). However, there was little expression of CsAP3 gene at dormancy and just after breaking of dormancy, while no expression was observed at floral transition, floral initiation and yellow stages. On the other hand CsNAP, a senescence gene, show an abrupt increase in

its expression in orange to scarlet transition. There was moderate expression of CsNAP gene during transition of shoot apical meristem to floral apical meristem which implies that there may be some role of CsNAP in this transition. There was little expression of CsNAP gene at dormancy stage (Fig. 3).

**Fig. 2** **a** Semi-quantitative expression levels of CsAP3 gene at different stages of flower development, **b** Melting curve of CsAP3 (red) and TUB (green), **c** Graphical Map representation of quantitative expression levels of PI gene at different stages of flower development



**Fig. 3** **a)** Semi-quantitative expression levels of CsNAP gene at different stages of flower development, **b)** Melting curve of CsNAP (green) and TUB (yellow), **c)** Graphical representation of quantitative expression levels of CsNAP gene at different stages of flower development



CsAP3 gene is involved in development of tepals and stamens in saffron flower. Its expression varies at different stages and different organs of the flower during the course of its development. It is an important regulator of many developmental genes and plays an active role in floral development pathway. CsAP3 expression is very high in developing petals and stamens (Gemma 2006). It has been reported that there has been expression of CsAP3 gene in stigma too (Tsaftaris et al. 2006).

AP1 and (AP3-PI) heterodimer have regulatory role in the expression of AP3 gene (Jens 2006). In present study, least expression of CsAP3 at initial stages of flower development can be attributed to the absence of AP3-PI heterodimers. AP3/PI gene expression is positively regulated by higher expression of AP1 gene (Jens 2006). At the early stage, AP3 and PI do not form heterodimer and activation is attributed to AP1 only (Lamb et al. 2002). The abrupt increase in CsAP3 expression from yellow to orange stage of stigma development can be elucidated by the fact that at the orange stage of stigma, CsAP3 and PI express and their products bind with each other to form a heterodimer. This heterodimer bind to the promoter of CsAP3 gene and activate it in an auto-regulated positive loop. At later stages when flower is open and stigmas are scarlet in color, CsAP3-PI heterodimer bind to the CArG

sequences of CsAP3 promoter and maintain the expression of CsAP3 by an autoregulatory feedback loop (Goto and Meyerowitz 1994). But at this stage the expression of AP1 gene is minimal due to inactivation by AP3-PI heterodimer and AG (Jens 2006). That may be the reason for moderate expression of CsAP3 gene during this stage. CsNAP gene plays a critical role in senescence and salt stress (Kalivas et al. 2010). It was earlier reported that AP3-PI heterodimer directly interact with the NAP gene and promote its activity (Sablowski and Meyerowitz 1998). However, we observed that from orange to scarlet transition CsAP3 gene expression declined from high to moderate levels and CsNAP gene expression had a steep increase from orange to scarlet stage. This implies that there may be some other stimuli or factor initiating the expression of CsNAP in addition to AP3-PI for activating the expression of CsNAP gene. Earlier it was observed that CsNAP gene expression has association with CsAP3-CsPI heterodimer formation (Kalivas et al. 2010). We may conclude that some other factor(s) may be involved in the initiation of CsNAP expression, whereas AP3-PI heterodimer assist the activation/initiation of CsNAP directly or indirectly.

This is the first study of the comparative expression analysis of floral homeotic genes of saffron in relation with

senescence at different flower development stages. Understanding the pathway completely and deciphering the complete mechanism of CsNAP activation can pave a way for prolonged flowering of saffron by activation of particular key genes, which in turn will give farmers ample time to collect the crop and also protect the flower from damage caused due to frost in November.

**Conflicts of interest** None.

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