



CstMYB1R1, a REVEILLE-8-like transcription factor, regulates diurnal clock-specific anthocyanin biosynthesis and response to abiotic stress in *Crocus sativus* L

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Abstract

Key message *CstMYB1R1* acts as a positive regulator of *Crocus* anthocyanin biosynthesis and abiotic stress tolerance which was experimentally demonstrated through molecular analysis and over-expression studies in *Crocus* and *Nicotiana*.

Abstract Regulatory mechanics of flavonoid/anthocyanin biosynthesis in *Crocus* floral tissues along the diurnal clock has not been studied to date. MYB proteins represent the most dominant, functionally diverse and versatile type of plant transcription factors which regulate key metabolic and physiological processes *in planta*. Transcriptome analysis revealed that MYB family is the most dominant transcription factor family in *C. sativus*. Considering this, a MYB-related REVEILLE-8 type transcription factor, *CstMYB1R1*, was explored for its possible role in regulating *Crocus* flavonoid and anthocyanin biosynthetic pathway. *CstMYB1R1* was highly expressed in *Crocus* floral tissues, particularly tepals and its expression was shown to peak at dawn and dusk time points. Anthocyanin accumulation also peaked at dawn and dusk and was minimum at night. Moreover, the diurnal expression pattern of *CstMYB1R1* was shown to highly correlate with *Crocus* ANS/LDOX gene expression among the late anthocyanin pathway genes. *CstMYB1R1* was shown to be nuclear localized and transcriptionally active. *CstMYB1R1* over-expression in *Crocus* tepals enhanced anthocyanin levels and upregulated transcripts of *Crocus* flavonoid and anthocyanin biosynthetic pathway genes. Yeast one hybrid (Y1H) and GUS reporter assay confirmed that *CstMYB1R1* interacts with the promoter of *Crocus* *LDOX* gene to directly regulate its transcription. In addition, the expression of *CstMYB1R1* in *Nicotiana* plants significantly enhanced flavonoid and anthocyanin levels and improved their abiotic stress tolerance. The present study, thus, confirmed positive role of *CstMYB1R1* in regulating *Crocus* anthocyanin biosynthetic pathway in a diurnal clock-specific fashion together with its involvement in the regulation of abiotic stress response.

Keywords *Crocus sativus* · MYB transcription factors · Circadian rhythm · Secondary metabolism · Anthocyanins · Abiotic stress

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Abbreviations

RVE8	REVIELLE-8
Y1H	Yeast one hybrid
LDOX	Leucoanthocyanidin oxygenase
qRT-PCR	Quantitative real-time PCR

Introduction

Crocus sativus L., a sterile triploid geophyte, is valued for its spice and medicinal properties which are a reflection of accumulation of secondary metabolites in its floral tissues (Moraga et al 2009). *Crocus* stigmas, in particular, exhibit enrichment of unique apocarotenoid moieties like crocin, picrocrocin, and safranal. However, the floral tissues also

constitute a rich source of other secondary metabolites including flavonoids and anthocyanins (Mykhailenko et al. 2019; Senizza et al. 2019). The *Crocus* stigmas and tepals, in particular, harbor a considerable diversity of flavonoids and anthocyanins (Moraga et al. 2009; Li 2014; Mykhailenko et al. 2019). Flavonoid biosynthesis is by far the best characterized secondary metabolite pathway in plants (Tohge et al. 2017). Anthocyanins are biosynthesized as an extension to the general flavonoid biosynthetic pathway involving a number of biocatalysts classified as the early and late biosynthetic enzymes. Chalcone synthase (CHS), chalcone isomerase (CHI), flavanone-3-hydroxylase (F3H), and flavonoid 3'-hydroxylase (F3'H) constitute early biosynthetic enzymes whereas dihydroflavonol 4-reductase (DFR), anthocyanidin synthase/leucoanthocyanidin oxygenase (ANS/LDOX), and UDP-glucoside: flavonoid glucosyltransferase (UFGT) are categorized as the late anthocyanin biosynthetic enzymes (Guo et al. 2014; Yonekura-Sakakibara et al. 2019). Flavonoid/anthocyanin biosynthesis is considered to be the best characterized secondary metabolite pathway in plants and its regulation has also been explored to a considerable level (Li et al. 2014). Moreover, a number of studies have figured out the involvement of plant MYB transcription factors in regulating biosynthesis of flavonoids and anthocyanins (Vimolmangkang et al. 2013; Schwinn et al. 2016; Chen et al. 2019a). For instance, CsMYB2 and CsMYB26 have been shown to regulate flavonoid biosynthesis in *Camelia sinensis* (Wang et al. 2018). In *Glycine max*, GmMYB100 acts as negative regulator of flavonoid biosynthesis (Yan et al. 2015). Interestingly, plant MYBs via their regulatory control over secondary metabolite biosynthesis have been shown to modulate host response to biotic and abiotic stress (Hussain et al. 2022). Recently, a well-known transcriptional regulator of *Arabidopsis* anthocyanin biosynthetic pathway, AtMYB111, was reported to modulate salt response of the host plant by modulating flavonoid biosynthesis (Li et al. 2019). Also, an R2R3 MYB transcription factor of *Scutellaria baicalensis*, SbMYB8, is credited for its role in flavonoid biosynthesis and improvement of drought stress tolerance in transgenic tobacco (Yuan et al. 2015). Again, the MYB transcription factors have also been shown to specifically regulate the anthocyanin biosynthesis. For example, a grape hyacinth R2R3 MYB, MaMybA, acts as activator of anthocyanin biosynthesis, and ectopic expression of *MaMybA* in tobacco exhibited severe magenta coloration (Chen et al. 2019b). Similarly, DcMYB113 regulates anthocyanin biosynthesis in carrot (Xu et al. 2020). In addition, MYB115 and MYB134 were shown to act as major activators of proanthocyanidin pathway in poplar (James et al. 2017).

Our laboratory has been working toward understanding regulation of secondary metabolism in *C. sativus*. Toward this, a few MYB transcription factors regulating carotenoid

and apocarotenoid biosynthesis were identified by our group previously (Bhat et al. 2021; Hussain et al. 2022). Keeping in view the frequent involvement of MYB transcription factors in regulating plant flavonoid and anthocyanin biosynthesis, we further aimed at identifying MYB genes which regulate *Crocus* anthocyanin biosynthesis. In *Crocus*, anthocyanins are predominantly synthesized in tepals (Goupy et al. 2013). From our transcriptome data, several MYB genes were shown to be upregulated in *Crocus* tepals, *CstMYB1R1* being one among them (Baba et al. 2015; Bhat et al. 2021). *CstMYB1R1* showed close homology to REVIELLE-8 (RVE8)-like MYB transcription factors which have been reported to modulate the plant circadian rhythm and to act as the positive regulators of anthocyanin biosynthesis (Pérez García et al. 2015; De Leone et al. 2019). This prompted us to investigate the role of *CstMYB1R1* in regulating the anthocyanin biosynthesis in *Crocus*. It was observed that *CstMYB1R1* exhibited diurnal expression peak at dawn and dusk time points which correlated with expression pattern of ANS/LDOX gene and anthocyanin accumulation in tepals. The *Crocus* floral tissues over-expressing *CstMYB1R1* showed upregulation of key flavonoid and anthocyanin pathway genes together with significant enhancement of anthocyanin levels. *Crocus* LDOX gene was confirmed to be direct regulatory target of *CstMYB1R1*. Over-expression of *CstMYB1R1* in tobacco plants increased the total anthocyanin content significantly and enhanced the abiotic stress tolerance. Thus, the present study confirms the positive regulatory role of *CstMYB1R1* in biosynthesis of *Crocus* anthocyanins and tolerance to abiotic stress.

Materials and methods

Plant material collection and treatments

For various treatments and transient over-expression studies, *C. sativus* corms weighing around 8 g were grown in pots under controlled conditions at a temperature of 16 °C. Hormone treatment was given after flower anthesis. Flowers were sprayed with 100 µM abscisic acid (ABA), gibberellic acid (GA₃), methyl jasmonate (MeJA), and salicylic acid (SA). The flowers were collected after 6 and 12 h post-treatment and stored at -80 °C till further use. Different abiotic stress treatments were given as previously described (Baba et al. 2017). Briefly, for cold treatment, plants were kept at 4 °C. For dehydration and salt stresses, 200 mM each of mannitol and NaCl were added into the soil in which *Crocus* corms were grown thrice at 3 h intervals, respectively, for 12 h. For oxidative stress, 100 µM methyl viologen was mist-sprayed to flowers at 3 h intervals for 12 h. The tissues were harvested post-24 h

of stress treatments and stored in $-80\text{ }^{\circ}\text{C}$. UV stress was given by irradiating plants with $1500\text{ }\mu\text{J}\cdot\text{m}^2$ for 2 h.

Agro-infiltration was done as described in Bhat et al (2021). *Crocus* flowers were agro-infiltrated and covered with polybags and the flowers were harvested 72 h post-agro-infiltration.

Nicotiana benthamiana plants were raised under the photoperiodic conditions of 8 h light and 16 h dark, diurnal temperatures of $21\text{ }^{\circ}\text{C}$ (day) and $16\text{ }^{\circ}\text{C}$ (night) with light intensity of $90\text{ }\mu\text{mol}/\text{m}^2\text{s}^{-1}$, and humidity of about 60% and stably transformed through *Agrobacterium tumefaciens*-mediated transformation.

In silico analysis and phylogeny

CstMYB1R1 nucleotide sequence was retrieved from the transcriptome database previously generated by Baba et al. (2015). Online software tools: InterPro (<https://www.ebi.ac.uk/interpro/>) and Pfam (<http://pfam.xfam.org/>) were employed for domain analysis of CstMYB1R1 protein. The 3D model of CstMYB1R1 was built using Swiss-Model online (<https://swissmodel.expasy.org/>). Amino acid alignment of CstMYB1R1 with orthologous MYB proteins was executed through the alignment tool, Muscle (<https://www.ebi.ac.uk/Tools/msa/muscle/>) and analyzed with the help of ESPript 3.0 online software (<http://espript.ibcp.fr/ESPrript/ESPrript/>). For constructing the Neighbor-Joining (NJ) phylogenetic tree, MEGA (Molecular Evolutionary Genetic Analysis) software version 11.0 was used with 1000 bootstrap replications (Tamura et al. 2013).

RNA extraction, cDNA synthesis, and quantitative RT-PCR analysis

For RNA isolation, 100 mg of flower tissue was ground using liquid nitrogen and RNA was isolated using RNeasy RNA isolation kit (Qiagen). RNA was further treated with DNase-I (Thermo Fisher Scientific) at $37\text{ }^{\circ}\text{C}$ and the purified RNA was subsequently used to synthesize cDNA through reverse transcription reaction with the help of RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific) following manufacturer's guidelines. Quantitative real-time PCR (qRT-PCR) was then performed using QuantStudio³ Real Time PCR system to analyze the relative gene expression. Geometric mean of C_t values of 18S rRNA and GAPDH genes as endogenous control were used to calculate results. The sequences of primers used for expression analysis are given in Table S1 in the supplementary file. For each sample, three biological replicates were used and the mean of three was given in the results.

Amplification of *CstMYB1R1* and its subcellular localization

The sequence of full-length *CstMYB1R1* was obtained from *Crocus* transcriptome database generated in house (Baba et al 2015). The forward and reverse primers were designed from this sequence. *CstMYB1R1* was amplified using cDNA as template; CstMYB1R1-F/R primers, and Taq polymerase (NEB). The amplified product was eluted from gel and purified using gel purification kit (Qiagen). The purified amplicon was cloned in pGEMT vector (Promega) and confirmed by sequencing.

For subcellular localization, the ORF of *CstMYB1R1* without stop codon was amplified, purified, and cloned in pEAQ-GFP vector in frame with GFP using primers (CstMYB1R1-GFP-F/R). Vector alone (pEAQ-GFP) and 35S::CstMYB1R1-GFP construct were transformed into *Agrobacterium tumefaciens* GV3101 cells and were further agro-infiltrated into onion epidermal cells as described in Bhat et al (2021) and Hussain et al (2022). The onion peels were then visualized under confocal microscope.

Transactivation assay

To examine the transactivation potential, the CDS of *CstMYB1R1* was cloned in frame with GAL4 DNA-binding domain of pGBKT7 (BD) vector. With the help of Yeastmaker Yeast Transformation System 2 (Takara, Japan), BD-CstMYB1R1 recombinant plasmid and pGBKT7 alone were transformed into Y187 yeast cells which were grown on L-tryptophan (SD-Trp) selective agar medium. Next, this auxotrophic medium was added with NaH_2PO_4 (3 mg/mL), $\text{Na}_2\text{HPO}_4\cdot 7\text{H}_2\text{O}$ (7 mg/mL), and bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal, 80 $\mu\text{g}/\text{mL}$). D-glucose (1.8%) was replaced with D-galactose (2%) and D-raffinose (1%), and streaked with transformed Y187 cells. The agar plates were incubated at $30\text{ }^{\circ}\text{C}$ till the appearance of blue coloration. Y187 cells transformed with pGBKT7 vector alone or co-transformed with pGADT7-T and pGBKT7-p53 plasmids were used as negative and positive controls, respectively.

For the quantitative assessment of β -galactosidase (β -gal) reporter enzyme activity, the transformed Y187 cells were cultured in liquid medium lacking L-tryptophan. Using ortho-nitrophenyl- β -galactoside (ONPG) as substrate, following equation was used to calculate the β -gal units according to the instructions of Yeast Protocols Handbook (Clontech):

$$\beta\text{-gal units} = \text{OD}_{420} \times 10^3 / (V \times t \times \text{OD}_{600}),$$

where OD_{420} and OD_{600} are absorbance values of the culture at 420 and 600 nm, respectively; V is volume in mL

(=0.5 mL, including concentration factor); and t is the time elapsed (min) to the production of yellow coloration.

Over-expression of *CstMYB1R1* in *C. sativus*

To transiently over-express *CstMYB1R1* in *Crocus* tepals, its CDS was cloned into pBI121 vector downstream of 35S *CaMV* promoter. pBI121 vector alone was used as negative control. The agro-infiltration protocol was followed as described in Bhat et al (2021). pBI121 control vector and pBI121-*CstMYB1R1* construct were transformed into *Agrobacterium tumefaciens* GV3101 cells and were subsequently transformed into the *Crocus* tepals through adaxial surfaces using syringe-driven agro-infiltration (Bhat et al 2021). The transformation was confirmed with the help of GUS staining assay and by measuring expression of *CstMYB1R1* using qRT-PCR. The flower tissues were harvested 72 h after infiltration, snap-frozen using liquid N₂, and stored at -80 °C till use. For each agro-infiltration experiment, a minimum of ten flowers were used.

Stable expression of *CstMYB1R1* in *Nicotiana benthamiana* and leaf disc assays

For stable transformation of *N. benthamiana*, plants were grown in growth chamber as mentioned above. The *Agrobacterium tumefaciens* strain GV3101 harboring pBI121-*MYB1R1* construct or pBI121 vector alone were cultured and *Nicotiana* infiltration medium was prepared as described in Bhat et al (2021); Hussain et al (2022). The agro-infiltration medium was agro-infiltrated into *N. benthamiana* leaves. Uniform sized leaf discs were cut from agro-infiltrated leaves, treated with cefotaxime (250 mg/ml) for 3 min. These discs were washed, dried, and used for callusing in MSG media containing 25 g/l sucrose, 1 mg/l BAP, 0.5 mg/l IBA and 1 X B5 vitamins and pH adjusted to 5.7. The callusing was followed by root and shoot induction and raising of whole plant. Shoot induction medium consisted of MS media with BAP (2 mg/l), NAA (0.2 mg/l), and calli were kept in this media for 4 weeks. The shoots were then transferred to root induction medium containing MS media with BAP (0.5 mg/ml), IAA (1 mg/ml) and kinetin (2 mg/ml) and grown for 4 weeks. The fully grown plants were then transferred to soil for hardening. Presence of transgene was confirmed by semi-quantitative PCR of *CstMYB1R1*. Five T_0 lines were generated which were subsequently grown till T_2 generation. The stress assays were performed using leaf discs from three (L1–L3) T_2 generation plants.

Disc assays were performed as previously described in Baba et al. (2017), Hussain et al (2022). Precisely, the leaf discs of equal diameter were excised from fully expanded leaves of 35S::*MYB1R1* *N. benthamiana* plants and vector-control-expressing plants with the help of a cork borer. The

leaf discs were subjected to various abiotic stress treatments. For UV stress, leaf discs were subjected to UV-B irradiation (1500 $\mu\text{J}/\text{m}^2$) for 6 h. Salt (200 mM NaCl), dehydration (200 mM Mannitol) and oxidative (100 μM MeV) stresses were given for 72 h at room temperature. The leaf discs were then harvested and used for the estimation of total chlorophyll and malondialdehyde (MDA) contents.

Determination of total flavonoids and anthocyanins

Total flavonoid content of tobacco leaf extracts was measured through colorimetric procedure as formulated by Chang et al. 2002. Total flavonoid content was quantified using the calibration curve and presented in terms of quercetin equivalents (mg of QE per gram of dry weight) ($R^2 = 0.998$).

Total anthocyanin content was measured as per Kang et al 2021. Approximately 100 mg tissue samples were crushed in liquid N₂ and anthocyanins were extracted using 1 mL extraction buffer (95% ethanol:1.5 M HCl=85:15, v/v) at room temperature in the dark for 12 h. Next, the absorbance readings were taken at 530, 620, and 650 nm wavelengths using a spectrophotometer (Thermo Fisher Scientific). The anthocyanin content was quantified by the equation:

Anthocyanin content = $\text{OD}_\lambda / \xi_\lambda \times V/m \times 10^6 \text{ nmol g}^{-1}$ fresh weight,

where V: volume; m: mass (in grams), $\text{OD}_\lambda = (A_{530} - A_{620}) - 0.1(A_{650} - A_{620})$, and $\xi_\lambda = 4.62 \times 10^4$

Determination of chlorophyll content and malondialdehyde (MDA) levels

To investigate stress-induced photosynthetic pigment degradation, total chlorophyll content of *N. benthamiana* leaf discs exposed to different stresses was determined in accordance with the method described previously (Baba et al. 2017). In brief, 0.5 g of leaf tissue was homogenized in 80% (w/v, 2 mL) cold acetone. The homogeneous suspension was then centrifuged at $3000 \times g$ for 10 min and the supernatant was collected. The absorbance for each sample was measured at 663 and 645 nm by the spectrophotometer using 80% (w/v) cold acetone as reference. The absorbance values of blank at respective wavelengths were subtracted from the corresponding absorbances of the sample and the total chlorophyll content was quantified as follows:

$$\text{Total Chl } (\mu\text{g/gFW}) = 0.020A_{663} + 0.00802A_{645},$$

where A_{663} and A_{645} are the absorbances at 663 and 645 nm, respectively.

The analysis of stress-mediated membrane damage was done by determining the malondialdehyde (MDA) content of the leaf samples as per the procedure formulated previously (Baba et al. 2017). Precisely, 0.5 g of leaf discs was

homogenized in 0.5 mL of 0.1% trichloroacetate (TCA) (w/v). The homogenates were then centrifuged for 15 min at $10,000\times g$. Next, 0.5 mL of the supernatant was harvested and mixed with 0.5% thiobarbituric acid (TIBA) (w/v), and the mixture was incubated for 40 min at 95 °C. Afterward, the samples were immediately cooled on ice and then centrifuged at $10,000\times g$ for 5 min. The supernatants were collected and their absorbance was then measured at 532 (A_{532}) and 600 (A_{600}) nm wavelengths. To exclude the non-specific turbidity, absorbance at 532 nm was subtracted with the absorbance at 600 nm. Finally, MDA content was calculated as:

$$\text{MDA content (nmol/g FW)} = (A_{532} - A_{600} \times V \times 1000) / (W \times 155),$$

where V = volume of the extract and W = sample fresh weight.

HPLC analysis

The extraction and HPLC-based quantification of anthocyanins from *Crocus* tepals collected at different diurnal stages and those transiently transformed with pBI121-MYB1R1 or pBI121 control vector was performed as per Peng et al. (2020). Briefly, 300 mg of freeze-dried *Crocus* tissue was homogenized with acidified methanol containing 0.1% HCl for 2.5 h at room temperature. Following centrifugation at $3000\times g$, the supernatant obtained was spin-dried and then resuspended in 20% methanol. The mixture was then passed through syringe-driven filter and further diluted with 20% methanol prior to HPLC analysis. The HPLC analysis was performed on Dionex UltiMate 3000 Series HPLC system (Thermo Fisher Scientific) fitted with photo-diode array at 520 nm and C18 column (Acclaim PA2, Thermo Fisher Scientific) at 35 °C. The solvent system used consisted of 100% of solvent A (0.1% HCOOH) ramped to 98% at 5 min, 85% A at 10 min and then 80% A at 20 min and 100% B (ACN + 0.1% HCOOH) at 35 min. Flow rate used was 0.5 mL/min for 30 min using 10 μ l of sample run. The HPLC standard delphinidin-3,5-diglucoside chloride was purchased from Millipore Sigma Company. The HPLC chromatograms are given in the Supplementary File as Fig. S1.

Yeast one hybrid (Y1H) assay

The *in vivo* protein–DNA interaction of CstMYB1R1 transcription factor with the promoter of *Crocus* LDOX gene was studied using the Y1H Gold yeast strain with the help of Matchmaker® Gold Yeast One-Hybrid Library Screening System in accordance with the manufacturer’s procedure (Clontech). First, core LDOX promoter was amplified de novo with gene-specific primers LDOX-GSP1 and LDOX-GSP2 (Table S1) using Universal Genome Walker™ 2.0

Kit (Clontech) as per the manufacturer’s recommendations. The PLACE online promoter analysis tool (<https://www.dna.affrc.go.jp/PLACE/?action=newplace>) was used to identify the MYB binding site(s) within the amplified LDOX promoter. The 100-bp promoter stretch carrying a putative MYB binding site was PCR amplified using LDOXpro forward and reverse primers (Table S1) and cloned into *HindIII* and *XhoI* sites of pAbAi yeast bait vector and transformed into Y1H yeast cells using Yeastmaker™ Yeast Transformation System 2 (Clontech) following the manufacturer’s instructions. Moreover, the MYB DNA-binding site within the selected stretch was mutated by replacing four consecutive nucleotides in the forward primer (Table S1) to generate mutant bait vector (LDOXproMut). On the other hand, *CstMYB1R1* CDS was cloned in frame with GAL4 into pGADT7 (AD) vector to generate the prey construct for Y1H assay. Afterward, the pGADT7-CstMYB1R1 prey construct or pGADT7 vector (negative control) was transformed into LDOXpro wild type (Wt) and mutant (Mut) Y1H bait cells. The synthetic defined (SD) medium deficient in L-uracil (-Ura) and L-leucine (-Leu) was used to screen the transformants. Next the bait Y1H cells were streaked on different concentrations of Aureobasidin A (AbA) and the minimal inhibitory concentration was recorded. To assess protein–DNA interaction, the bait strains transformed with prey constructs were cultured on selection medium containing 200 ng/mL AbA, the concentration marginally higher than least concentration (175 ng/mL) for inhibiting bait strains. In addition, the p53 bait strain Y1HGold [p53-AbAi] was generated by homologous integration of p53-AbAi vector and used as a positive control in Y1H assay.

Interaction between CstMYB1R1 and LDOX promoter using GUS reporter assay

The 0.5 Kb LDOX promoter amplicon was cloned into the linearized pBI101 binary vector at *HindIII/XmaI* sites, upstream of the beta-glucuronidase (GUS) gene. *CstMYB1R1* was cloned into pBI121 vector downstream of CaMV 35S promoter. pBI101-LDOX, 35S:CstMYB1R1, and control pBI101 were transformed into *Agrobacterium tumefaciens* GV3101 strain as described by Bhat et al (2021) and Hussain et al (2022). Interaction between LDOX promoter and CstMYB1R1 through quantitative assessment of GUS activity was performed as described in Yang et al (2000). pBI101, 35S:CstMYB1R1, and pBI101-LDOX were agro-infiltrated into onion epidermal cells alone or in combination. For GUS assay, onion peels from infiltration sites were collected and ground in 100 μ l of a GUS extraction buffer (50 mM sodium phosphate buffer at pH 7.0, 40 mM 2-mercaptoethanol, and 10 mM Na₂EDTA). This was followed by centrifugation at 14,000 rpm for 7 min at 4 °C. To initiate enzyme activity, 50 μ l of the supernatant was

added to a tube containing 450 µl of GUS extraction buffer and 1 mM 4-methylumbelliferyl-d-glucuronide (MUG). The GUS activity was determined by tracking the cleavage of the MUG substrate using spectrophotometer to quantify fluorescence with a 365 nm excitation wavelength and a 455 nm emission wavelength. Protein concentration was determined using Bradford reagent (Biorad, USA). For each construct, three biological replicates from independently transformed plants were used in each measurement.

Statistical analysis

Three replicates were used to perform each experiment. The final values were representative of mean ± standard deviation. GraphPad Prism 7.0 offline software was used for performing the statistical analysis. The paired two-tailed Student's *t* test was used to analyze the statistical difference

between two treatment groups. The *p* values < 0.05 were taken to represent statistically significant difference.

Results

CstMYB1R1 is a MYB-related (1R) type of MYB transcription factor

The in silico analysis showed that CstMYB1R1 protein carries a single conserved amino acid MYB repeat in its DNA-binding domain. CstMYB1R1 shares highest conservation with orthologous MYB plant transcription factors in only the DNA-binding domain, while the rest of its amino acid sequence shows significant variation (Fig. 1A). The CDS of *CstMYB1R1* consisted of 903 bp coding for a protein of 300 amino acids (Fig. 1B). The phylogenetic analysis revealed that CstMYB1R1 is evolutionary closer to a REVEILLE-8

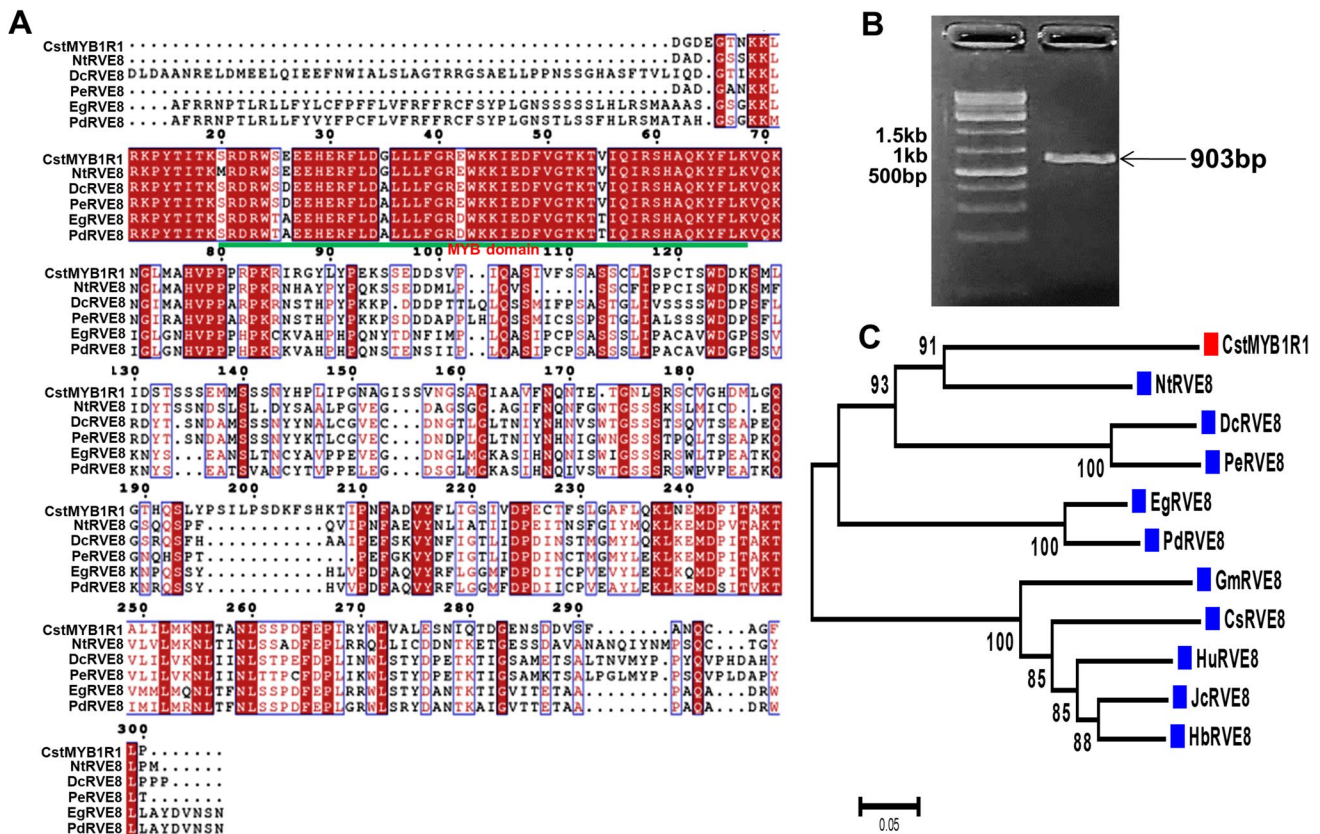


Fig. 1 Sequence analysis, amplification, and phylogeny of CstMYB1R1. **A** Sequence alignment **B** PCR amplification, and **C** phylogeny of CstMYB1R1 with its orthologous proteins. The amino acid sequences of MYB orthologs were obtained from NCBI database: NtRVE8 (AGO33167.1) [MYB transcription factor [*Narcissus tazetta* subsp. chinensis]], DcRVE8 (XP_020673865.1) [protein REVEILLE-8 [*Dendrobium catenatum*]], (PeRVE8) XP_020591224.1 [protein REVEILLE-8 [*Phalaenopsis equestris*]], EgRVE8 (XP_010920945.1) [protein REVEILLE-8 [*Elaeis guineen-*

sis]], PdRVE8 (XP_008792393.1) [protein REVEILLE-8 [*Phoenix dactylifera*]], JcRVE8 (XP_012082262.2) [protein REVEILLE-8 [*Jatropha curcas*]], HbRVE8 (XP_021666869.1) [protein REVEILLE-8-like [*Hevea brasiliensis*]], GmRVE8 (XP_003520640.1) [protein REVEILLE-8 [*Glycine max*]], CsRVE8 (ACH87169.1) [MYB transcription factor [*Camellia sinensis*]] and HuRVE8 (XP_021289952.1) [protein REVEILLE-8-like [*Herrania umbratica*]]

type of MYB-related transcription factor from *Narcissus tazetta* (NtRVE8) among the orthologous plant MYB transcription factors (Fig. 1C).

CstMYB1R1 localizes to nucleus and is transcriptionally active

For examining subcellular localization of CstMYB1R1, its CDS (without stop codon) was cloned into pEAQ-GFP in frame with GFP and transformed into onion epidermal cells by agro-infiltration and visualized under confocal microscope. The control vector was also agro-infiltrated into onion epidermal cells. We observed that the control vector was present throughout the cell, while CstMYB1R1-GFP was present only in nucleus (Fig. 2A). To investigate

the transcriptional activity of CstMYB1R1, it was cloned in pGBKT7 vector and transformed into yeast strain Y187. Y187 cells harboring BD-MYB1R1 construct, negative control (Y187 cells transformed with pGBKT7 vector alone), and positive control (Y187 cells co-transformed with pGBKT7-p53 and pGADT7-T) were grown on X-gal SD/-Trp. It was seen that Y187 cells harboring BD-CstMYB1R1 and positive control could develop blue color resulting from the breakdown of X-gal by the action of host reporter β -galactosidase (Fig. 2B). Moreover, by evaluating the host cell β -galactosidase activity using ONPG as substrate, it was confirmed that the Y187 cells transformed with BD-CstMYB1R1 exhibited significantly higher β -galactosidase enzyme activity in comparison to negative control cells, the activity being highest for the positive control cells (Fig. 2C).

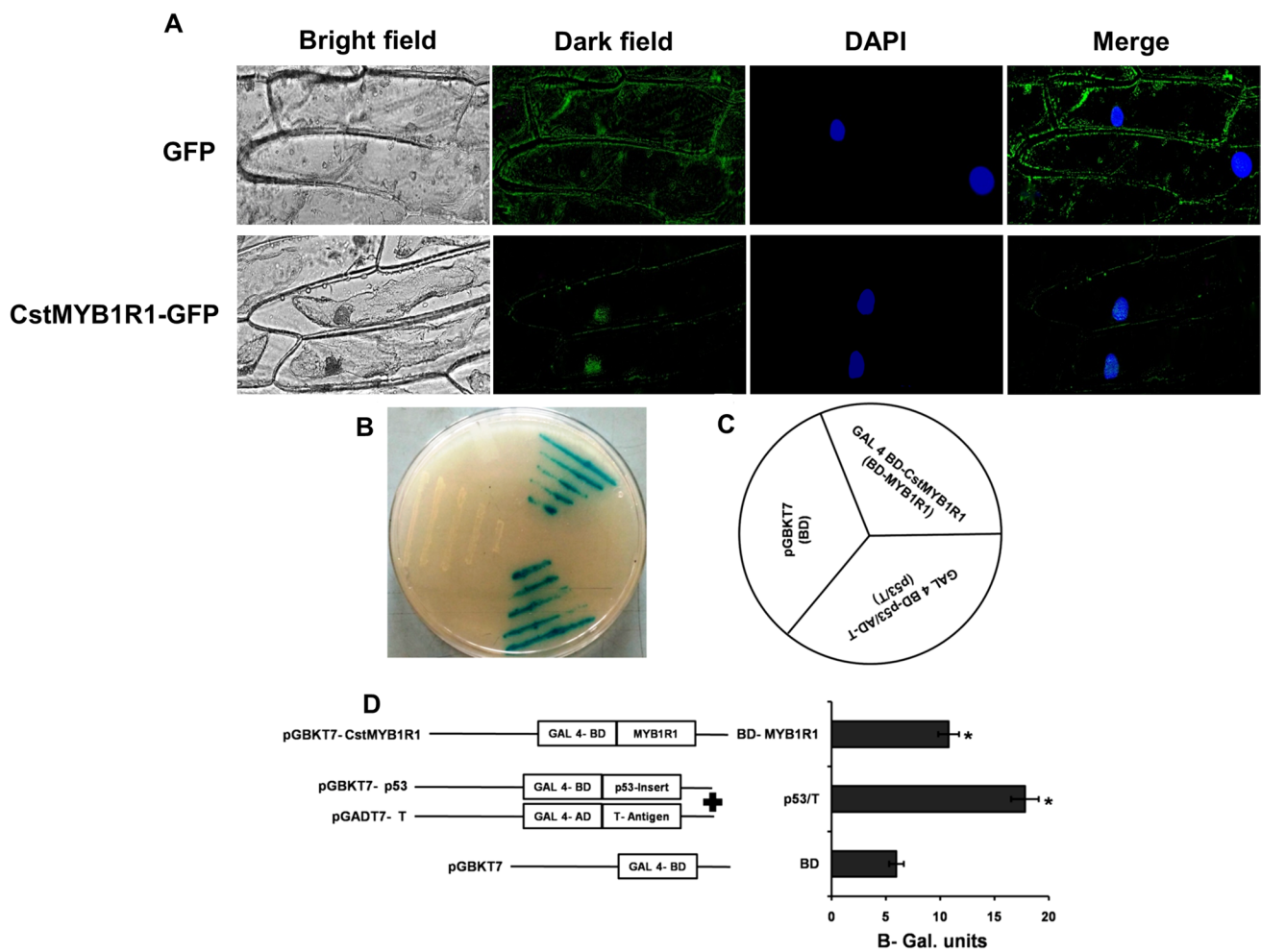


Fig. 2 Assessment of subcellular localization and transactivity analysis of CstMYB1R1. **A** subcellular localization of pEAQ and pEAQ-CstMYB1R1 transformed onion epidermal cells and DAPI counterstaining of the transfected cells, **B** *in vivo* transactivation assay of Y187 yeast cells harboring BD-CstMYB1R1, Y187 cells containing pGBKT7 vector alone as negative control, and Y187 cells co-transformed with pGBKT7-p53 and pGADT7-T plasmids as positive

control. The BD-CstMYB1R1 and positive control transformed cells produced blue color due to β -galactosidase reporter enzyme activation, **C** quantification of β -galactosidase activity with ONPG as substrate. The enzyme quantification was performed in triplicates and final values are representative of mean \pm standard deviation. The *p* values < 0.05 were indicative of statistically significant difference (*)

Taken together, the *CstMYB1R1* is localized to nucleus and is transcriptionally active.

CstMYB1R1 is upregulated in floral tissues of *C. sativus*

The qRT-PCR was used to determine the expression of *CstMYB1R1* in *Crocus* floral tissues including tepals, stamens, and stigmas using corm as reference. The results indicated that *CstMYB1R1* is significantly upregulated (p value < 0.05) in all the floral tissues (Fig. 3A). Tepals were shown to express the highest level of *CstMYB1R1* transcripts. The expression analysis was also performed at different *Crocus* flowering stages like pre-anthesis, anthesis, and post-anthesis. The expression of *CstMYB1R1* was shown to be highest at anthesis (Fig. 3B). The latter is considered to be the metabolically most active stage of flowering where floral tissues

reportedly exhibit the highest accumulation of secondary metabolites (Moraga et al 2009).

CstMYB1R1 expression is affected by hormone and abiotic stress treatments

To assess whether *CstMYB1R1* has any role in regulating the hormonal and stress signaling pathways, the *Crocus* plants were treated with plant growth regulators and different abiotic stresses, and qRT-PCR was performed to analyze the effect of corresponding treatments on *CstMYB1R1* expression. The results showed that *CstMYB1R1* expression was significantly upregulated (p value < 0.05) under ABA, GA_3 , and SA treatments (Fig. 3C). However, it showed significant downregulation (p value < 0.05) when the plants were treated with MeJA. Interestingly, the expression of *CstMYB1R1* increased markedly under different abiotic stress treatments like dehydration, salt, MeV, and UV (Fig. 3D).

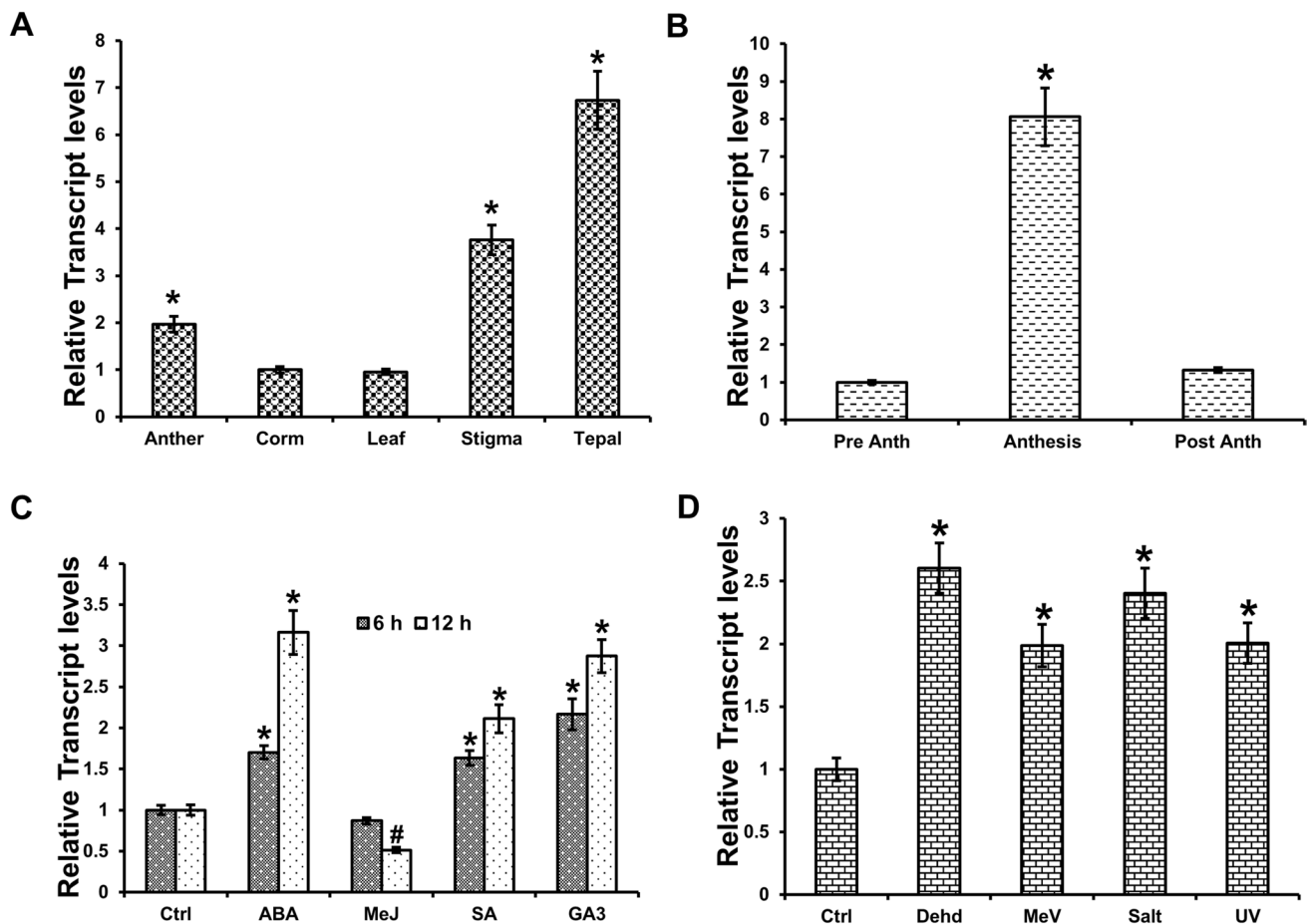


Fig. 3 Relative transcript levels of *CstMYB1R1* (A) in different *Crocus* tissues (B) at different stigma developmental stages (C) under hormonal and (D) abiotic stress treatments. Tissues were harvested after 6 and 12 h of hormonal treatments and 24 h after stress treatments except for UV where tissues were harvested after 2 h of con-

tinuous UV exposure. The expression analyses were performed with the help of qRT-PCR using three independent biological replicates. The final values are representative of mean \pm standard deviation. *indicates significant differences as compared to control conditions (Student's t test: $*p < 0.05$)

To prove that the stress treatments were successfully applied to *Crocus* plants, expression of few known stress response genes in these plants was checked by qRT-PCR. These genes showed upregulation in stressed plants as compared to control (Supplementary Fig. S1A). We also investigated the effect of these stresses on anthocyanin content and observed that most of the stresses enhanced anthocyanin content in *Crocus* plants (Supplementary Fig. S1B). The modulation of *CstMYB1R1* transcript levels under phyto-hormone and abiotic stress treatments, thus, suggests regulatory role of *CstMYB1R1* in corresponding signaling pathways.

***CstMYB1R1* exhibits diurnal expressional correlation with *LDOX/ANS* and anthocyanin accumulation in *Crocus* tepals**

CstMYB1R1 exhibited significant level of sequence similarity with REVEILLE-8 type MYB transcription factors which play role in the regulation of circadian rhythm (Gray et al. 2017). Besides, the REVEILLE-8 genes reportedly act as positive regulators of anthocyanin biosynthesis (Pérez-García et al 2015). To see if anthocyanins are

synthesized in circadian manner in *Crocus*, we quantified total anthocyanins and delphinidin content at different diurnal stages, viz., dawn, noon, dusk, and night. We observed higher concentration of anthocyanins (p value < 0.05) in *Crocus* tepals at dawn and dusk diurnal stages (Fig. 4A). The HPLC analysis of delphinidin-3,5-diglucoside showed a similar accumulation pattern (Fig. 4B). To investigate if *CstMYB1R1* has any role in regulating circadian-mediated anthocyanin biosynthesis, we checked the expression of *CstMYB1R1* along with anthocyanin pathway genes (*DFR*, *LDOX*, and *UFGT*) at different diurnal stages, viz., dawn, noon, dusk, and night. The results showed that *CstMYB1R1* transcript level peaked around dawn and dusk, while its expression reached the lowest at night (Fig. 4C). The anthocyanin pathway genes also exhibited variation in their expression patterns along diurnal clock. Among these, the expression of *LDOX* was seen to positively correlate with *CstMYB1R1* expression profile (Fig. 4C) suggesting that *LDOX* might be the possible transcriptional regulatory target of *CstMYB1R1*. The diurnal floral anthocyanin levels are, thus, positively regulated by *CstMYB1R1* in *C. sativus*.

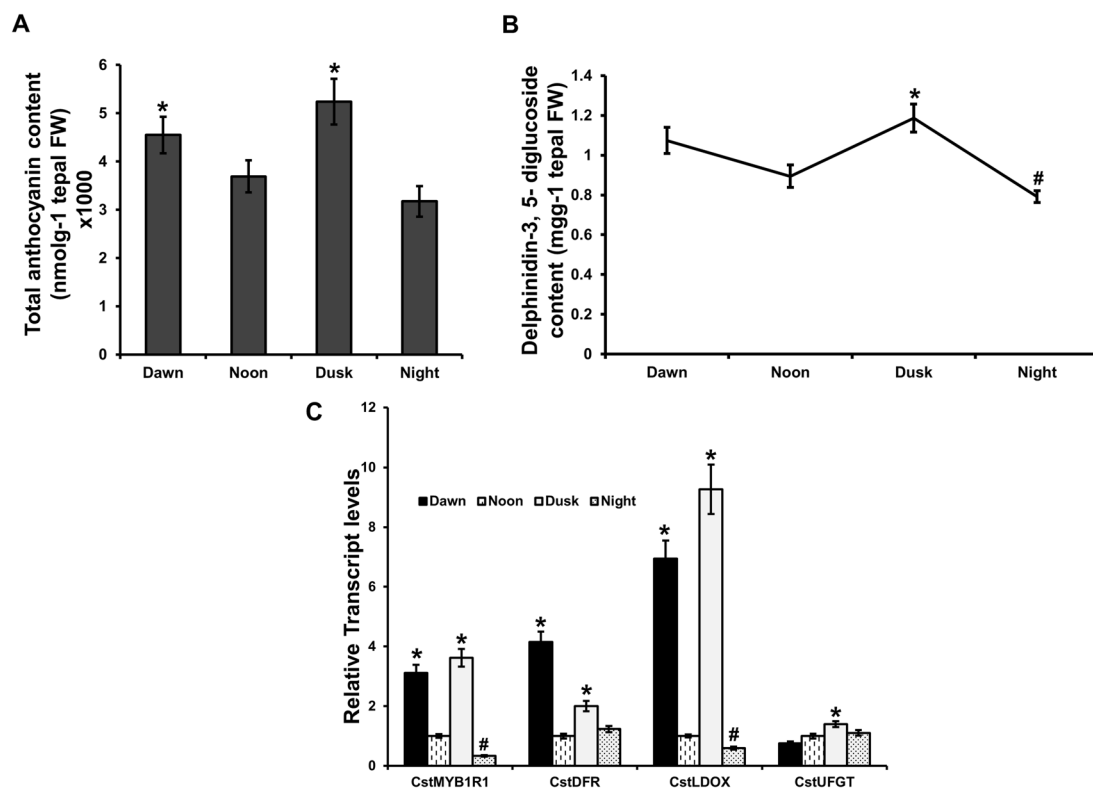


Fig. 4 Diurnal stage-specific metabolite profiling and gene expression analysis. **A** Total anthocyanin content. **B** HPLC analysis of delphinidin-3,5-diglucoside levels in *Crocus* tepals collected at different diurnal stages. **C** Relative transcript levels of *CstMYB1R1* and anthocyanin biosynthetic pathway genes (*DFR*, *LDOX*, and *UFGT*) at

different diurnal stages. The expression and metabolite analyses were performed using three independent biological replicates and the final values are representative of mean \pm standard deviation. Significant differences were calculated using Student's t test: * $p < 0.05$

CstMYB1R1 acts as an activator of anthocyanin biosynthesis in *C. sativus*

To confirm role of *CstMYB1R1* in regulating anthocyanin pathway in *Crocus*, the gene was transiently over-expressed in *Crocus* tepals. The transformation was standardized using control pBI121 vector and GUS assay, and further, the over-expression of *CstMYB1R1* in transformed tepals was confirmed using qRT-PCR (Fig. 5A). To know about the effect of *CstMYB1R1* on anthocyanins, we quantified anthocyanin content in plants over-expressing vector control and in those over-expressing *CstMYB1R1*. We observed higher total anthocyanin content in *CstMYB1R1*-over-expressing plants (Fig. 5B). Further, HPLC-based quantification of one of the dominant anthocyanins (delphinidin-3,5-diglucoside)

in 35S::*CstMYB1R1* plants showed significant increase as compared to plants transformed with vector control (Fig. 5C). The expression analysis of early (*CHS*, *CHI*, *C3H*, and *FLS*) and late anthocyanin biosynthetic genes (*DFR*, *LDOX*, and *UFGT*) was also performed in *Crocus* tepals transiently over-expressing *CstMYB1R1* using qRT-PCR. The results indicated that *CstMYB1R1* over-expression induced significant (p value < 0.05) upregulation of *CHS*, *CHI*, and *C3H* (early genes) and *DFR*, *LDOX*, and *UFGT* (late genes) (Fig. 5D). The *Crocus LDOX* gene, in particular, exhibited highest upregulation by *CstMYB1R1* over-expression. The results suggested that *CstMYB1R1* positively regulates the anthocyanin biosynthetic pathway while *LDOX* gene might possibly be the direct regulatory target of *CstMYB1R1*. Taken together, these results confirm

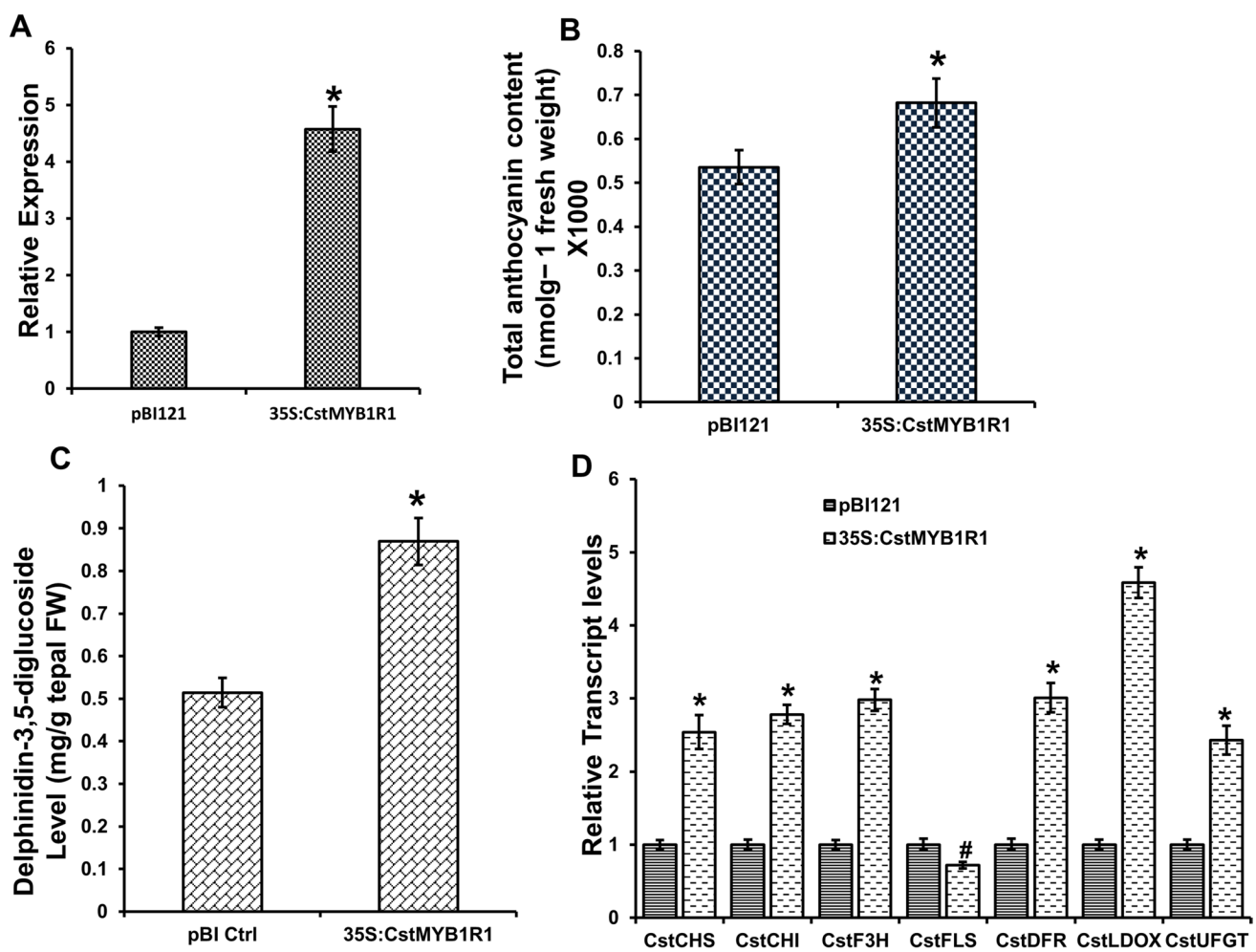


Fig. 5 Effect of *CstMYB1R1* over-expression on flavonoid and anthocyanin pathways. **A** Confirmation of transformation of *Crocus* tepals by GUS assay and qRT-PCR of *CstMYB1R1*. **B** Estimation of total anthocyanin content in *CstMYB1R1* over-expressing tepals with reference to corresponding control vector transformed tissues. **C** HPLC analysis of delphinidin-3,5-diglucoside levels in *CstMYB1R1* over-expressing tepals with reference to corresponding control vector

transformed tissues. **D** Expression analysis of flavonoid and anthocyanin biosynthetic pathway genes. The expression analyses were performed through qRT-PCR using three independent biological replicates. The final values are representative of mean \pm standard deviation. The p values < 0.05 were indicative of statistically significant difference (*, #)

that CstMYB1R1 acts as positive regulator of *Crocus* anthocyanin biosynthesis.

CstMYB1R1 directly interacts with the promoter of *LDOX* gene

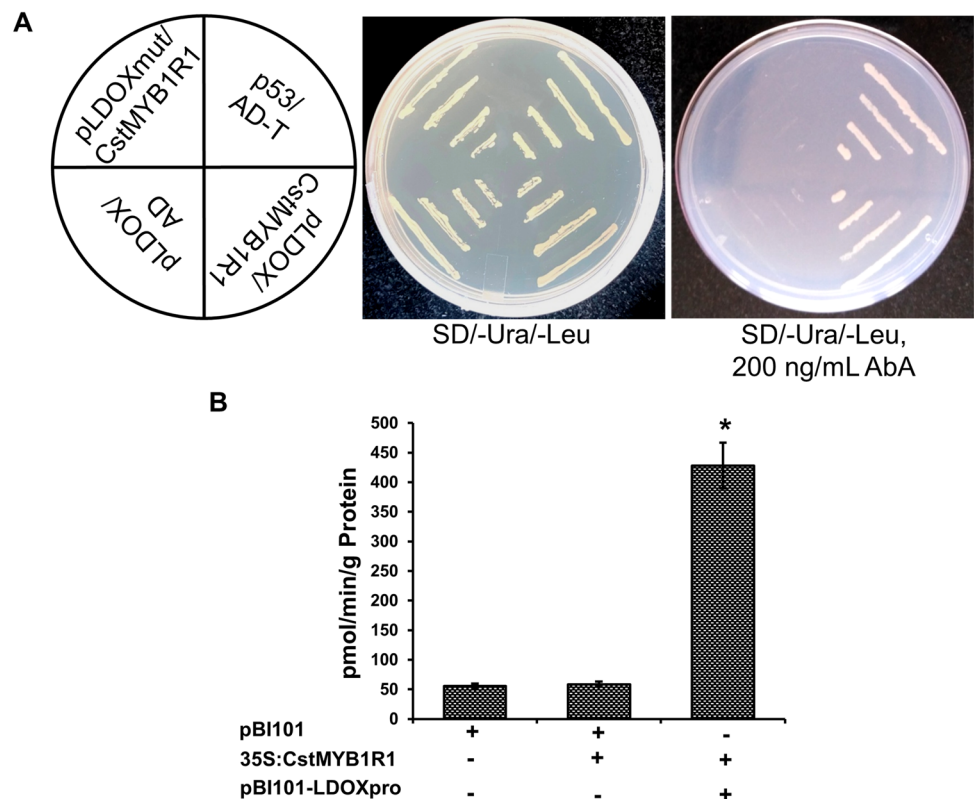
To confirm whether CstMYB1R1 regulates the *Crocus* anthocyanin biosynthesis directly by interacting with the promoter elements of pathway genes, Y1H assay was performed. Approximately, 0.5 kb core region of *LDOX* promoter was amplified through genome walking and analyzed for MYB binding sites. The *LDOX* promoter stretch carrying a MYB binding site was used for the interaction analysis. We found that co-transformation of yeast cells with *LDOX* promoter bait and CstMYB1R1 prey constructs led to the activation of reporter gene for Aureobasidin A (AbA) resistance and the host cells were able to grow on selection medium with AbA which confirmed the direct interaction of CstMYB1R1 with the MYB binding site within *LDOX* gene promoter (Fig. 6A). Moreover, the mutations in the MYB-binding site within the selected stretch of *LDOX* promoter were shown to abort this protein–DNA interaction (Fig. 6A). The results, thus, confirmed that CstMYB1R1 interacts with *LDOX* promoter in a sequence-specific manner and directly modulates its expression to regulate the anthocyanin biosynthesis in *C. sativus*.

To further confirm the binding of CstMYB1R1 to *LDOX* promoter, we performed GUS reporter assay. We already had CstMYB1R1 cloned in pBI121 vector downstream of CaMV 35S promoter. We then cloned 0.5 Kb long *LDOX* promoter stretch in pBI101 upstream of GUS gene. The two constructs (35S::CstMYB1R1 and pBI101-*LDOX*pro) were transformed into *Agrobacterium* GV3101 strain and subsequently co-infiltrated into onion epidermal cells. The two constructs were also agro-infiltrated individually into onion epidermal cells. This was followed by performing GUS assay of the infiltrated tissue samples. We observed many fold increase in GUS activity in cells co-infiltrated by 35S::CstMYB1R1 and pBI101-*LDOX*pro (Fig. 6B). This again confirmed binding of CstMYB1R1 to *LDOX* promoter.

CstMYB1R1 enhances flavonoid/anthocyanin levels and modulates stress response in *Nicotiana benthamiana*

To gain further understanding about the role of CstMYB1R1, the pBI-CstMYB1R1 was expressed in *Nicotiana benthamiana* plants and stable transgenic lines were developed. The expression of CstMYB1R1 transgene in *N. benthamiana* plants was confirmed by semi-quantitative PCR (Supplementary Fig. 3). To analyze the effect of CstMYB1R1 expression on secondary metabolite accumulation in 35S::CstMYB1R1 *Nicotiana* plants, we estimated total

Fig. 6 *In vivo* interaction assay of CstMYB1R1 with *LDOX* promoter. **A** Yeast one hybrid assay showing interaction of CstMYB1R1 protein with *LDOX* promoter (wild type–wt and mutant–mut) transformed in Y1H cells. SD/-Ura/-Leu medium carrying 200 ng/mL Aureobasidin A (AbA) was used for the screening of transformants with positive interactions. **B** GUS reporter assay depicting GUS activity in onion peels transiently transformed with pBI101 control vector, 35S::CstMYB1R1, and pBI101-*LDOX*pro individually and in combination. Interaction between CstMYB1R1 and *LDOX* promoter increases the GUS activity many fold. For each transformation experiment, three replicates were used



flavonoid and anthocyanin contents in vector control and three 35S::CstMYB1R1 *N. benthamiana* lines. The flavonoid and anthocyanin contents were significantly higher (p value < 0.05) in 35S::CstMYB1R1 *Nicotiana* plants (Fig. 7A and B). To investigate the role of CstMYB1R1 in stress response, the leaf discs from 35S::CstMYB1R1 *N. benthamiana* lines and control plants were subjected to different stress treatments and their chlorophyll and MDA contents were determined. It was observed that the chlorophyll content of leaf discs collected from 35S::CstMYB1R1 plants was significantly higher (p value < 0.05) than the respective discs from vector control plants (Fig. 7C). Conversely, leaf discs from 35S::CstMYB1R1 plants exhibited significantly lower (p value < 0.05) MDA content than leaf discs from vector control plants (Fig. 7D). Higher chlorophyll and lower MDA levels indicate that 35S::CstMYB1R1 plants are more

tolerant to stresses. To further confirm this, we determined the expression of a few stress responsive marker genes in vector control and 35S::CstMYB1R1 plants. We observed significant upregulation of these genes in 35S::CstMYB1R1 plants. This again confirmed the role of CstMYB1R1 in stress tolerance which might have resulted from higher flavonoid/anthocyanin accumulation in *CstMYB1R1* over-expressing plants.

Discussion

Plant MYB transcription factors perform a wide range of functions including regulation of secondary metabolism and mediating plant stress response (Vimolmangkanet al. 2013; Ambawatet al. 2013; Schwinn et al. 2016; Mmadi et al.

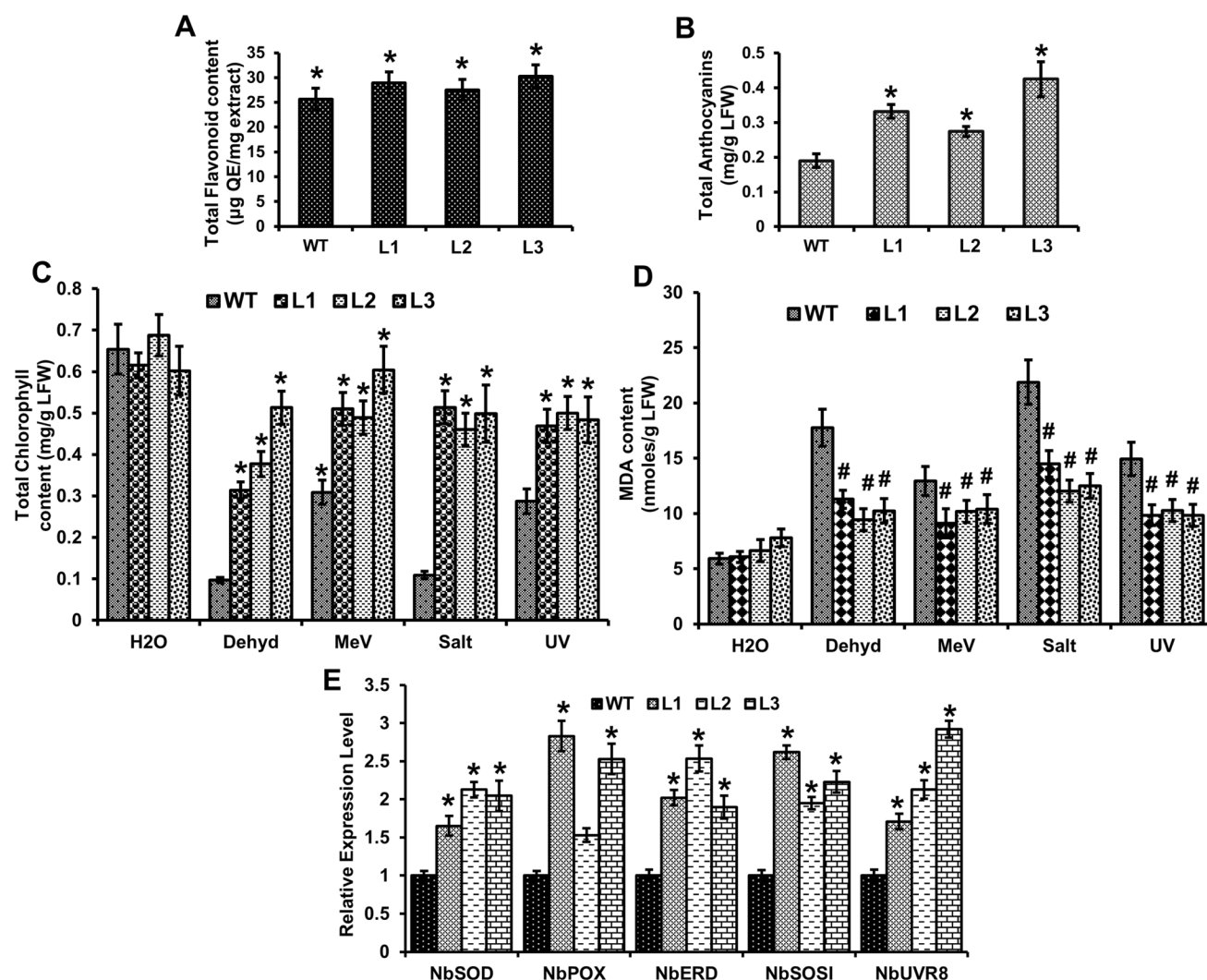


Fig. 7 Estimation of secondary metabolites, chlorophyll, and MDA content in vector control and CstMYB1R1 over-expressing *N. benthamiana* plants under various stress conditions. The estimation

was carried out using three replicates and results are presented as mean \pm standard deviation. The p values < 0.05 were indicative of statistically significant difference (*, #)

2017; Chen et al. 2019c). Different studies have proved that MYB transcription factors regulate biosynthesis of flavonoids/anthocyanins as exemplified by the discovery of first plant MYB transcription factor, C1 from maize (Paz-Ares et al. 1987). MYB transcription factors coordinate multiple cues to activate anthocyanin biosynthetic pathway in plants by enhancing expression of pathway genes at multiple enzymatic steps (Niu et al. 2010). Number of studies have implicated that MYB transcription factors act as potential activators of plant anthocyanin biosynthetic pathway. The *Arabidopsis* MYB75/PAP1 (PRODUCTION OF ANTHOCYANIN PIGMENT 1) transcription factor is well recognized for its role in regulating the anthocyanin accumulation (Zuluaga et al. 2008). The transgenic tomato plants expressing MYB75/PAP1 possessed significantly higher anthocyanin content in stem, leaves, flowers, and fruits when grown under normal environmental conditions. Moreover, the over-expression of *Arabidopsis* MYB113 or MYB114 led to higher anthocyanin accumulation as demonstrated by Zhao (2007).

In plants, the physiological and metabolic processes are sustained in a synchronized manner along the 24 h rhythmic diurnal day/night cycles (Levi and Schibler 2007). During recent times, regulation of this rhythmic behavior has been worked out to a great extent. There is an increasing support that internal and external cues feed stress signals to molecular clock to modulate the circadian rhythms and enable host plants to defend themselves against the detrimental effects of abiotic/biotic stresses. The plants respond by modulating their rhythmic behavior at cellular, physiological, and molecular levels to maintain the synchronized state with the changing environmental conditions (Xu et al. 2022). Interaction of environmental and genetic factors in shaping the plant secondary metabolism in diurnal rhythmic fashion is poorly understood. Nevertheless, REVEILLE-8 (RVE8) transcription factor has been shown to play a key regulatory role in controlling the anthocyanin biosynthesis along the diurnal clock in *Arabidopsis* (Pérez García et al., 2015). Our laboratory has been working on the role of MYB genes in regulating secondary metabolism in *Crocus*. Toward this, we have identified a few MYB genes which regulate apocarotenoid biosynthesis in *Crocus* (Bhat et al 2021; Hussain et al 2022). We also wanted to expand our understanding on role of MYB genes in *Crocus*. So we started exploring the role of these genes in regulating anthocyanin biosynthesis in *Crocus*. Since anthocyanins are accumulated mainly in tepals, we looked at the expression profile of MYB genes and shortlisted those which were upregulated in tepals. One of the tepal upregulated genes was *CstMYB1R1* and it clustered with plant *RVE8* genes which are known to be the regulators of anthocyanin biosynthesis (Bhat et al. 2021). Therefore, we selected *CstMYB1R1* for further characterization. The gene was localized in nucleus and was transcriptionally active

(Fig. 2). It was also induced in response to various stress and hormone treatments (Fig. 3C, D). Here we observed that in case of hormones, *CstMYB1R1* showed differential expression. It was upregulated in response to most of the hormones except JA. We know that hormone and stress signaling pathways are interconnected in complex ways. Different genes might behave in different ways in response to various signals in different plants and in different tissue types (Colebrook et al. 2014).

An interesting feature about secondary metabolite production in *Crocus* is that it accumulates different specialized metabolites in different floral tissues. Stigmas are rich in apocarotenoids and biosynthesis of these compounds peaks during night owing to higher *carotenoid cleavage dioxygenase 2* (*CsCCD2*) and *lycopene- β -cyclase 2a* (*CsLycB2a*) transcription at lower temperatures and under darkness (Ahrazem et al. 2016). Anthocyanin accumulation is comparatively higher in tepals (Goupy et al. 2013). To understand if anthocyanins are also accumulated in diurnal rhythmic manner, we estimated total anthocyanins and delphinidin diglucoside, a major anthocyanin in *Crocus*, at dawn, noon, dusk, and night time points. We observed that anthocyanins were highest at dusk and lowest during night (Fig. 4A and B). Many reports have shown that anthocyanin biosynthesis decreases during night/darkness while it is induced by light exposure, in particular the blue and red lights (Xu et al. 2014; Liu et al. 2015). However, diurnal changes in anthocyanins in *Crocus* have not been studied yet. Current study presents the first such report on *Crocus*. Our next objective was to see if *CstMYB1R1* regulates anthocyanins as per diurnal changes. Expression analysis showed that *CstMYB1R1* exhibited diurnal expression peaks at dawn and dusk and its expression pattern correlated with the diurnal expression profile of *Crocus ANS/LDOX* gene (Fig. 4C). The positive regulatory effect of *CstMYB1R1* expression on anthocyanin accumulation was confirmed by its transient over-expression study in *Crocus*. *CstMYB1R1* over-expression was confirmed by GUS assay as well as by quantifying *CstMYB1R1* expression using qRT-PCR (Fig. 5A). The *Crocus* tepals which transiently over-expressed *CstMYB1R1* showed enhanced total anthocyanin and delphinidin content (Fig. 5B, C). These results were further confirmed by measuring expression of pathway genes. Most of the pathway genes were upregulated in plants over-expressing *CstMYB1R1* (Fig. 5D). Among the pathway genes, *LDOX* showed highest expression. This prompted us to see if *CstMYB1R1* protein binds to *LDOX* promoter. We amplified the promoter using genome walking and its PLACE analysis showed the presence of MYB binding elements. Y1H and GUS reporter assay confirmed that *CstMYB1R1* binds to *LDOX* promoter and drives its expression (Fig. 6).

Since *CstMYB1R1* was induced in response to abiotic stresses and hormones (Fig. 3), we were inquisitive

to know if this gene plays any role in stress tolerance. For this, *CstMYB1R1* was expressed in *N. benthamiana* and stable transgenic lines were raised. Further, to assess these transgenic plants for stress tolerance, we performed leaf disc stress assays. Here we present data on three T₂ transgenic *N. benthamiana* lines. It is further important to mention that we performed stress assays in *Nicotiana* and not in *Crocus* because stable transformation protocol for *Crocus* is not available and transiently transformed *Crocus* tepals would not have been a good choice for stress assays. Under stress conditions, chlorophyll starts to degrade and membrane lipid per-oxidation occurs and these processes are used as makers of stresses (Baba et al 2017). Therefore, we measured chlorophyll and malondialdehyde (MDA) content in wild type and 35S::*CstMYB1R1* plants. We observed that 35S::*CstMYB1R1* plants had higher chlorophyll and lower MDA content when grown under various stress conditions which was indicative of the fact that 35S::*CstMYB1R1* plants are more tolerant to stresses. To investigate if there is any correlation between stress tolerance and anthocyanin content, we measured total flavonoid and anthocyanin content in wild type and 35S::*CstMYB1R1* plants and we observed significant increase in both these metabolites (Fig. 7). Flavonoids and anthocyanins act as antioxidant molecules and have potential role in free-radical scavenging (Ali et al. 2014; Muniyandi et al. 2019). These inferences reveal that enhanced stress tolerance in transgenic *Nicotiana* plants expressing *CstMYB1R1* might have resulted via higher flavonoid/anthocyanin accumulation which in turn act as ROS scavengers (Xu et al. 2018). There are many reports where abiotic stresses tolerance has been achieved by modulating anthocyanin accumulation (Li et al 2017). Increase in anthocyanin accumulation might, thus, be seen as one of the promising strategies for abiotic stress tolerance in plants and as a versatile approach of crop improvement (Sun et al. 2021).

Summing up, the present study revealed that *CstMYB1R1* positively controls the anthocyanin biosynthesis in *C. sativus* and modulates the accumulation of *Crocus* anthocyanins in diurnal rhythmic mode by regulating the pathway gene expression directly or indirectly. Further, it also enhances stress tolerance in transformed *Nicotiana* plants.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00299-023-03082-x>.

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Author contributions The current study was conducted under the supervision of NA. She along with ZYB wrote the manuscript. ZYB

and JAM carried out the experimentation and performed their analyses. AKY and DS performed HPLC analysis.

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Data availability The sequence of *CstMYB1R1* gene and *CstLDOX* promoter sequence used in this study have been submitted to NCBI Gene Bank through BankIt sequence submission tool under the accession numbers OQ436455 and OQ436454, respectively. All other data is given in manuscript and supplementary material.

Declarations

Conflict of interest The authors declare no conflict of interest.

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