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ORIGINAL ARTICLE

Transcriptome wide identification, phylogenetic analysis, and expression profiling of zinc-finger transcription factors from *Crocus sativus* L.

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Abstract *Crocus sativus* belongs to *Iridaceae* family and is the only plant species which produces apocarotenoids like crocin, picrocrocin, and safranal in significant quantities. Besides their organoleptic properties, *Crocus* apocarotenoids have been found to possess remarkable pharmacological potential. Although apocarotenoid biosynthetic pathway has been worked out to a great degree, but the mechanism that regulates the tissue and developmental stage-specific production of *Crocus* apocarotenoids is not known. To identify the genes regulating apocarotenoid biosynthesis in *Crocus*, transcriptome wide identification of zinc-finger transcription factors was undertaken. 81 zinc-finger transcription factors were identified which grouped into eight subfamilies. C2H2, C3H, and AN20/AN1 were the major subfamilies with 29, 20, and 14 members, respectively. Expression profiling revealed *CsSAP09* as a potential candidate for regulation of apocarotenoid biosynthesis. *CsSAP09* was found to be highly expressed in stigma at anthesis stage corroborating with the accumulation pattern of apocarotenoids. *CsSAP09* was nuclear localized and activated reporter gene transcription in yeast. It was highly induced in response to oxidative, salt and dehydration stresses, ABA and methyl jasmonate. Furthermore, upstream region of *CsSAP09* was found to contain stress and light responsive elements. To our knowledge, this is the first report on the study of a gene family in *C. sativus* and may provide basic insights into the putative role of *zinc finger genes*. It may also serve as a valuable resource for functional characterization of these genes aimed towards unraveling their role in regulation of apocarotenoid biosynthesis.

Keywords Saffron · Transcriptome · AN20/AN1 · Zinc finger · Apocarotenoid

Introduction

Crocus sativus L. is a sterile triploid plant belonging to *Iridaceae* family (Fernandez et al. 2011). The dehydrated red stigmas of the flower of *Crocus* comprise the world's costliest spice, saffron (Rakesh et al. 2009). The distinctive feature on account of which *Crocus* stands out from other plants is the massive and unparalleled accumulation of apocarotenoids like crocin, picrocrocin, and saffranal (Frusciante et al. 2014). These apocarotenoids impart typical color, outstanding taste, and inimitable aroma to saffron because of which it has been cherished since millennia in different cuisines (Kyriakoudi et al. 2015). Saffron apocarotenoids also exhibit multiple biological actions including anticancer, anti-inflammatory, antimicrobial, and antioxidant activities (Kaefer and Milner 2008). The most noteworthy health benefits relate to its activity against various types of cancers (Gutheil et al. 2012; Zhang et al. 2013), gastric disorders (Kianbakht and Mozaffari 2009), cardiovascular diseases (Zheng et al. 2006), alzheimer disease

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(Moshiri et al. 2015), and mild-to-moderate depression (Lopresti and Drummond 2014).

The apocarotenoids build up throughout the growth of *Crocus* stigmas, reaching a maximum at fully developed scarlet stage and decreasing thereafter (Rubio et al. 2010; Mir et al. 2012). The market value of saffron is exceptionally high and the demand is soaring for various purposes. However, the production is dismally low, rather on the decline (Jain et al. 2016). Therefore, a great interest has surfaced with regards to understanding and eventually manipulating the biosynthesis and production of saffron apocarotenoids. However, so far, most of the studies have focused only on the spice-related properties and very little attention has been paid to the regulation of biosynthesis and accumulation of apocarotenoids in *C. sativus* (Ahrazem et al. 2015). Although some of the genes involved in the biosynthetic pathway have been identified and characterized (Baba et al. 2015a; Frusciante et al. 2014), very little is known about the genes that regulate tissue and developmental stage-specific production of these compounds. There is only a single report where an ultrapetala transcription factor has been shown as a potential candidate regulating apocarotenoid biosynthesis in *Crocus* (Ashraf et al. 2015).

To gain a comprehensive understanding about apocarotenoid biosynthetic pathway and its regulation, we generated a transcriptome map of *Crocus* (Baba et al. 2015b). A search for genes encoding transcription factors resulted in identification of many transcription factor families among which zinc-finger gene family was an abundant one. Furthermore, differential expression profiling between stigma and rest of the *Crocus* flower suggested that many zinc-finger genes were induced in stigma which is the actual site of apocarotenoid biosynthesis. This prompted us to design a detailed study on this class of transcription factors. In this context, we did transcriptome wide identification, phylogenetic analysis, and expression profiling of zinc-finger family of transcription factors from *Crocus* so as to elucidate their possible role in regulation of stigma development and apocarotenoid biosynthesis.

Zinc-finger domains are ubiquitously dispersed throughout the eukaryotic genomes (Gourcilleau et al. 2011) and mediate important regulatory roles (Iuchi 2001). Zinc-finger family of transcription factors is one of the largest and versatile transcription factor gene families in plants (Hall 2005). Based on the number and order of Cys and His residues, zinc-finger proteins have been categorized into at least 14 families (Klug and Schwabe 1995; Arnaud et al. 2007). On the basis of plant transcription factor database (http://planttfdb.cbi.edu.cn/) (Zhang et al. 2011), it has been shown that out of the total transcription factors, the percentage of zinc fingers (ZNFs) in different plant species such as *Arabidopsis*, potato, rice, maize, and wheat is almost similar (16–18%). It is speculated that ZNFs are probably conserved in plant evolution, and their key biological functions are most likely comparable in different plant species (Li et al. 2013). ZNFs mediate important regulatory functions by interacting with DNA or chromatin, RNA and other proteins (Iuchi 2001; Yang et al. 2006). ZNFs also affect various other functions, such as plant growth and development (Luo et al. 1999; Yun et al. 2002), phytohormone response (Molnar et al. 2002), and biotic and abiotic stresses responses (Tian et al. 2010).

In this study, we identified 81 ZNFs from *Crocus* transcriptome data-set developed in our laboratory (Baba et al. 2015b). We performed comparative phylogenetic analysis of *CsZNFs* with *Arabidopsis* zinc-finger homologs, and investigated the transcript levels of selected genes in different tissues, flower developmental stages, and in response to different phytohormones and various abiotic stresses using qRT-PCR. *CsSAP09* was found to be highly expressed in stigma, the site of apocarotenoid biogenesis, at anthesis stage. Since *Crocus* apocarotenoids are synthesised only in stigma in a developmental stage-specific fashion reaching their pleatue at anthesis stage, therefore, *CsSAP09* appears a credible candidate from the point of regulation of apocarotenoid biosynthesis. Therefore, *CsSAP09* was cloned and characterized. The gene was found to be localized in nucleus and showed activated reporter gene transcription in yeast. These results provide insights about the phylogenetic relation and functions of *Crocus* zinc fingers in general and *CsSAP09* in particular. Furthermore, the study may act as a foundation towards elucidation of functionality of *Crocus* zinc-finger genes.

Materials and methods

Identification of zinc-finger transcription factors from *C. sativus* transcriptome

Based on BLAST homology results, 173 putative zinc-finger transcripts were identified from a transcriptome data-set of *C. sativus* published from our laboratory (Baba et al. 2015b). The EMBOSS package of Transeq program was used to translate zinc-finger transcription factor DNA sequences. The amino-acid sequences of the longest open reading frame (ORF) in six ORFs were selected. Based on the domain search in NCBI CDD and Interproscan domain search, 81 sequences containing the ZnF domain were finally sorted out for analysis. The sequences of *Arabidopsis* zinc-finger proteins were obtained from (http://www.Arabidopsis.org).

Phylogeny and classification of *Crocus* zinc-finger proteins

To analyze the phylogenetic relationship amongst *CsZNF* gene family members, zinc-finger domains from all *CsZNFs* were aligned by CLUSTAL W (Thompson et al. 1994). Multiple sequence alignment and phylogenetic analyses were carried out by MEGA software version 6 (Tamura et al. 2013). The evolutionary history was deduced by the neighbour-joining (NJ) method. The phylogenetic tree with a bootstrap of 1000 replications was finally used to represent the evolutionary history of the genes.

To infer the phylogenetic relationship between major ZNF gene subfamilies of *Crocus sativus* and corresponding *Arabidopsis thaliana* zinc-finger gene families, separate phylogenetic trees based only on zinc-finger domains of the two plants were constructed for *C2H2, C3H* and *A20*/*AN1* (SAPs) using the MEGA 6 software (Tamura et al. 2013).

Plant material and treatments

Crocus sativus L., plants grown in an experimental plot at the Indian Institute of Integrative Medicine (IIIM), Srinagar, India (longitude: 34°5′24″N; latitude: 74°47′24″ and altitude 1585 m amsl), were used as the source of plant material. Flowers were harvested at different developmental stages, viz., 3 days prior to anthesis, on the day of anthesis and 2 day post-anthesis. Further different tissues including tepals, stigmas, anthers, leaves, and corms were also collected, wrapped in silver foil, snap frozen in liquid nitrogen, and stored in −80 until required.

Phytohormone and stress treatments were given as described earlier (Baba et al. 2015a). Briefly, *C. sativus* corms were grown in pots containing soil under greenhouse conditions at 26 ± 2 °C till the flowering stage. For phytohormone treatments, plants were sprayed with 0.1 mM salicylic acid (SA), 50 µM 2, 4-D, 0.1 mM methyl jasmonate (JA), and 0.1 mM gibberellic acid (GA3). For oxidative, salt and dehydration stresses, 5 µM methylviologen (MV), 200 mM NaCl, and 200 mM mannitol were added to soil intermittently till the soil got saturated. Cold and UV stresses were given by transferring the plants to 4 °C and UV-B light of 1500 J/m^2, respectively. Control plants were grown under optimal conditions. Tissue samples were collected after 24 h of the treatments and stored till further use.

Isolation of nucleic acids and cDNA synthesis

RNA isolation was done by RNeasy RNA isolation kit (Qiagen) following the manufacturer's instructions. RNA quality was assessed by separation on 1% agarose gel. Furthermore, RNA was quantified using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies). The samples were then treated with DNase I (Fermentas) to remove any genomic DNA contamination. cDNA was synthesised by reverse transcription kit (Fermentas) as per manufacturer's guidelines. Genomic DNA was isolated from corms using DNeasy Plant mini kit (QIAGEN) according to the manufacturer's protocol.

qRT-PCR analysis

Quantitative RT-PCR was used to analyze the transcript levels of *Crocus* zinc-finger proteins. qRT-PCR was performed in triplicates in ABI Step One Real time (Applied Biosystems). The reaction was performed in a total volume of 20 µl which included 10 µL of 2X SYBR Green Master Mix, 0.2 µM gene-specific primers, and 100 ng of template cDNA. The cycling conditions were 95 °C for 20 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Transcript abundance of zinc-finger genes was detected in different tissues including corms, stigmas, tepals, anthers, and leaves; flower development stages; and also in plants exposed to different stresses and phytohormones. The sequence of primers used is given in supplementary table S1. The analysis was done by relative quantification method ddCT ($\Delta\Delta^{\Delta\Delta CT}$). The amplification of *Crocus* actin gene was used as an endogenous control.

Cloning and sequence analysis of *CsSAP09* gene

Gene-specific primers (*CsSAP09* F and *CsSAP09* R) based on the gene sequence from the transcriptome data (Baba et al. 2015b) were used to clone *CsSAP09* gene. The cycling parameters were 3 min at 94 °C, 30 cycles (30 s at 94 °C, 30 s at 55 °C and 45 s at 72 °C) and a final extension of 7 min at 72 °C. The amplified clone was purified with gel extraction kit (Qiagen), cloned in pGEM-T Easy vector (Promega), and sequenced. A search for homologous nucleic acid and protein sequences was performed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/). A multiple sequence alignment was generated by ClustalW (http://www.ebi.ac.uk/clustalw/). The deduced amino-acid sequence was analyzed by ProtParam Programs at ExPASy proteomics server (http://ca.expasy.org/) to calculate pI/Mw. The phylogenetic tree was constructed using the MEGA 6.06 software (Tamura et al. 2013).

Subcellular localization

The subcellular localization of *CsSAP09* was determined by transient expression assay in onion epidermal cells. *CsSAP09* with restriction sites for *XhoI* and *EcoRI* was amplified using *CsSAP09*YFP-F and *CsSAP09*YFP-R primer pair and fused in frame with YFP reporter gene in

PAM-PAT-35S. The cycling parameters were the same as described elsewhere. The fusion construct of *CsSAP09*-YFP was bombarded on to the onion peels using biolistic gene delivery device PDS-1000/He (Bio-Rad). The onion peels were incubated for 24 h in dark before visualization under confocal microscope.

Transactivation assay

CsSAP09 was cloned in yeast (*Saccharomyces cerevisiae*) expression vector pGBKT7 (Clontech) at *NdeI-EcoRI* site to express the *CsSAP09* protein fused to GAL4 DNA-binding domain (GAL4-BD). The primers used were CsSAP-09PGBKT-F and CsSAP09PGBKT-R and cycling conditions were 3 min at 94 °C, 30 cycles (30 s at 94 °C, 30 s at 55 °C, and 45 s at 72 °C), and final extension for 7 min at 72 °C. The resulting construct was transformed into yeast strain Y187. The positive transformants were selected onto synthetic media lacking tryptophan, adenine, and histidine.

Amplification of the core promoter of *CsSAP09*

CsSAP09 promoter sequence was isolated by the genome-walking method using the GenomeWalker Universal Kit (Clontech) following the manufacturer's instructions. For library preparation, genomic DNA was digested with four different blunt-end generating restriction endonucleases (*DraI, PvuII, EcoRV*, and *StuI*). Each set of digested genomic DNA samples was purified and ligated to the GenomeWalker AP adaptor (provided with the kit) independently to generate four adapter-ligated libraries. To amplify the promoter region of *CsSAP09*, two-round PCR was performed using the adapter primer (provided with the kit) and gene-specific primers *Cs*SAP- SP1 and *Cs*SAP-SP2. *In silico* analysis was performed through the Plant-Care database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Results

Identification of zinc-finger proteins from *Crocus* transcriptome and their phylogenetic analysis

In this study, we provide information on the zinc-finger (ZNF) proteins of *Crocus sativus*. Initially a total of 173 unigenes were predicted from *Crocus* transcriptome (Baba et al. 2015b), possibly containing ZNF domains. These sequences were further analyzed by InterProScan and NCBI CDD search which confirmed the presence of ZNF domains in only 81 sequences and these were taken for further analysis.

To study the phylogenetic relationship amongst the *C. sativus* zinc-finger proteins (*CsZNFs)*, a neighbour-joining unrooted tree with bootstrap analysis of 1000 replicates was constructed by MEGA6 from the alignment of zinc-finger domains of the protein sequences. The phylogenetic analysis divided the proteins into eight subfamilies with *C2H2, C3H*, and *A20/AN1* as the major subfamilies containing 29, 20, and 14 sequences, respectively (Fig. 1). The other subfamilies included GRF, NAM, B box, Swim, and Zn-HD containing proteins and all these had less than five gene sequences. Since *C2H2, C3H*, and *A20/AN1* emerged as the major subfamilies, we focussed our study on these three classes. Generic names (*C2H201-C2H229, C3H01-C3H20*, and *SAP01-SAP14*) were assigned to distinguish each member of the three major subfamilies for the purpose of further analysis.

Phylogenetic and evolutionary relationship of *Crocus* zinc-finger proteins with *Arabidopsis* homologs

To understand the phylogenetic relationships of *CsZNFs* with *Arabidopsis* homologs and to gain insights about their putative functions, unrooted phylogenetic trees were built based on the alignment of zinc-finger domains of proteins from *Crocus* and those from *Arabidopsis*. To simplify the comparison, phylogenetic trees were generated separately for the *C2H2, C3H*, and *A20/AN1* (also called SAPs) subfamilies (Figs. 2, 3, 4). The results showed that although in some branches, *Crocus* and *Arabidopsis* homologs are very closely related, but this was not the case in most of the other branches. For example, in a comparative phylogenetic analysis between *Crocus* and *Arabidopsis C2H2* zinc fingers, it was observed that ten *CsC2H2* genes including *CsC2H203, CsC2H204, CsC2H205, CsC2H206, CsC2H208, CsC2H210, CsC2H211, CsC2H213*, and *CsC2H214* formed a separate clad, while others grouped with their *Arabidopsis* homologs (Fig. 2). Likewise, in case of *C3H* subfamily, many *Crocus C3H* genes grouped with their *Arabidopsis* counterparts. For example, *CsC3H05* and *CsC3H06* shared a common clad with *AT3G12680.1* and *AT1G10320.1*, while *CsC3H01, CsC3H01, CsC3H02, CsC3H03*, and *CsC3H04* grouped with *AT2G32930.1* and *AT3G48440.1*. However, many others like *CsC3H16, CsC3H19*, and *CsC3H20* formed a separate clad (Fig. 3). Furthermore, phylogenetic analysis of *Crocus* and *Arabidopsis* SAP proteins showed that while most of the genes from *Crocus* grouped with *Arabidopsis* genes, *CsSAP012* formed a separate clad (Fig. 4).

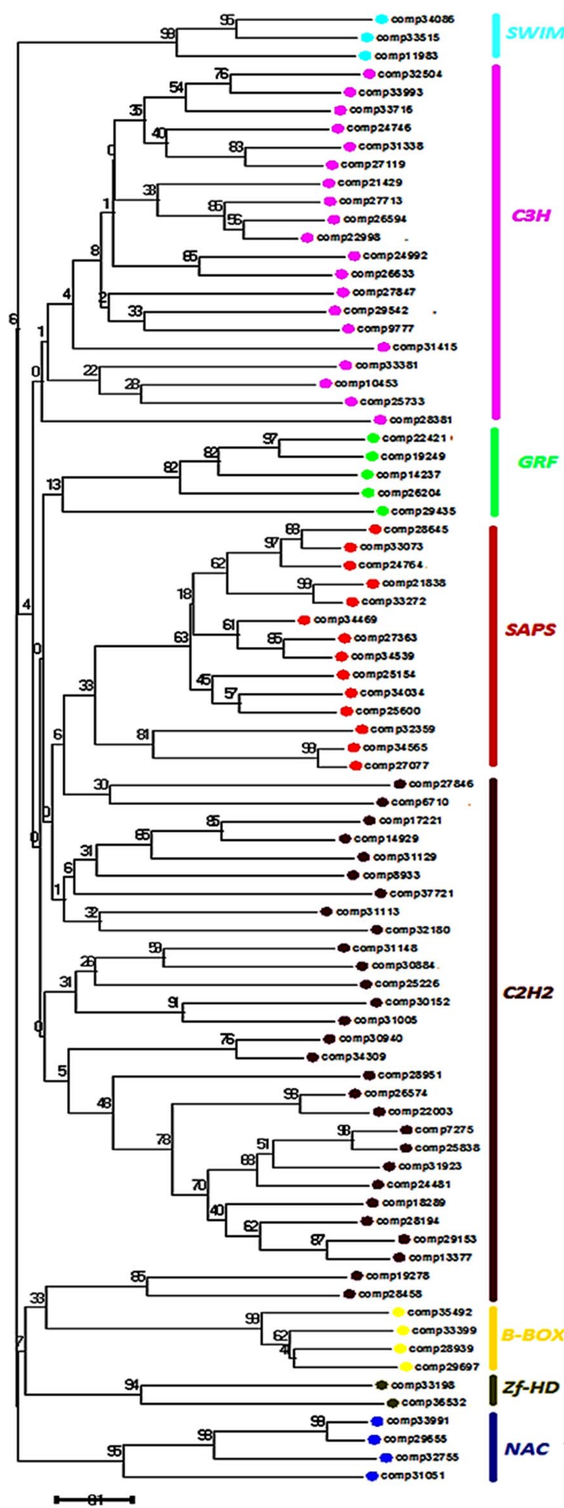


Fig. 1 Phylogenetic analysis of *CsZNF* genes. The phylogenetic tree was generated by the neighbour-joining (NJ) algorithm using Molecular Evolutionary Genetics Analysis (MEGA) software version 6.06 (Tamura et al. 2013). The *numbers* on major branches indicate bootstrap percentages for 1000 replicate analyses. *CsZNF* genes have been divided into eight subfamilies

Fig. 2 Phylogenetic tree of C2H2 proteins of *Crocus sativus* and *Arabidopsis thaliana*. A phylogenetic tree was generated from zinc-finger domains of C2H2 proteins of *C. sativus* and *A. thaliana* using MEGA software version 6.06. The *numbers* on major branches indicate bootstrap percentages for 1000 replicate analyses

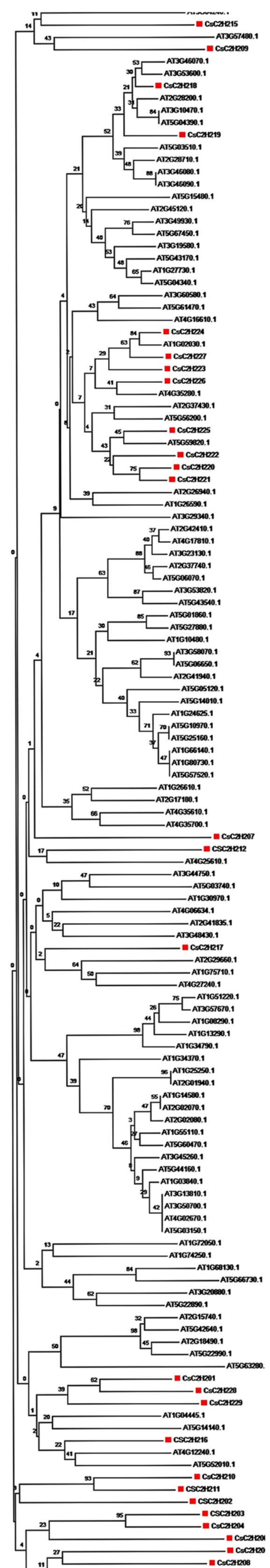
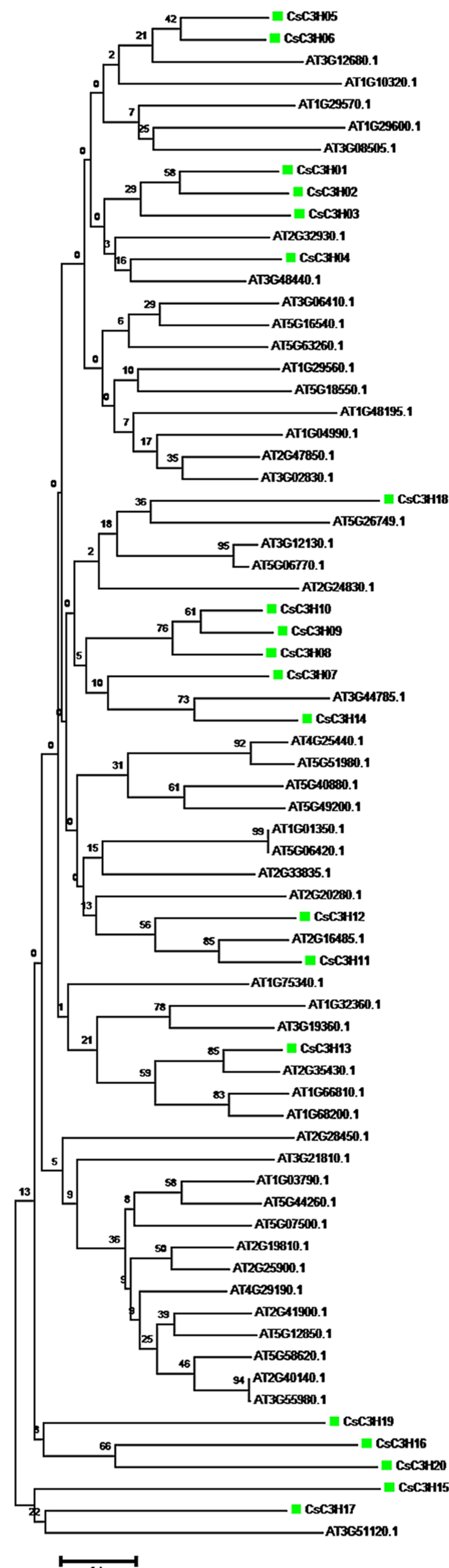


Fig. 3 Phylogenetic tree of C3H proteins of *Crocus sativus* and ▸ *Arabidopsis thaliana*. A phylogenetic tree was constructed from zinc-finger domains of C3H proteins of *C. sativus* and *A. thaliana* using MEGA software version 6.06. The *numbers* on major branches indicate bootstrap percentages for 1000 replicate analyses



Tissue and developmental stage-specific expression of *CsZNF* genes

To get first round information about the biological function of *CsZNFs*, the expression patterns of one representative gene from three major subfamilies (*CsC2H216, CsC3H13, and CsSAP09)* were analyzed by quantitative RT-PCR. The selected *CsZNFs* showed varying expression levels in different tissues and flower development stages as depicted in (Fig. 5a–f). *CsC2H216* exhibited highest expression in tepal at post-anthesis stage of flower development (Fig. 5a, d). For *CsC3H13*, the highest expression was observed in anther at post-anthesis stage of flower development (Fig. 5b, e). Furthermore, *CsSAP09* displayed peak expression in stigma and anthesis stage of flower development alluding to its probable role in apocarotenoid production (Fig. 5c, f).

Stress inducible expression of *CsZNF* genes

To evaluate the role of *CsZNF* genes in abiotic stresses, the expression of three selected genes was analyzed by qRT-PCR under various stress treatments. Overall, transcript levels of all the three genes tested were detected to respond to more than one treatment. *CsC2H216* was significantly induced by cold and dehydration stress (Fig. 6a); *CsC3H13* was highly induced by cold and salt stress (Fig. 6b), whereas *CsSAP09* displayed enhanced expression in response to methylviologen, dehydration, and salt stress (Fig. 6c).

Hormone responsive expression of *CsZNF* genes

The expression kinetics of *CsZNF* genes was also investigated in response to various phytohormones. Transcript abundance revealed that the genes exhibited differential expression in response to various phytohormones. All the three genes responded to abscisic acid and methyl jasmonate (Fig. 6d–f). *CsC2H216* showed maximum induction in response to methyl jasmonate followed by ABA (Fig. 6d). *CsC3H13* highly responded to auxin followed by gibberellic acid and ABA (Fig. 6e), while *CsSAP09* exhibited highest induction in response to ABA followed by salicylic acid and methyl jasmonate (Fig. 6f).

Fig. 4 Phylogenetic tree of *AN1/A20* proteins of *Crocus sativus* and *Arabidopsis thaliana*. A phylogenetic tree was constructed from AN1 zinc-finger domains of *SAPs* of *C. sativus* and *A. thaliana* using MEGA software version 6.06. The *numbers* on major branches indicate bootstrap percentages for 1000 replicate analyses

Cloning and analysis of *CsSAP09*

As mentioned in the above sections, *CsSAP09*, was highly expressed in stigma of *Crocus* flower and its expression surged upto the day of anthesis and later showed a decline. This expression pattern matched with the accumulation pattern of apocarotenoids, therefore, indicating probable role of this gene in regulating apocarotenoid metabolism. To move further towards characterizing the gene, we cloned full-length *CsSAP09*. The complete cDNA of *CsSAP09* was 0.489 kb long. The open reading frame (ORF) encoded for a protein of 162 amino acids with an A20 and AN1 zinc-finger domain at N and C termini. The predicted molecular weight and pI of the protein was 17.4 kDa and 7.9, respectively. The ProtParam analysis suggested that the protein is hydrophilic with GRAVY value of −0.396.

The database search using the amino-acid sequence as query showed homology to several AN1/ A20-ZNFs from plants including *Musa acuminata* subsp. *malaccensis, Phoenix dactylifera, Sesamum indicum*, etc. All these proteins showed homology to *CsSAP09* in the AN1-type and A20-like zinc-finger region (Fig. 7b). Phylogenetic analysis of *CsSAP09* with other plant A20/AN1-ZNFs revealed that *CsSAP09* showed close similarity to *Musa acuminata* subsp. *malaccensis* stress-associated protein 5-like (MASAP5), *Phoenix dactylifera* stress-associated protein 5-like (PdSAP5), and *Sesamum indicum* stress-associated protein 4 (SiSAP4) (Fig. 7c). Furthermore, the comparison of full-length cDNA of *CsSAP09* with a genomic DNA fragment generated by PCR amplification and sequencing revealed the coding region of the genomic clone of *CsSAP09* to be continuous, without any intron.

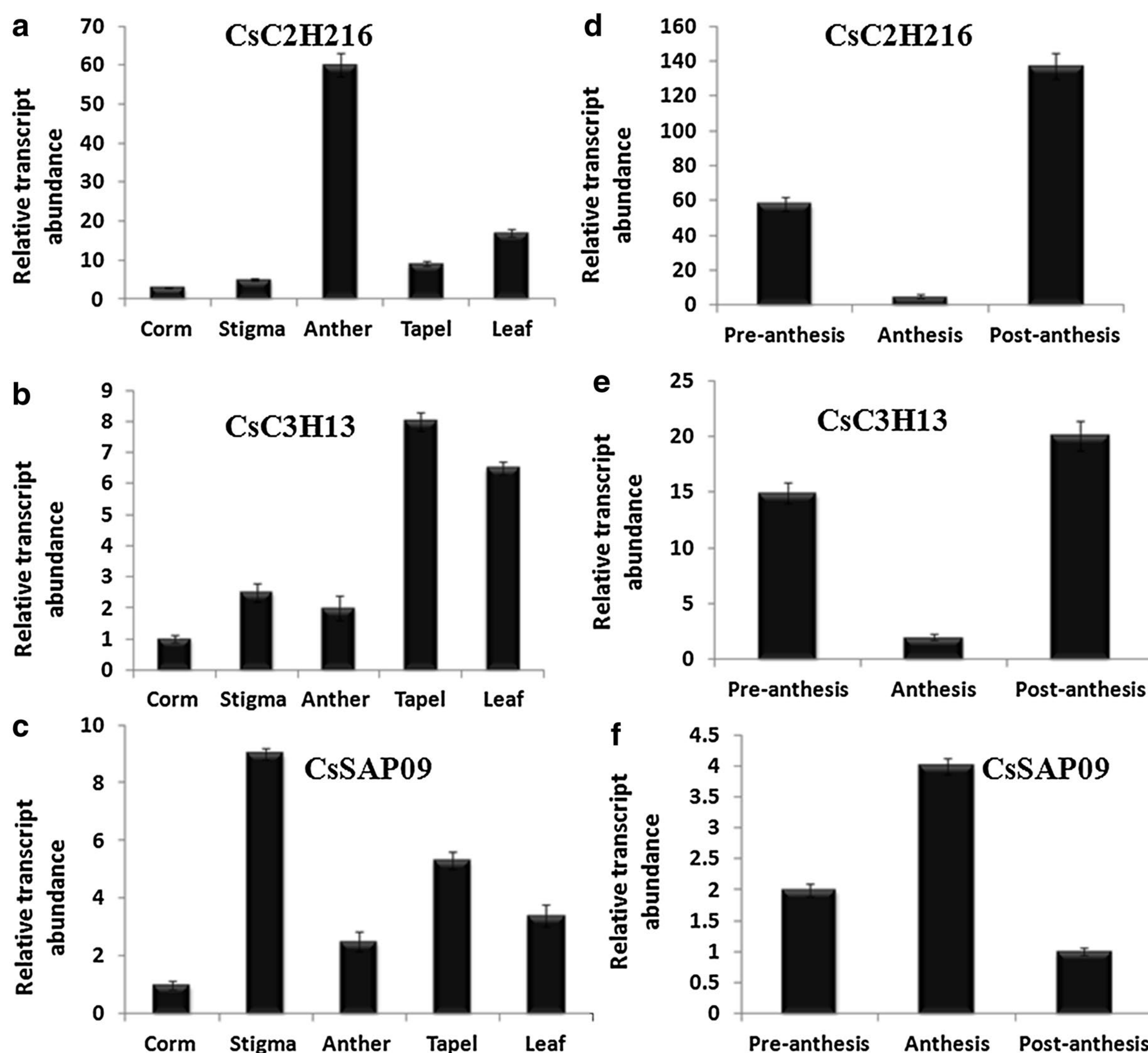


Fig. 5 Spatial and temporal expression profiling of *CsZNFs*. Relative expression ratio of three *CsZNF* genes analyzed by qRT-PCR (**a**–**c**): in five different tissues and (**d**–**f**): at three different flower development stages. The relative expression level of each gene was calculated relative to its expression in control sample. qRT-PCR data were normalized using *Crocus* actin gene. Experiment was done in triplicates. *Error bars* indicate standard deviation

CsSAP09 localizes in the nucleus

To understand the mechanism underlying the regulatory role of *CsSAP09*, its subcellular localization was examined. Programs like NucPred and NLStradamus (Brameier et al. 2007; Nguyen et al. 2009) predicted a nuclear localization signal in *CsSAP09*. To ascertain the nuclear localization, *CsSAP09* was fused in frame to the N-terminus of the YFP reporter gene of PAM-PAT-35S vector. *CsSAP09-YFP* fusion driven by the 35 S promoter of cauliflower mosaic virus (CaMV35S) promoter was bombarded onto onion (*Allium cepa*) epidermal cells by gold particle bombardment and visualized under confocal microscope. While the control YFP fluorescence was observed throughout the cell, *CsSAP09-YFP* fluorescence was localized only to the nucleus (Fig. 8a) confirming nuclear localization of *CsSAP09*.

CsSAP09 functions as a transcriptional activator in yeast

The transcriptional activity assay of *CsSAP09* was examined in yeast strain Y187. For this, ORF of *CsSAP09* was fused in frame to the GAL4 DNA-binding domain (BD) to produce pGBKT7-*CsSAP09-BD* construct. The GAL4 DNA-binding domain-*CsSAP09* fusion protein was expressed in yeast strain Y187. Yeast cells transfected with the pGBKT7-BD vector, lacking the activation domain, as the negative control did not grow on the SD medium deficient in His and Trp (SD/-Trp-Ade-His). However,

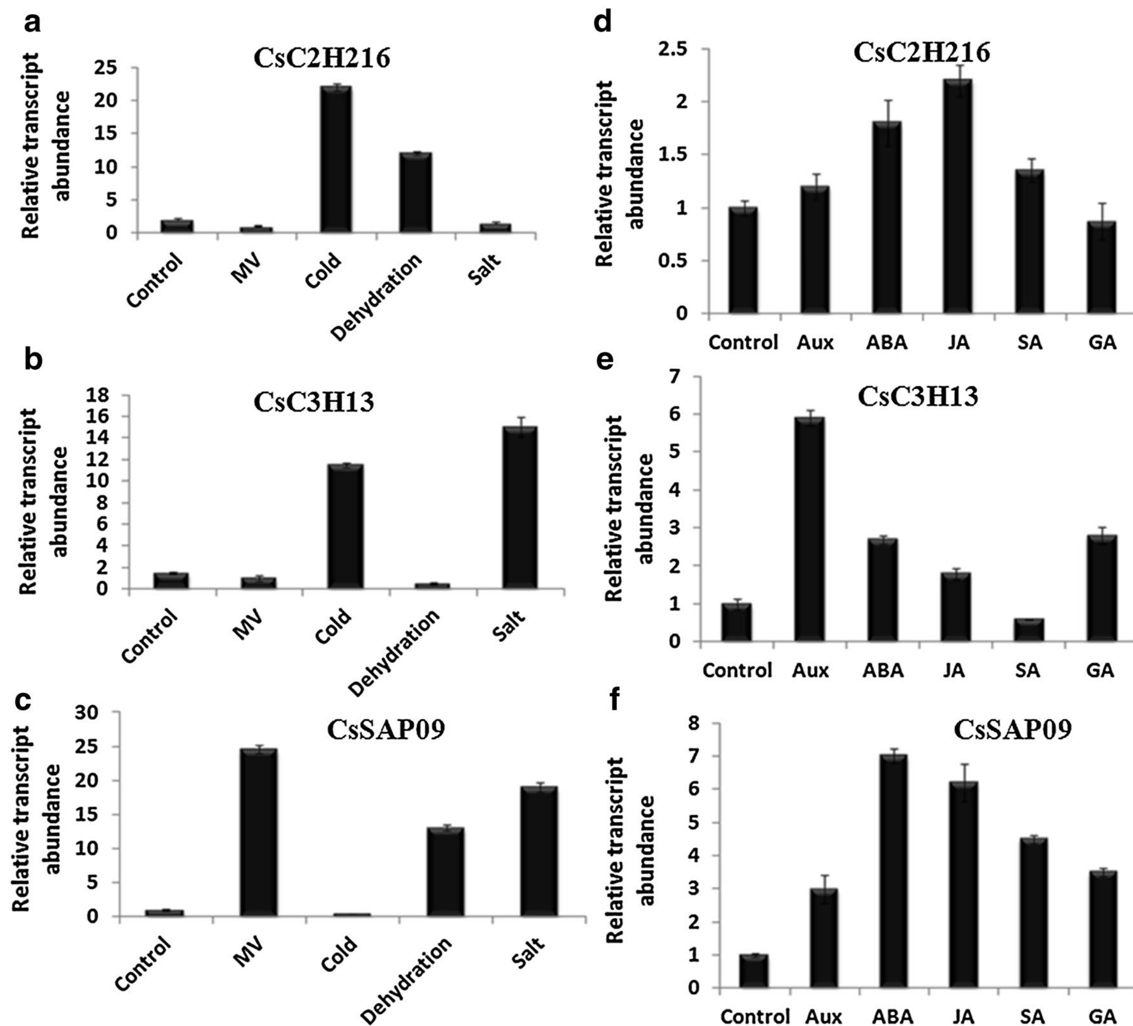


Fig. 6 Stress induced and hormone responsive expression profiling of *CsZNFs*. Relative expression levels of three *CsZNF* genes analyzed by qRT-PCR (**a**–**c**): under different stresses and (**d**–**f**): in response to phytohormone treatments. The relative expression ratio of each gene was calculated relative to its expression in control sample. qRT-PCR data were normalized using *Crocus* actin gene. (*Error bars* indicate standard deviation)

the cells carrying pGBKT7-*BD*- *CsSAP09* or pGBKT7-53 + pGADT7-T (the positive control) grew well on the SD/-Trp-Ade-His medium (Fig. 8b). *CsSAP09* provided the activation domain and the cells exhibited activity of β-galactosidase reporter gene upon addition of X-gal on Whatman filter paper (Fig. 8b). Thus, these results confirm that *CsSAP09* is transcriptionaly active in yeast.

Isolation of core promoter of *CsSAP09*

The expression of SAP genes is known to be induced by various abiotic factors including drought, salt, cold, light/UV light, mechanical wounding, and plant hormones (Giri et al. 2011, 2013). These factors modulate the gene expression by interacting with the cis-regulatory elements in the upstream regions of *SAPs* genes. To get an idea about the putative cis elements in the upstream region of *CsSAP09*, the region flanking the 5′ end was isolated. Genome-walking approach isolated a 565 bp fragment upstream of the start codon, corresponding to the putative promoter region of *CsSAP09* (Fig. 9). Computational analysis using PlantCare database revealed several important cis-acting regulatory elements within the promoter region of *CsSAP09*, as shown in Table 1. Consistent with the established role of *SAPs* in abiotic stress, a cis-acting regulatory element essential for the anaerobic induction (ARE element) was found at the position 93. An MBS cis element which is an MYB-binding site involved in drought inducibility (Urao et al. 1993) is also present at positions 65,262 and 460. Besides, many light responsive elements were also found.

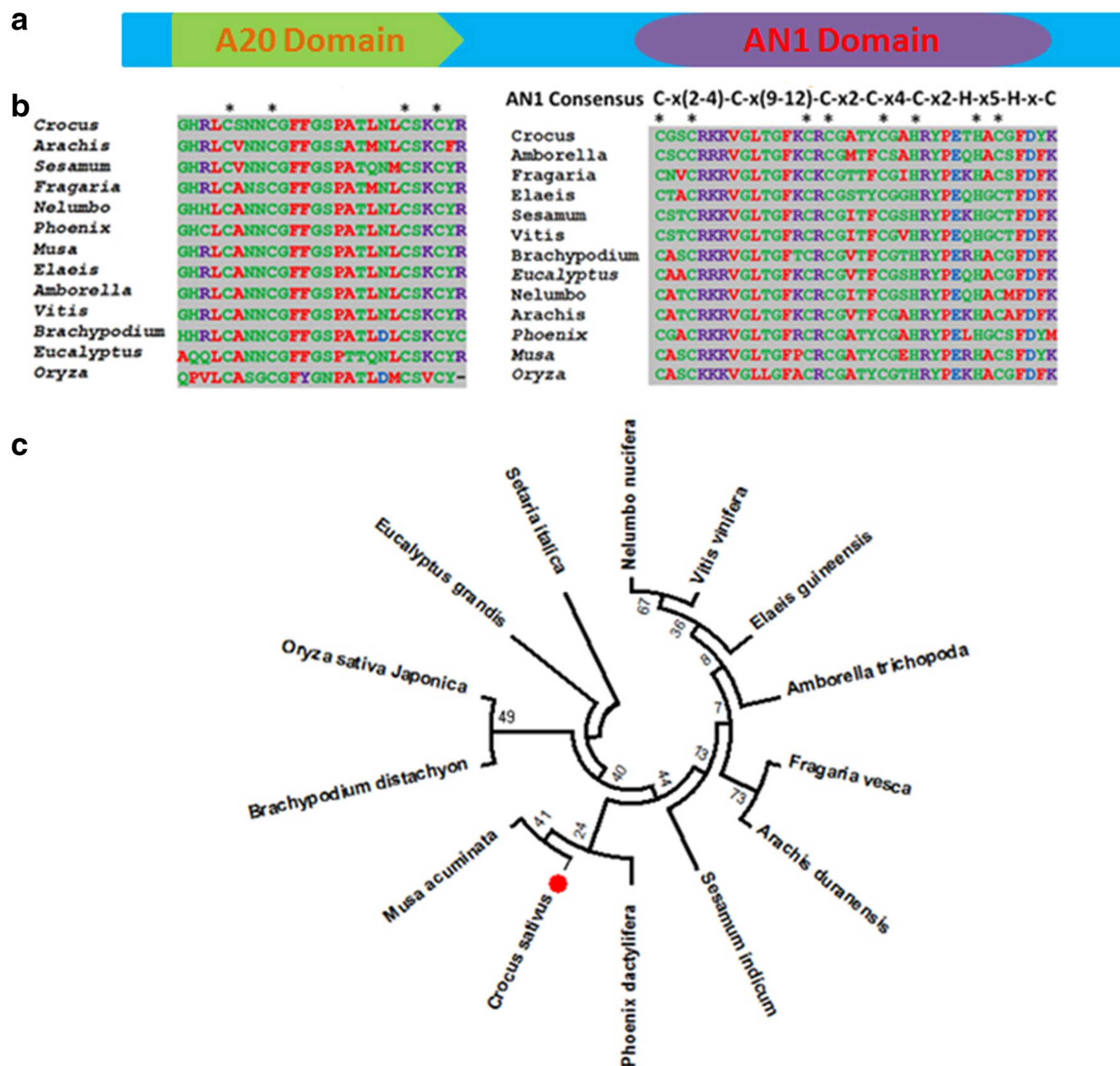


Fig. 7 Sequence alignment and phylogeny of *CsSAP09*. **a** Diagrammatic representation of A20 and AN1 zinc-finger domains, **b** multiple sequence alignment and **c** phylogenetic tree of CsSAP09 and other A20/AN1-zinc-finger protein homologs. The neighbor-joining tree was constructed using MEGA 6.06 with 1000 bootstrap replications. The GenBank accession numbers of different sequences used are as follows: *Phoenix dactylifera* (XP_008812241.1), *Musa acuminata* subsp. *malaccensis (*XP_009393382.1), *Elaeis guineensis* (XP_010911244.1), *Brachypodium distachyon* (XP_003575065.1), *Eucalyptus grandis* (XP_010031502.1), *Oryza sativa* Japonica (XP_015639390.1), *Fragaria vesca* subsp. *vesca* (XP_004303001.1), *Setaria italic* (XP_004969762.1), *Nelumbo nucifera* (XP_010271706.1), *Sesamum indicum* (XP_011094369.1), Arachis duranensis (XP_015951458.1), *Amborella trichopoda* (XP_006851496.1), and *Vitis vinifera* (XP_010654069.1). Values at the node denote percentage bootstrap value. The genetic distances are indicated by the *horizontal bar*

Discussion

Transcription factors are central elements that regulate spatio-temporal expression of genes, thus ensuring their accurate functioning in an organism. Proper identification and classification are necessary for functional characterization of any new gene. In this study, 81 ZNFs belonging to 8 subfamilies were identified from *Crocus* transcriptome. *C2H2* was the largest family with 29 genes followed by *C3H* with 20 genes and *A20*/*AN1* or *SAP* with 14 members (Fig. 1). Since these families formed the most abundant classes, our further study was focused on them. In *Arabidopsis*, 176 *C2H2* and 68 *C3H* genes, while in rice, 189 *C2H2* and 67 C3H genes have been identified (Agarwal et al. 2007; Ciftci-Yilmaz and Mittler 2008; Wang et al. 2008). Furthermore, *A20*/*AN1*-type zinc-finger genes are represented by 14 members in *Arabidopsis* and 18 in rice. In our study, the number of genes identified was lesser than other plants. This may be because an initial search of transcriptome may underestimate the number of *CsZNF* genes

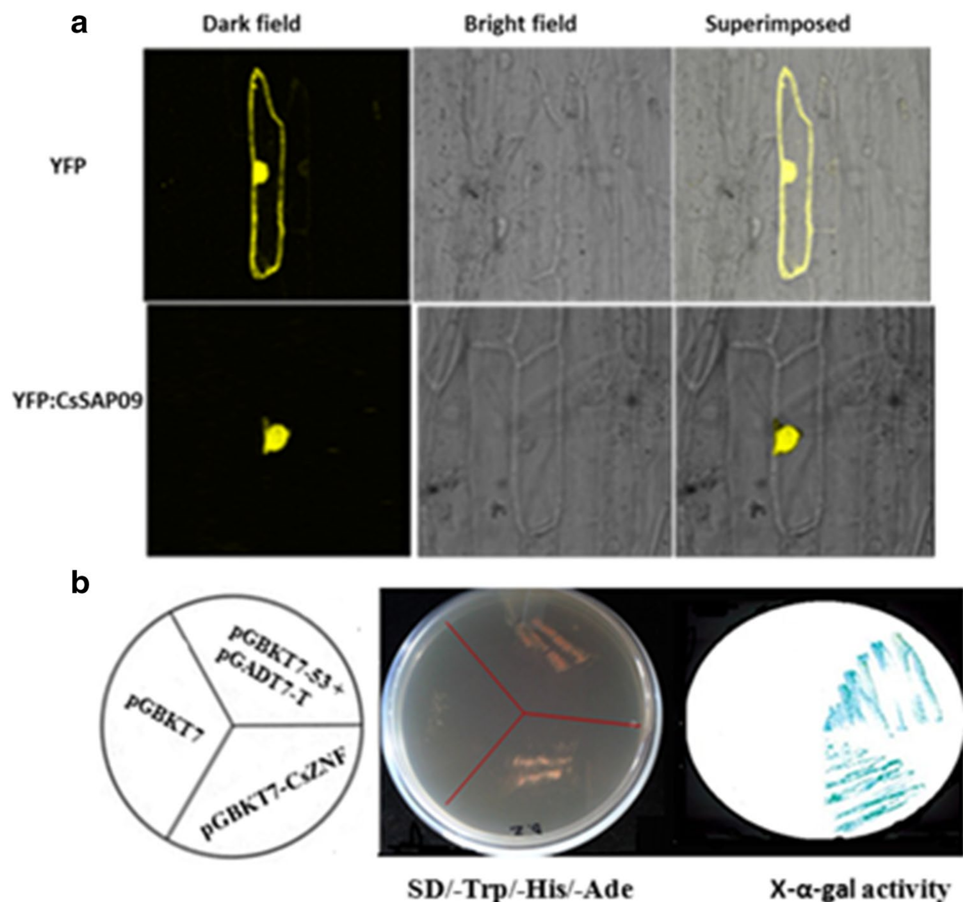


Fig. 8 Subcellular localization and transactivation assay of *CsSAP09*. **a** Upper panel shows YFP accumulation throughout the cell, while the lower one shows CsSAP09-YFP is localized to the nucleus. **b** Fusion protein of the GAL4 DNA-binding domain and *CsSAP09* were expressed in yeast strain Y187. The empty pBD (pGBKT7) vector (negative control) and the pGAL4 vector (positive control) were used. The culture solution of the transformed yeast was dropped onto SD/-Trp-ade-his plates. The plates were incubated for 3 days and then subjected to β-galactosidase assay

for these being transcription factors, are often expressed at low levels (Arora et al. 2007). Another reason might be the fact that, in this study, only flower tissue was used for generation of transcriptome and hence does not include the genes expressed in other plant parts. Therefore, more genes containing the ZNF domain may come to the fore as more transcriptome and genome sequence information is made available.

To know about the evolutionary relationship of *CsZNFs* with their *Arabidopsis* homologs, comparative phylogenetic analysis was done. In case of all the three families, while many *Crocus* ZNFs clustered with their *Arabidopsis* homologs, others either formed a separate clad or some of the genes were seen as outliers (Figs. 2, 3, 4). This might be because of the fact that *Arabidopsis* and *Crocus* belong to distant families. Furthermore, less number of sequences from *Crocus* might also be a reason for this pattern.

Gene expression patterns are an essential aspect of study of gene functions (Gu 2004). The expression profile of a gene is usually indicative of its function. We studied expression pattern of three representative genes (*CsC2H216, CsC3H36*, and *CsSAP09)* one each from *C2H2, C3H*, and *SAP* classes. The three genes were chosen, because we had the longest sequence available for them among all the members present in each of these classes. These selected genes displayed a complex expression pattern in different tissues and at different flower developmental stages (Fig. 5). Among the five tissue types tested, *CsC2H216* showed highest expression in tepal; *CsC3H13* exhibited peak expression in anther while *CsSAP09* was induced more in stigma. This expression pattern may indicate their tissue specific roles. Furthermore, *CsSAP09* displayed peak expression at anthesis stage of flower development. The expression pattern of *CsSAP09* corroborates with accumulation pattern of apocarotenoids which are produced in stigma and their levels increase upto the anthesis stage and then show a decline (Ashraf et al. 2015). This indicates possible role of this gene in regulating apocarotenoid biosynthesis in *Crocus*.

Growth, development, and metabolism of plants are usually challenged by different stresses from the environment, such as salinity, drought, osmotic, and oxidative stresses during their life cycle. A myriad of stress-related genes is induced whose expression products are required to adapt to these environmental insults (Kasuga et al. 1999; Coram and Pang 2006). Zinc-finger proteins are also known to mediate various biotic and abiotic stress-related adaptations in different plants (Tian et al. 2010). In this context,

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+ TCCGGTATCG CGATGCATCT AGATTCCTCC GGCGAGGAAG AGGACGAGGC GGAAGAAGTG ATGGCAGTTA 70
- AGGCCATAGC GCTACGTAGA TCTAAGGAGG CCGCTCCTTC TCCTGCTCCG CCTTCTTCAC TACCGTCAAT

+ CCATTCTCGT CGCCCCTCGT CCTGGTTTAG TGTTGTTGGA TTCTGGGACA ACACATTCCT TATAGTTGTC 140
- GGTAAGAGCA GCGGGGAGCA GGACCAAATC ACAACAACCT AAGACCCTGT TGTGTAAGGA ATATCAACAG

+ GGCTTTTGGT CGTCGGCATC ATATAAGATT AGAGACCTTA GTGAGACCGA TGAGAGTGTT CACGGGGAAT 210
- CCGAAAACCA GCAGCCGTAG TATATTCTAA TCTCTGGAAT CACTCTGGCT ACTCTCACAA GTGCCCCTTA

+ GGGCCGGTTC GTATCGTTGG GGTGGTGAGA GCGTGCCCAG TCGTGATTCC GTAACTGGAG TTCAAGGCTA 280
- CCCGGCCAAG CATAGCAACC CCACCACTCT CGCACGGGTC AGCACTAAGG CATTGACCTC AAGTTCCGAT

+ GGATTTGTCT TCCCTCTTCC GGGTTACGAT GTAGTATACA GGATGGATTG GTTGGGTCTC TCCTATGCCA 335
- CCTAAACAGA AGGGAGAAGG CCCAATGCTA CATCATATGT CCTACCTAAC CAACCCAGAG AGGATACGGT

+ CGATCCACTA CCGTGAGCGG CGTGTGGTGT TCCGACTGCC TCTAATACCA GAGGCAGTAA TTGGTGGCGC 420
- GCTAGGTGAT GGCACTCGCC GCACACCACA AGGCTGACGG AGATTATGGT CTCCGTCATT AACCACCGCG

+ GTTTGGTTCG AAGAAGGTTG AACGCCCTAC ACTCACGGTT AACTGAGAGT CGGGTTGTCC AGCTCCTAGG 490
- CAAACCAAGC TTCTTCCAAC TTGCGGGATG TGAGTGCCAA TTGACTCTCA GCCCAACAGG TCGAGGATCC

+ GTGGTGCAGG AGTTCTTGGA TGTGTCTCCA GAGTATAGCC TGGTCTTCCG CCTCGTCCTC TTCCTCGCCG 560
- CACCACGTCC TCAAGAACCT ACACAGAGGT CTCATATCGG ACCAGAAGGC GGAGCAGGAG AAGGAGCGGC

+ GAGGTGTTT 569
- CTCCACAAA
      *
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Fig. 9 Isolation and analysis of CsSAP09 promotor. DNA sequence of *C. sativus CsSAP09* promoter (565 bp) amplified from the *StuI* genome-walking library. The putative TSS, TATA box, CAAT box, and other important cis-regulatory elements are marked out

we investigated the expression of the selected *CsZNFs* in response to various stresses. We observed increase in expression of *CsC2H216* in response to cold and dehydration (Fig. 6a) and of *CsC3H13* in case of cold and salt stress (Fig. 6b). Earlier reports have also demonstrated involvement of *C2H2* and *C3H* family proteins in various abiotic stresses (Li et al. 2013). This suggests that role of these gene families in stress tolerance remains conserved across plant species. Furthermore, expression of *CsSAP09* was also induced in response to salt, methylviologen, and dehydration stresses (Fig. 6c). Almost all *SAP* family genes have been implicated in various stress responses (Giri et al. 2011, 2013). Overexpression of *SAPs* has been shown to enhance tolerance to multiple abiotic stresses in a number of transgenic plants (Mukhopadhyay et al. 2004).

The stress responses in plants are mediated by phytohormones. Therefore, we investigated expression of *CsZNFs* in response to various phytohormone treatments. Our results showed that all the three genes were induced by different phytohormones, albeit to varied levels. *CsC2H216* showed maximum induction in response to methyl jasmonate followed by ABA (Fig. 6d). *CsC3H13* was highly elicited in response to auxin followed by gibberellic acid and ABA (Fig. 6e), while *CsSAP09* exhibited highest expression in response to ABA followed by salicylic acid and methyl jasmonate (Fig. 6f). All these hormones play role in controlling and fine tuning various stress responses. Moreover, induction of *CsSA09* expression in response to ABA further establishes its role in abiotic stress, since ABA is known to regulate tolerance to abiotic stress (Wani and Kumar 2015).

AN20/AN1, an emerging class of zinc-finger proteins, also called as stress-associated proteins (SAPs), shows considerable levels of structural conservation and functional homology from diverse plant species. These proteins were, for the first time, identified in rice as products of multiple stress responsive genes (Mukhopadhyay et al. 2004; Vij and Tyagi 2006), but their functional repertoire has been expanding. Since expression profile of *CsSAP09* suggested its role in regulating apocarotenoid metabolism, its full-length gene was cloned and its sequence analyzed. It has an AN1-type zinc-finger region at the C terminus of the protein spanning amino acids 87 to 157. It has a consensus sequence of Cx2-4Cx9-12Cx2Cx4Cx2Hx5HxC, where x represents any amino acid. The conserved cysteine and histidine residues form and stabilize the zinc finger. There are four conserved cysteine residues at the amino-acid positions 14, 18, 30, and 33 toward the N-terminus of the protein (Fig. 7). This region is akin to the A20-like

Table 1 Putative cis-acting regulatory elements identified in the promoter of *CsSAP09* by PLANTCARE databases (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/)

Cis-element	Position	Signal sequence	Putative function	References
4 cl-CMA2b	230 (−)	TCTCACCAACC	Light responsive element	PlantCare
ARE	93 (+)	TGGTTT	Cis-acting regulatory element essential for the anaerobic induction	PlantCare
CAAT box	283 (−)	CAAAT	Common cis-acting element in promoter and enhancer regions	Shirsat et al. (1989)
	409 (−)			
	327 (−)			
	410 (−)			
G-box	250 (−)	CACGAC	Cis-acting regulatory element involved in light responsiveness	PlantCare
GT1-motif	45 (+)	GGTTAA	Light responsive element	PlantCare
L-box	23 (−)	TCTCACCAACC	Part of a light responsive element	
MBS	65 (−)	TAACTG	MYB-binding site involved in drought inducibility	Urao et al. (1993)
	460 (+)			
	26 (+)			
MNF1	243 (+)	GTGCCC(A/T)(A/T)	Light responsive element	PlantCare
Sp1	230 (−)	CC(G/A)CCC	Light responsive element	PlantCare
	489 (−)			
TATA box	130 (−)	TATAA	Core promoter element	Tjaden et al. (1995)
	315 (−)	TATA		
	46 (+)	TATA		
	161 (+)	ATATAA		
	393 (+)	TAATA		
	131 (+)	TATA		
	162 (+)	TATA		

zinc-finger protein (Dixit et al. 1990). Comparison of full-length cDNA of *CsSAP09* with a genomic fragment generated by PCR amplification and sequencing revealed that the genomic clone is continuous, without any intron. The *O. sativa* stress-associated protein 1(*Os*Sap1), identified as the first plant A20/AN1 zinc-finger as well as tea, *Camellia sinensis*, and *Cs*Zfp were also without any intron (Mukhopadhyay et al. 2004; Paul and Kumar 2015).

Recently, various research groups have reported that A20/AN1-like proteins localize to various subcellular compartments (Hishiya et al. 2006; Kanneganti and Gupta 2008). The NLStradamus and NucPred Programs (Brameier et al. 2007; Nguyen et al. 2009) were used to predict the nuclear localization signal in *CsSAP09*. This analysis revealed the presence of a putative nuclear localization sequence. Congruently, our study also revealed that *CsSAP09* is localized in the nucleus (Fig. 8a). *At*SAP3 and *At*SAP5 are also nuclear localized (Kim et al. 2015). Furthermore, it was found that *CsSAP09* was transcriptionally active in yeast strain Y187 (Fig. 8b).

The promoter isolation led to the amplification of 565 bp long fragment which contained many cis elements including MBS, a Myb-binding site; ARE; and some light responsive elements (Fig. 9). Myb genes are known to regulate gene expression in response to many developmental cues as well as biotic and abiotic stress responses (Liu et al. 2015; Li et al. 2016). Furthermore, Myb genes are also involved in regulating plant secondary metabolism (Chezem and Clay 2016). Since expression profile of *CsSAP09* hinted at its involvement in apocarotenoid metabolism and stress response, the presence of MBS and ARE elements in its promoter region further supports its role. Furthermore, a number of reports have shown that light regulates carotenoid metabolism in a number of plants (Ahrazem et al. 2016). The presence of light responsive elements in *CsSAP09* promoter suggests that the gene might be involved in light responsive regulation of apocarotenoid metabolism.

In conclusion, we did transcriptome wide identification and phylogenetic analysis of zinc-finger genes in *Crocus* in which *C2H2, C3H*, and *A20/AN1* or *SAPs* emerged as major classes. The transcript abundance of one representative member from each of these classes was investigated which showed that the genes are induced in response to different abiotic stresses and phytohormones. The transcript profile of *CsSAP09* corroborated with known metabolite profile of apocarotenoids, therefore, indicating its possible role in regulating apocarotenoid metabolism. Further studies will elucidate the exact role of CsZNFs

in general and *CsSAP09* in particular in *Crocus* apocarotenoid metabolism.

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Compliance with ethical standards

Conflict of interest Authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies performed with animals by any of the authors.

References

Agarwal P, Arora R, Ray S, Singh AK, Singh VP, Takatsuji H et al (2007) Genome-wide identification of C2H2 zinc-finger gene family in rice and their phylogeny and expression analysis. Plant Mol Biol 65:467–485

Ahrazem O, Rubio-Moraga A, Nebauer SG, Molina RV, Gómez-Gómez L (2015) Saffron: its phytochemistry, developmental processes, and biotechnological prospects. J Agric Food Chem 63:8751–8764

Ahrazem O, Rubio-Moraga A, Argandoña-Picazo J, Castillo R, Gómez-Gómez L (2016) Intron retention and rhythmic diel pattern regulation of carotenoid cleavage dioxygenase 2 during crocetin biosynthesis in saffron. Plant Mol Biol 91:355–374

Arnaud D, Dejardin A, Leple JC, Lesage Descauses M, Pilate G (2007) Genome-wide analysis of LIM gene family in *Populus trichocarpa, Arabidopsis thaliana*, and *Oryza sativa*. DNA Res 14:103–116

Arora R, Agarwal P, Ray S, Singh AK, Singh VP, Tyagi AK et al (2007) MADS-box gene family in rice: genome-wide identification, organization and expression profiling during reproductive development and stress. BMC Genomics 8:242

Ashraf N, Jain D, Vishwakarma RA (2015) Identification, cloning and characterization of an ultrapetala transcription factor CsULT1 from Crocus: a novel regulator of apocarotenoid biosynthesis. BMC Plant Biol 15:25

Baba SA, Jain D, Abbas N, Ashraf N (2015a) Overexpression of Crocus carotenoid cleavage dioxygenase, CsCCD4b, in Arabidopsis imparts tolerance to dehydration, salt and oxidative stresses by modulating ROS machinery. J Plant Physiol 189:114–125

Baba SA, Mohiuddin T, Basu S, Swarnkar MK, Malik AH, Wani ZA et al (2015b) Comprehensive transcriptome analysis of Crocus sativus for discovery and expression of genes involved in apocarotenoid biosynthesis. BMC Genomics 16:698

Brameier M, Krings A, MacCallum RM (2007) NucPred - Predicting nuclear localization of proteins. Bioinformatics 23:1159–1160

Chezem WR, Clay NK (2016) Regulation of plant secondary metabolism and associated specialized cell development by MYBs and bHLHs. Phytochemistry 131:26–43

Ciftci-Yilmaz S, Mittler R (2008) The zinc finger network of plants. CMLS 65;1150–1160.

Coram TE, Pang ECK (2006) Expression profiling of chickpea genes differentially regulated during a resistance response to Ascochyta rabiei. Plant Biotechnol J 4:647–666

Dixit VM, Green S, Sarma V, Holzman LB, Wolf FW, O'Rourke K et al (1990) Tumor necrosis factor-alpha induction of novel gene products in human endothelial cells including a macrophage-specific chemotaxin. J Biol Chem 265:2973–2978

Fernández JA, Santana O, Guardiola JL, Molina RV, Heslop-Harrison P, Borbely G et al (2011) The World Saffron and Crocus collection: strategies for establishment, management, characterization and utilization. Genet Resour Crop E 58:125–137

Frusciante S, Diretto G, Bruno M, Ferrante P, Pietrella M, Prado-Cabrero A et al (2014) Novel carotenoid cleavage dioxygenase catalyzes the first dedicated step in saffron crocin biosynthesis. Proc Natl Acad Sci USA 111:12246–12251

Giri J, Vij S, Dansana PK, Tyagi AK (2011) Rice A20/AN1 zinc-finger containing stress-associated proteins (SAP1/11) and a receptor-like cytoplasmic kinase (OsRLCK253) interact via A20 zinc-finger and confer abiotic stress tolerance in transgenic Arabidopsis plants. N Phytol 191:721–732

Giri J, Dansana PK, Kothari KS, Sharma G, Vij S, Tyagi AK (2013) SAPs as novel regulators of abiotic stress response in plants. Bioessays 35:639–648

Gourcilleau D, Lenne C, Armenise C, Moulia B, Julien JL, Bronner G et al (2011) Phylogenetic study of plant Q-type C2H2 zinc finger proteins and expression analysis of poplar genes in response to osmotic, cold and mechanical stresses. DNA Res Int J Rapid Publ Rep Genes Genom 18:77–92

Gu X (2004) Statistical framework for phylogenomic analysis of gene family expression profiles. Genetics 167:531–542

Gutheil WG, Reed G, Ray A, Anant S, Dhar A (2012) Crocetin: an agent derived from saffron for prevention and therapy for cancer. Curr Pharm Biotechnol 13:173–179

Hall TMT (2005) Multiple modes of RNA recognition by zinc finger proteins. Curr Opin Struct Biol 15:367–373

Hishiya, Iemura S, Natsume T, Takayama S, Ikeda K, Watanabe K (2006) A novel ubiquitin-binding protein ZNF216 functioning in muscle atrophy. EMBO J 25:554–564

Iuchi S (2001) Three classes of C2H2 zinc finger proteins. CMLS Cell Mol Life Sci 58:625–635

Jain M, Srivastava PL, Verma M, Ghangal R, Garg R, Fernandez JA et al (2016) De novo transcriptome assembly and comprehensive expression profiling in *Crocus sativus* to gain insights into apocarotenoid biosynthesis. Sci Rep 6:22456

Kaefer CM, Milner JA (2008) The role of herbs and spices in cancer prevention. J Nutr Biochem 19:347–361

Kanneganti V, Gupta AK (2008) Overexpression of OsiSAP8, a member of stress associated protein (SAP) gene family of rice confers tolerance to salt, drought and cold stress in transgenic tobacco and rice. Plant Mol Biol 66:445–462

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat Biotechnol 17:287–291

Kianbakht S, Mozaffari K (2009) Effects of saffron and its active constituents, crocin and safranal, on prevention of indomethacin induced gastric ulcers in diabetic and nondiabetic rats. J Med Plants 1:30–38

Kim GD, Cho YH, Yoo SD (2015) Regulatory functions of evolutionarily conserved AN1/A20-like Zinc finger family proteins in Arabidopsis stress responses under high temperature. Biochem Biophys Res Commun 457:213–220

Klug A, Schwabe JW (1995) Protein motifs 5. Zinc fingers. FASEB J 9:597–604

Kyriakoudi A, Ordoudi S, Roldán M, Medina MZ, Tsimidou (2015) Saffron, a functional spice. Austin J Nutri Food Sci 3:1059–1

Li WT, He M, Wang J, Wang YP (2013) Zinc finger protein (ZFP) in plants—a review. POJ 6:474–480

Li MJ, Qiao Y, Li YQ, Shi ZL, Zhang N, Bi CL, Guo JK (2016) A R2R3-MYB transcription factor gene in common wheat (namely TaMYBsm1) involved in enhancement of drought tolerance in transgenic Arabidopsis. J Plant Res 129:1097–1107

Liu J, Osbourn A, Ma P (2015) MYB transcription factors as regulators of phenylpropanoid metabolism in plants. Mol Plant. 8:689–708

Lopresti AL, Drummond PD (2014) Saffron (*Crocus sativus*) for depression: a systematic review of clinical studies and examination of underlying antidepressant mechanisms of action. Hum Psychopharmacol Clin Exp 29:517–527

Luo M, Bilodeau P, Koltunow A, Dennis ES, Peacock WJ, Chaudhury AM (1999) Genes controlling fertilization-independent seed development in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 96:296–301

Mir JI, Ahmed N, Wafai AH, Qadri RA (2012) Relative expression of CsZCD gene and apocarotenoid biosynthesis during stigma development in *Crocus sativus* L. Physiol Mol Biol Plants Int J Funct Plant Biol 18:371–375

Molnár G, Bancoş S, Nagy F, Szekeres M (2002) Characterisation of BRH1, a brassinosteroid-responsive RING-H2 gene from *Arabidopsis thaliana*. Planta 215:127–133

Moshiri M, Vahabzadeh M, Hosseinzadeh H (2015) Clinical applications of saffron (*Crocus sativus*) and its constituents: a review. Drug Res 65:287–295.

Mukhopadhyay A, Vij S, Tyagi AK (2004) Overexpression of a zinc-finger protein gene from rice confers tolerance to cold, dehydration, and salt stress in transgenic tobacco. Proc Natl Acad Sci USA 101:6309–6314

Nguyen BAN, Pogoutse A, Provart N, Moses AM (2009) NLStradamus: a simple Hidden Markov Model for nuclear localization signal prediction. BMC Bioinform 10:202

Paul A, Kumar S (2015) An A20/AN1-zinc-finger domain containing protein gene in tea is differentially expressed during winter dormancy and in response to abiotic stress and plant growth regulators. Plant Gene 1:1–7

Rakesh K, Virendra Singh V, Devi K, Sharma M, Singh MK, Ahuja PS (2009) State of art of saffron (*Crocus sativus* L.) agronomy: a comprehensive review. Food Rev Int 25:44–85

Rubio-Moraga A, Trapero A, Ahrazem O, Gómez-Gómez L (2010) Crocin transport in *Crocus sativus*: the long road from a senescent stigma to a newborn corm. Phytochemistry 71:1506–1513

Shirsat A, Wilford N, Croy R, Boulter D (1989) Sequences responsible for the tissue specific promoter activity of a pea legumin gene in tobacco. Mol Gen Genet 215:326–331

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.06. Mol Biol Evol 30:2725–2729

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Tian ZD, Zhang Y, Liu J, Xie CH (2010) Novel potato C2H2-type zinc finger protein gene, StZFP1, which responds to biotic and abiotic stress, plays a role in salt tolerance. Plant Biol (Stuttgart, Germany) 12:689–697

Tjaden G, Edwards JW, Coruzzi GM (1995) cis elements and trans-acting factors affecting regulation of a nonphotosynthetic light-regulated gene for chloroplast glutamine synthetase. Plant Physiol 108:1109–1117

Urao T, Yamaguchi-Shinozaki K, Urao S, Shinozaki K (1993) An *Arabidopsis* myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. Plant Cell 5:1529–1539

Vij S, Tyagi AK (2006) Genome-wide analysis of the stress associated protein (SAP) gene family containing A20/AN1 zinc-finger(s) in rice and their phylogenetic relationship with *Arabidopsis*. MGG 276;565–575

Wang D, Guo Y, Wu C, Yang G, Li Y, Zheng C (2008) Genome-wide analysis of CCCH zinc finger family in *Arabidopsis* and rice. BMC Genom 9:44

Wani SH, Kumar V (2015) Plant Stress Tolerance: Engineering ABA: A Potent Phytohormone. Transcriptomics 3:113

Yang L, Liu Z, Lu F, Dong A, Huang H (2006) SERRATE is a novel nuclear regulator in primary microRNA processing in *Arabidopsis*. Plant J Cell Mol Biol 47:841–850

Yun JY, Weigel D, Lee I (2002) Ectopic expression of SUPERMAN suppresses development of petals and stamens. Plant Cell Physiol 43:52–57

Zhang H, Jin JP, Tang L, Zhao Y, Gu XC, Gao G, Luo JC (2011) PlantTFDB 2.0: update and improvement of the comprehensive plant transcription factor database. Nucleic Acids Res 39:1114–1117

Zhang C, Zhang H, Zhao Y, Jiang H, Zhu S, Cheng B, Xiang Y (2013) Genome-wide analysis of the CCCH zinc finger gene family in *Medicago truncatula*. Plant Cell Rep 32:1543–1555

Zheng S, Qian Z, Sheng L, Wen N (2006) Crocetin attenuates atherosclerosis in hyperlipidemic rabbits through inhibition of LDL oxidation. J Cardiovasc Pharmacol 47:70–76