RELATIVE EXPRESSION ANALYSIS OF CSAG GENE DURING DIFFERENT STAGES OF STIGMA DEVELOPMENT IN CROCUS SATIVUS.L (SAFFRON)

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Abstract: Saffron is one of the most expensive spices in the world and its flower is unique in having stigma filled with apocarotenoids. The flower development in saffron is governed by MADS box homeotic genes. The stigma development in saffron flower is promoted by a C-class floral homeotic gene AGAMOUS (CsAG). The expression of CsAG began in yellow stigma and there was a considerable growth in its expression (16 folds) as stigma turned from yellow to Orange stage. The expression of CsAG gene continued to increase from orange stage to scarlet stage, when the stigma had fully developed. It was observed that expression of CsAG gene was in concordance with the development of stigma in saffron flower.

Keywords: AGAMOUS, MADS box, Stigma.

Introduction:

Saffron flower development is governed by specific floral homeotic genes belonging to MADS box family (Wafai et al., 2015). The MADS-box motif is a conserved 56-amino-acid region found within the DNA-binding domain of numerous eukaryotic transcription factors. The MADS-box is a conserved motif found within the DNA binding domains of these proteins and the name refers to four of the originally identified members: -MC M1, AG, QEFA and SRF (Dolan & Fields, 1991). MADS-box genes take their name from the *MINICHROMOSOME MAINTENANCE 1* (*MCM1*) genes in yeast, *AGAMOUS* (*AG*) in *Arabidopsis*, *DEFICIENS* (*DEF*) in *Antirrhinum* and serum response factor (*SRF*) in humans (Riechmann & Meyerowitz, 1997). *AGAMOUS is a C-class gene* involved in stamen and carpel development (Gregis et al., 2008). The AGAMOUS subfamily of MADS-box genes was named after the AGAMOUS gene of A. thaliana and is actively involved in floral development (Yanofsky et al., 1990), which is the only C function gene in this species. In the classical ABC model, the C function defines three different roles: carpel identity, stamen identity and floral meristem determinacy (FMD). The C class TF genes that are involved in the formation of the stamen and carpel also control floral meristem (FM) activity to ensure a definite number of floral organs. In A. thaliana a fourth role for the C function, has been assigned which is the prevention of the faulty expression of A- function genes in the two whorls of reproductive organs (Dreni & Kater, 2013). This is shown by the *A. thaliana* ag mutant, where there is a homeotic conversion of stamens into petals, as a result of the loss of stamen identity and faulty expression of the A function (Dreni & Kater, 2013). In the fourth innermost whorl, in place of a carpel, a new ag flower develops, which in turn develops in its centre another new ag flower. The D class (ovule identity) genes also belong to the AGAMOUS subfamily. FLORAL BINDING PROTEIN 7 (FBP7) and FBP11 of petunia were the first genes to be identified as master regulators of ovule identity. (Colombo et al., 1995). In angiosperms several AGAMOUS subfamily genes have been reported and also characterized, such as the monocots rice (Oryza sativa) and maize (Zea mays) which imply that these genes have functional conservation within flowering plants and they function as master regulators of stamen, carpel and ovule identity (Pinyopich et al., 2003). The number of AGAMOUS subfamily genes may differ between different species and typically show various degrees of redundancy. In saffron CsAG plays an important role in development of stigma and three AP1-, Five PI-, two AP3-, two AG- and two SEP-like MADS Box genes from *Crocus sativus* L were isolated (Tsaftaris et al., 2005). In this study, we have studied the relative expression of AGAMOUS gene during different stages of stigma development.

Methodology:

Sample Collection and storage

The samples were collected at three different stages of saffron flower on the basis of stigma development. The three stages were yellow stigma, orange stigma and scarlet stigma (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^{o}C.

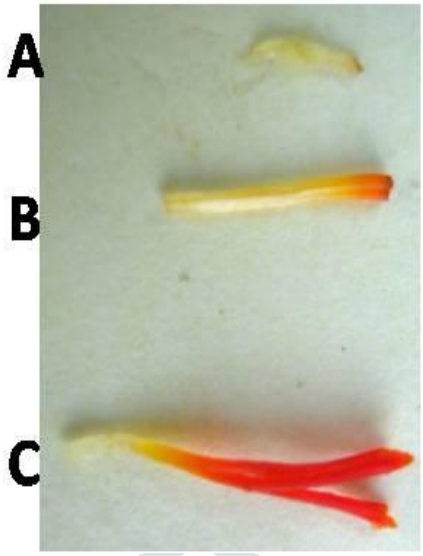


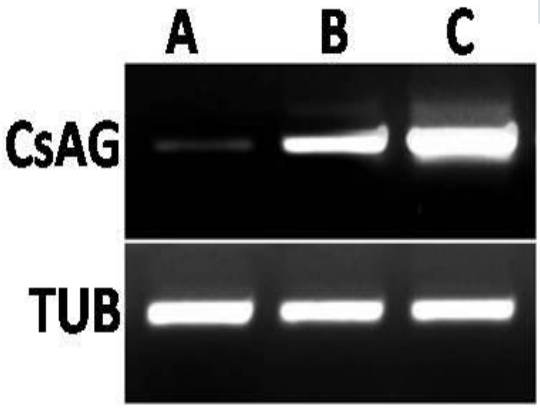
Fig 1: Three different stages of stigma development – A)yellow, B) Orange, C) Scarlet

RNA Extraction and Quantitative Real time PCR

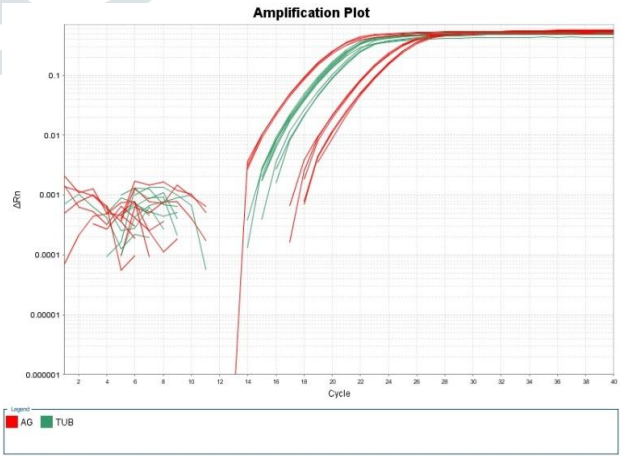
RNA was extracted from 100mg stigma in all three stages using Trizol reagent. 1µg/mL total RNA was used for synthesis of first strand cDNA using first strand cDNA synthesis kit (Bangalore Genei). The semi quantitative expression analysis was performed by Reverse transcription PCR using the primers CsAG-F **5´-GGCGGATCCATAGCAATAAGGTACCCAGTCAC-3´** and CsAG-R **5´-CGCCTCGAGAAGCTTCCTTCAAGCGAACTTG-3'** for CsAG gene and (5'-TGATTTCCAACTCGACCAGTGTC-3') and (5'-ATACTCATCACCCTCGTCACCATC-3') for tubulin gene. The amplification was carried out at 94C for 2 min, 25 cycles of 94C for 45sec, 54C for 45sec, 72C for 1 min, final extension of 72C for 5 mins. The PCR products were run on 2% agarose gel and the bands were visualized using EtBr stain. The real time PCR was done by same primers as in reverse transcription PCR. Tubulin gene expression was used as an internal control for normalization. The amplification was carried using SybrGreen at 94C for 2 min, 40 cycles of 94C for 15sec, 54C for 15sec, 72C for 20 sec, final extension of 72C for 2 mins. Advanced Relative quantification between different stages was done through $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Result and discussion:

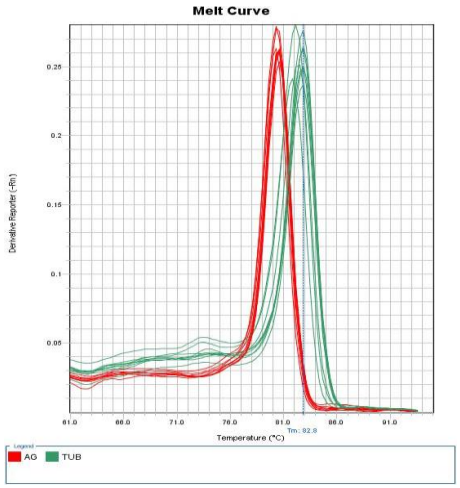
Expression analysis of CsAG gene during 3 stages of stigma development i.e., yellow, orange, and scarlet was done through reverse transcription PCR and real time PCR to find out the relative change in expression during three stages of stigma development. The reverse transcription semi quantitative PCR revealed that the expression of CsAG gene was little at yellow stage and there was a gradual increase in its expression in orange and scarlet stage (Fig 2A). Real time PCR showed that there was an increase in expression (16 folds) of CsAG gene from yellow to orange stage of stigma development. The expression level of CsAG gene further increased (6.25 folds) from orange to scarlet stage of stigma development. Hence the expression level during three stages of stigma development (yellow:orange:scalet) was observed to be 1:16:100. (Fig. 2D)



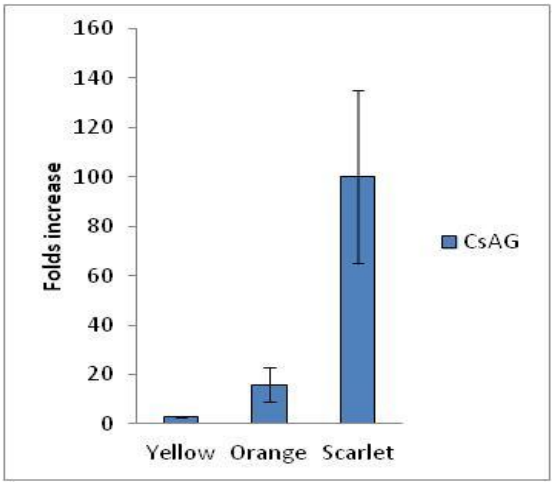
A)



B)



C)



D)

Fig 2: (A) Reverse Transcription PCR products of CsAG and TUB; (B)Amplification curve of CsAG and tubulin gene (C) Melting curve for CsAG and TUB, (D) Relative expression of CsAG gene during different stages of stigma development.

AGAMOUS gene is involved in development of stamens and stigma in saffron flower (Tsaftaris et al., 2005). Its expression varied at different stages and different organs of the flower during the course of its development. AG expression has been reported to be very high in developing stamens and stigma (Meyerowitz et al., 1991). In the present study, least expression of CsAG in yellow stage can be explained by the fact that the expression of AP1 gene is high at this stage, which negatively regulate the CsAG expression (Lamb et al., 2002). The abrupt increase in CsAG expression from yellow to orange stage of stigma development can be explained on the fact that, at the orange stage, the stigma development takes a high pace and AP3-PI heterodimers positively regulate the CsAG expression (Wafai et al., 2015). At Later stage, when flower is open and stigma are scarlet in color, the expression of AP1 gene is minimal due to inactivation by AP3-PI heterodimer which, in turn, results in highest expression of CsAG gene (Jens. 2006).

This is a preliminary work on the expression of floral homeotic genes in saffron. More work need to be done on the expression pattern of other homeotic genes like AP1, LFY, PI, AP3, CAL and SEP, which will give a holistic picture of the regulation of these floral genes and their effect on morphology and development of the saffron flower organs. This study can pave a way for *in vitro* flowering of saffron by activation of particular key genes. We have already studied the expression of CsAP3 gene and CsNAP gene earlier and with complete analysis of expression pattern of all floral homeotic genes, we may understand the flower development pathway in saffron in detail.

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