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Expression Analysis and Interaction Protein Screening of CRY1 in Strawberry

Yuyun Ye [1], Ruiling Li [2], Wenchao Pu [1], Yunting Zhang [1,3], Leiyu Jiang [1], Hao Li [1], Yongqiang Liu [1], Yuntian Ye [1], Maolan Yue [1], Yuanxiu Lin [1,3], Qing Chen [1], Yong Zhang [1], Ya Luo [1], Mengyao Li [1], Xiaorong Wang [1,3] and Haoru Tang [1,3,*]

[1] College of Horticulture, Sichuan Agricultural University, Chengdu 611134, China; yyycd92@163.com (Y.Y.); pwc8900855@163.com (W.P.); asyunting@gmail.com (Y.Z.); jiangsicau@163.com (L.J.); 18892893862@163.com (H.L.); liuyq0129@163.com (Y.L.); yeyuntian@sicau.edu.cn (Y.Y.); maolanyue@outlook.com (M.Y.); linyx@sicau.edu.cn (Y.L.); supnovel@sicau.edu.cn (Q.C.); zhyong@sicau.edu.cn (Y.Z.); luoya945@163.com (Y.L.); limy@sicau.edu.cn (M.L.); wangxr@sicau.edu.cn (X.W.)
[2] Department of Agricultural, Forest and Food Science, University of Turin, 10095 Turin, Italy; ruiling.li@unito.it
[3] Institute of Pomology and Olericulture, Sichuan Agricultural University, Chengdu 611134, China
* Correspondence: htang@sicau.edu.cn

Abstract: Cryptochrome 1 (CRY1), a main blue light receptor protein, plays a significant role in several biological processes. However, the expression patterns and function of *CRY1* in strawberry have not been identified. Here, the expression profile of *CRY1* in different tissues and developmental stages of strawberry fruit, and expression patterns response to abiotic stresses (low temperature, salt and drought) were analyzed. Its subcellular localization, interaction proteins and heterologous overexpression in tobacco were also investigated. The results showed that *CRY1* was mainly expressed in leaves and fruits with an expression peak at the initial red stage in strawberry fruit. Abiotic stresses could significantly induce the expression of *CRY1*. The CRY1 protein was located in both nucleus and cytoplasm. Five proteins (CSN5a-like, JAZ5, eIF3G. NF-YC9, and NDUFB9) interacting with CRY1 were discovered. Genes related flowering times, such as *HY5* and *CO*, in three overexpressed *FaCRY1* tobacco lines, were significantly upregulated. Taken together, our results suggested CRY1 have a broad role in biological processes in strawberry.

Keywords: strawberry; CRY1; blue light receptor; heterologous expression; abiotic stress; flowering time; photomorphogenesis

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1. Introduction

The strawberry (*Fragaria* × *ananassa*) is an important economic fruit crop world-wide, which has rich nutrition and health-care functions [1]. Breeders have long been working in increasing the yield and quality of strawberry fruit [2,3], which were constrained by numerous factors; For this reason, photomorphogenesis [4–6], flowering time regulation [6,7], and adaptability to various stresses [8,9] of strawberry were also the focus of researchers.

CRY1 (Cryptochrome 1), as one of the main blue light receptors, senses blue light and transmits signals, which plays a key role in multiple biological processes in plants. In *Arabidopsis*, CRY1 showed the function of inhibiting hypocotyl elongation; the latest research showed that the nuclear CRY1, but not cytoplasmic CRY1, mediates blue light inhibition of hypocotyl elongation [10]. AtCRYs directly interacted with E3 ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) and inhibited the degradation of downstream ELONGATED HYPOCOTYL 5 (HY5) and CONSTANS (CO) protein, mainly resulting in positive regulation of photomorphogenesis and flowering [11,12]. The latest researches showed that AtCRY1 promotes H2A.Z deposition to regulate HY5 target gene expression and photomor phogenesis in blue light through the enhancement of both SWR1 complex activity and HY5 recruitment of SWR1 complex to HY5 target loci, which was

likely mediated by interactions of CRY1 with SWC6 and ARP6, and CRY1 stabilization of HY5, respectively [13]. AtCRY1 undergoes COP1 and LRBs-dependent degradation in response to high blue light [14]. In addition, CRY1 had been identified in responding to multiple abiotic stresses, such as low temperature [15], drought [16,17], and salt stress [17–19]. Impaired response of *cry1* mutants reflected participation of these photoreceptors in acquiring freezing tolerance in *Arabidopsis* [15]. Meanwhile, *Arabidopsis cry1cry2* double mutants, which lack CRY proteins, showed higher drought tolerance compared to controls, while the opposite was found in plants overexpressing *CRY1* gene due to the excessive water loss through stomata [20]. In rice, suppression of *CRY1b* decreases both melatonin and expression of brassinosteroid biosynthetic genes resulting in salt tolerance [18]. For strawberry researchers and breeders, application of varieties with stronger tolerance to various abiotic stresses holds a great potential in production.

In recent years, several interacting proteins of CRY1 involving various biological processes had been identified, such as PIFs [21], BES1 [22], SWC6, ARP6 [13], and GID1 and DELLA proteins [23,24], which were involved in plant development and hormone signal regulation. Even in *Arabidopsis*, the protein interacting with CRY1 identified in previous studies could only reveal a small part of mechanism. CRY1 was becoming more and more important as the intersection of light, hormone and stress signal pathway. However, little was known about the role of CRY1 in photomorphogenesis, stress response and flowering time regulation in strawberry.

In this study, we found that *CRY1* was mainly expressed in functional leaves and fruits of strawberry. With the highest expression level in fruits at the initial red stage; Low temperature, drought and salt stress could all significantly induce the expression of *CRY1* in strawberry. The CRY1 protein was localized in cytoplasm and nucleus. Five interacting proteins (CSN5a-like, JAZ5, NDUFB9, eIF3G, NF-YC9) of CRY1 were identified through yeast two-hybrid library screening. Three independent tobacco mutants with heterologous overexpression of strawberry CRY1 were obtained. The qRT-PCR of mutants showed that *CRY1* can promote the expression of downstream transcription factor *HY5* and flowering gene *CO*. These results will help to further improve the mechanism of CRY1 in horticultural crops. All of the efforts were carried out to have a better understanding of the role of CRY1 in the various biological processes in strawberry.

2. Materials and Methods

2.1. Plant Materials

The octoploid cultivated strawberry 'Benihoppe' (*Fragaria* × *ananassa* Duch.), diploid strawberry 'Ruegen' (*Fragaria vesca*), and tobacco (*Nicotiana benthamiana*) were cultured in the plant tissue culture room and greenhouse under long day condition (16 h of light, 5000 lx, 8 h of darkness, 22 °C. Plants were grown in plastic basin with a mixture of peat soil, perlite and vermiculite (Volume ratio = 4/1/1), regular irragation, fertilization with compound fertilizer solution. In vitro plants were cultured in 1/2 MS solid medium. The plants were cultured in 1/2 MS solid medium for 40 d before transplant.

2.2. Gene Cloning, Sequence Analysis and Phylogenetic Tree Construction

The full length of coding sequences (CDS) of *FaCRY1* were cloned from octoploid cultivated strawberry 'Benihoppe' with primers (Table S1) designed referring to its homologs (FvCRY1, XM_011465426.1; NCBI). The sequencing results were spliced by software CLC Genomics Workbench (version 3.6.1, CLC Bio, Midtjylland, Denmark) and compared with FvCRY1 by DNAMAN (version 8.0, LynnonBiosoft, San Ramon, CA, USA). Download the sequences of the direct homologous genes of strawberry *CRY1* in other plants from NCBI (https://www.ncbi.nlm.nih.gov/, accessed on 26 November 2020) databases. A phylogenetic tree was constructed using Mega (version 7.0, Sudhir Kumar, Glen Stecher and Koichiro Tamura, Philadelphia, PA, USA) based on amino acid sequences. Neighbor-joining phylogenetic was used for phylogenetic tree analysis. Both bootstrap test and approximate likelihood ratio test were set as 1000 times. The conserved domains of FaCRY1 protein

were predicted by NCBI-CDD (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, accessed on 6 June 2020).

The cis-acting elements in the promoter region were predicted with Plant-Care (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 23 December 2019).

2.3. The Expression Pattern of CRY1 Analyzed with qRT-PCR

Different tissues (roots, stems, young leaves, function leaves, flowers, mature full red fruit and runner) of 'Benihoppe' were collected from the plants in soil; They were used as materials for tissue-specific expression profile analysis. Meanwhile, different development stages of fruits: little green (LG, seven days after fruit set), big green (BG, 15 days after fruit set), white (W), turn red (TR,1/4 red), half red (HR, 1/2 red) and mature full red (FR) [7,25]; They were collected as materials for fruits development periods expression profile analysis. All tissues were set up with three biological replicates, all replicates were taken from plants that cultured under the same conditions. Each biological replicate contained at least six samples, and three to four samples were collected from each plant.

The in vitro plants of diploid strawberry 'Ruegen' were put into the artificial climate chamber at 4 °C for low temperature treatment [26,27], the relative humidity was 75%. Plants grown at 25 °C were used as the control. The leaf tissues were sampled at 0 h, 3 h, 6 h, 9 h and 12 h interval respectively. As for drought and salt stress treatment, the tissue culture seedlings of diploid strawberry 'Ruegen' were put into Hogland nutrient solution. After acclimatization for four days, plants were divided into three groups, and transferred into either 10% PEG-6000 + Hogland solution, 200 mm NaCl + Hogland solution or Hogland solution respectively to mimic drought stress [28,29], salt stress [29,30], or regular grown condition. The leaf and root tissues were sampled at 0 h, 3 h, 6 h, 12 h, and 24 h interval respectively.

The total RNA of all samples were extracted with a modified CTAB method [31]. The quality of RNA was evaluated by electrophoresis on a 1% agarose gel and scanned using a NanoDrop spectrophotometer [7]. A reverse transcription PCR reaction was conducted with Prime-Script^{TM} RT Reagent Kit with gDNA Eraser (Takara, Dalian, China) (Confirmed: Takara is a Japanese company. The kit we used was produced by its subordinate company in Dalian, China) using 1 μg total RNA, following the manufacturer's protocol. The cDNA was used as the template in sequential 20 μL qRT-PCR reaction system as the template on the basis of a SYBR Premix ExTaqTM Kit (Takara, Dalian, China).

The relative gene expression was determined by qRT-PCR. The qRT-PCRs were performed on the CFX96 real-time PCR system (Bio-Rad, CFX connect). Each reaction was performed with three technical replicates. The calculation method for qRT-PCR was $2^{-\Delta\Delta CT}$. The *Actin* was used as housekeeping gene for the calibration. IBM SPSS Statistics (Version 23) was used for one-way ANOVA test, Duncan test and multiple comparison ($p < 0.05$). Origin (Version 9) was used for drafting Figures. Primers were listed in Table S1.

2.4. Subcellular Localization Analysis

The CDS of *FaCRY1* was amplified and homologous recombinated into a plasmid vector (pYTSL-16), which was modified from pMDC83-35S and pSITE-2NB [7]. The *FaCRY1* gene was inserted frame with the *GFP* (Green fluorescent protein) gene on the vector. The fusion protein contained FaCRY1 and GFP (FaCRY1::GFP) was driven by the 35S promoter. The HY5-mCherry vector driven by the same 35S promoter was used as a nucleus maker [32]. The plasmid was further transformed into Agrobacterium tumefaciens strain GV3101. The plasmids were transiently expressed in the epidermal cells of tobacco (*Nicotiana benthamiana*) leaves as previously described [7,33]. These two vectors were transiently co-expressed in tobacco leaf epidermal cells. All of the fluorescence signals of the samples were detected by a confocal laser scanning microscopy system (FV3000 Olympus, Tokyo, Japan).

2.5. Yeast Two-Hybrid Screening Assay

The yeast two-hybrid cDNA library was constructed based on pGADT7 was entrusted to Shanghai Ouyi Biomedical Technology Co., Ltd., Shanghai, China. Yeast two-hybrid screening assay and the validation of potential interacting proteins analysis was conducted according to the manual of consulting instructions of Matchmaker™ Gold Yeast Two-Hybrid Screening Systems (Shanghai Yubo Biotechnology Co., Ltd., Shanghai, China). The SD/-Trp and SD/-Trp/-His/-Ade culture medium were used for auto-activation and toxicity test of bait plasmid.

Referring to the method in the previous studies [34,35], the sequences of CRY1 were divided into segments in order to find the region, which lacks auto-activation. A total of 5 truncated bait plasmids were constructed respectively: CRY1-BD-2 (amino acids 1 to 507), CRY1-BD-3 (amino acids 1 to 563), CRY1-BD-4 (amino acids 88 to 646), CRY1-BD-5 (amino acids 174 to 646) and CRY1-BD-6 (amino acids 284 to 646).

About 260 monoclonal yeast strains screened by SD/-Leu/-Trp/-His/-Ade/X-α-Gal/AbA (QDO/X/A) culture medium were sequenced. The plasmid containing 68 gene fragments was extracted and checked by rotary experiments. Ten genes were selected, cloned and inserted into pGADT7 vector for the second rotary experiments. Primers were listed in Table S1.

2.6. Agrobacterium-Mediated Heterologous Expression of the FaCRY1 in Tobacco

A pCambia2301::d35S-FaCRY1-NOS vector, carrying the *Kanamycin* and *GUS* gene for later selection, was constructed for heterologous expression of the *FaCRY1* in tobacco (*Nicotiana benthamiana*). Tobacco leaf disk was transformed with *A. tumefaciens* strain GV3101 according to the reported protocols [36], which carried out with minor modifications. The obtained plantlets were selected using 100 mg/L Kanamycin as antibiotic. After bacteriostatic culture, the explants were cultured in the medium containing 100 mg/L Kanamycin, 250 mg/L carboxybenzyl, 250 mg/L Timentin, 6-BA 2.0 mg/L + NAA 0.01 mg/L.

The transgenic lines were identified with the GUS histochemical staining. Genomic DNA were extracted from the plantlets. Two independently confirmed transformants were further used to detect the *Kanamycin* gene integration using PCR to validate insertion of the T-DNA region. The young leaves from tobacco were selected as samples. PCR amplicons were detected in a 1% agarose gel (Figure S1). The qRT-PCR was used to detect the expression level of relative genes. Primers were listed in Table S1.

3. Results

3.1. Gene Cloning, Protein Property and Phylogenetic Analysis of FaCRY1

The full length of coding sequences of *FaCRY1* were cloned with primers (Table S1). The analysis of conserved domain showed that CRY1 protein contained two conserved domains: the N-terminal PHR photolyase domain (contain MTHF region and FAD region) and the C-terminal CCE domain (Figure 1a).

The phylogenetic tree showed that FaCRY1 had a close relationship with FvCRY1, the similarity in the amino acid sequence of FaCRY1 and FvCRY1 was 99.85%. FaCRY1 clustered with homologous proteins from other Rosaceae species, and the CRY1 from the octoploid cultivated strawberry (FaCRY1) was distantly related to the *Arabidopsis* (AtCRY1) and tomato (*Solanum lycopersicum*, SlCRY1).

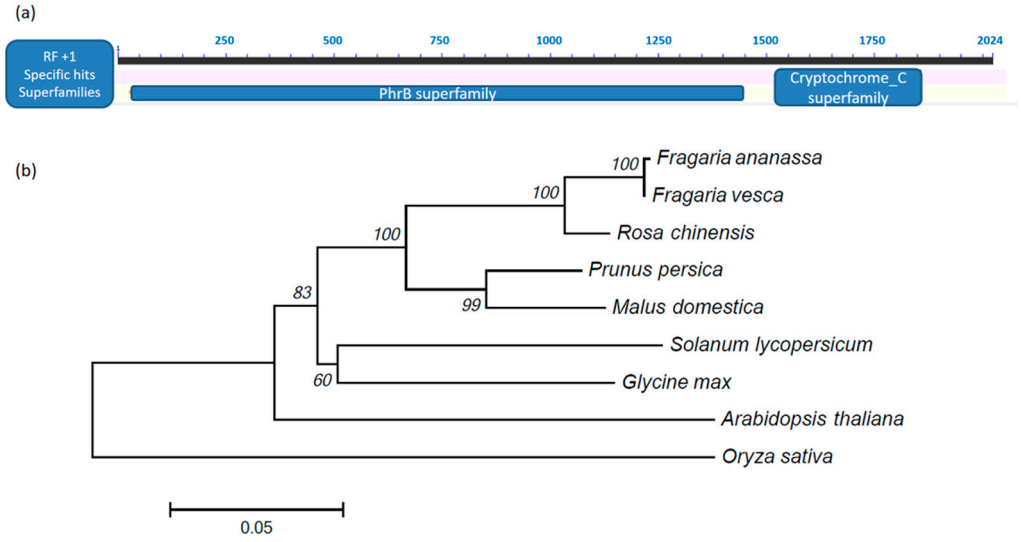

Figure 1. CRY1 protein conserved domain and phylogenetic tree. (**a**) An analysis of CRY1 conserved domain. (**b**) A phylogenetic tree of CRY1 proteins sequences from cultivated strawberry, wild strawberry, and other species.

3.2. Cis-Reglatory Elements in the Promoters of FaCRY1 Genes

The promoter sequences of CRY1, which were 1981 bp upstream of transcription start sit, were cloned from strawberry genomic DNA with primers listed in Table S1. In addition to the core elements of the basic transcription and regulation mechanism of higher plants, such as CAAT-box, TATA-box and A-box, as well as some elements with unknown functions. Twenty-six types of cis-regulatory elements were predicted in the CRY1 promoter sequences from octoploid cultivated strawberry (Figure 1). We further classified them into four groups according to their functions annotated by Plant-Care and previous studies [7,37–40]. The light response element was the largest category, followed by hormone response and stress-inducible element (Table 1), which indicates that the potential gene function of CRY1 in strawberry.

Table 1. Cis-regulatory elements in promoters of FaCRY1 genes.

Classify	Site Name	Numbers	Annotation
Light	Box 4	2	part of a conserved DNA module involved in light responsiveness
	G-Box	1	involved in light responsiveness
	G-box	1	involved in light responsiveness
	GATA-motif	2	part of a light responsive element
	GT1-motif	2	light responsive element
	I-box	1	part of a light responsive element
	MRE	1	MYB binding site involved in light responsiveness
	TCCC-motif	1	part of a light responsive element
	TCT-motif	1	part of a light responsive element
Hormone	AAGAA-motif	1	involved in the abscisic acid responsiveness
	ABRE	1	involved in the abscisic acid responsiveness
	AuxRR-core	1	involved in auxin responsiveness
	GARE-motif	1	gibberellin-responsive element
	P-box	2	gibberellin-responsive element
Stress	STRE	3	Osmotic stress response element
	TC-rich repeats	1	involved in defense and stress responsiveness
	TCA	1	associated with salt stress
	W-box	1	stress response element
Others	MYB	4	N/A
	MYB-like sequence	2	N/A
	Myb-binding site	2	N/A
	CCGTCC motif	1	related to meristem-specific activation
	CCGTCC-box	1	related to meristem-specific activation
	MYC	1	N/A
	Myc	1	N/A
	ARE	1	cis-acting regulatory element essential for the anaerobic induction

3.3. The Expression Pattern of CRY1 in Strawberry

The qRT-PCR results showed the highest expression of *CRY1* was detected in leaf and full red fruits tissues, with a relatively high expression level in young leaves, flowers and runner, but the lowest expression in the root and stem (Figure 2). During strawberry fruit development, the expression of *CRY1* peaked at TR stage, and then decreased. The expression of *CRY1* did not change significantly in the early or late stages in strawberry fruit development.

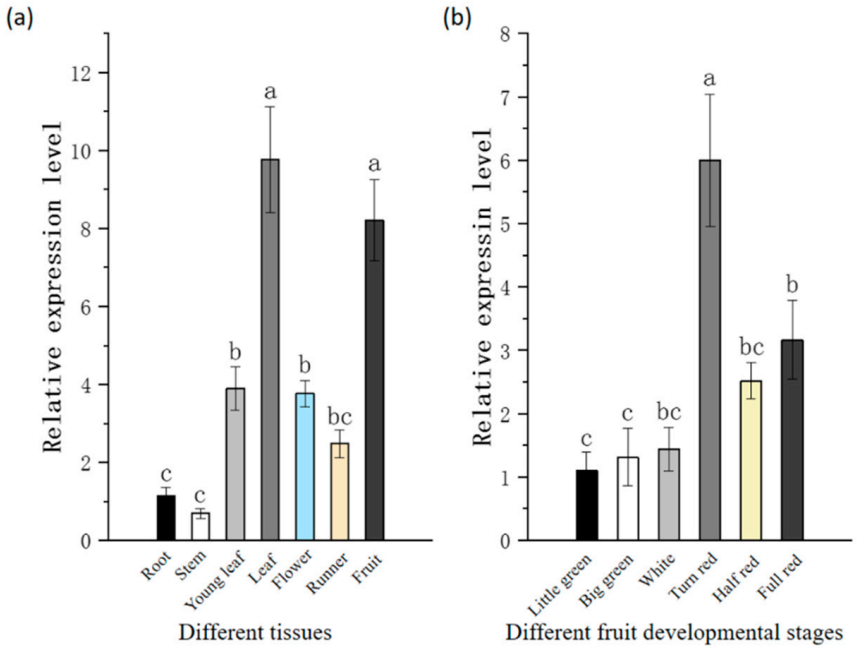

Figure 2. Relative expression level of *CRY1*. (**a**) Different tissues. (**b**) Different developmental stages of strawberry fruit. The significance was annotated by letters. Different letters represent significant differences. $p < 0.05$.

3.4. Effects of Abiotic Stress on CRY1 Expression

The expression patterns of *CRY1* response to abiotic stresses (low temperature, salt and drought) were analyzed. The qRT-PCR showed that the transcript abundance of *CRY1* in strawberry leaf exposed to 4 °C was significantly induced after 3 h, and then decreased significantly at 6 h. There was no significant difference of expression level between 4 °C and control from 6 to 12 h (Figure 3).

The qRT-PCR showed that both salt stress and drought stress could significantly induce the expression of *CRY1*. In this study, the expression of *CRY1* in strawberry roots was significantly up-regulated from 12 to 24 h (Figure 4a) and reached the peak at 6 h in leaf, and then decreased gradually, but there was no difference with control at 24 h under salt stress (Figure 4b). The expression of *CRY1* in strawberry roots was significantly up-regulated only after 12 h (Figure 4c), and then had no difference compared to the control group thereafter. The expression of *CRY1* in leaves was significantly induced in 3 h, and then tended to be stable, with no significant difference comparing to the control group.

These results showed that cold stress, salt stress, and drought stress can significantly induce the transcription of *CRY1* in a short time, but they will gradually decline and tend to be stable in the end.

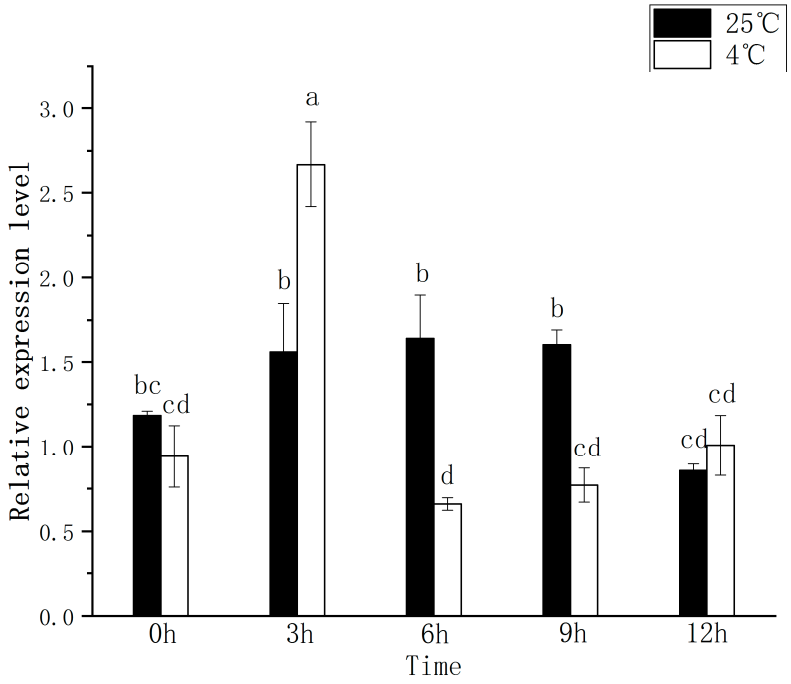

Figure 3. Relative expression level of *CRY1* in strawberry leaf under low temperature stress. The significance was annotated by letters. Different letters represent significant differences. $p < 0.05$.

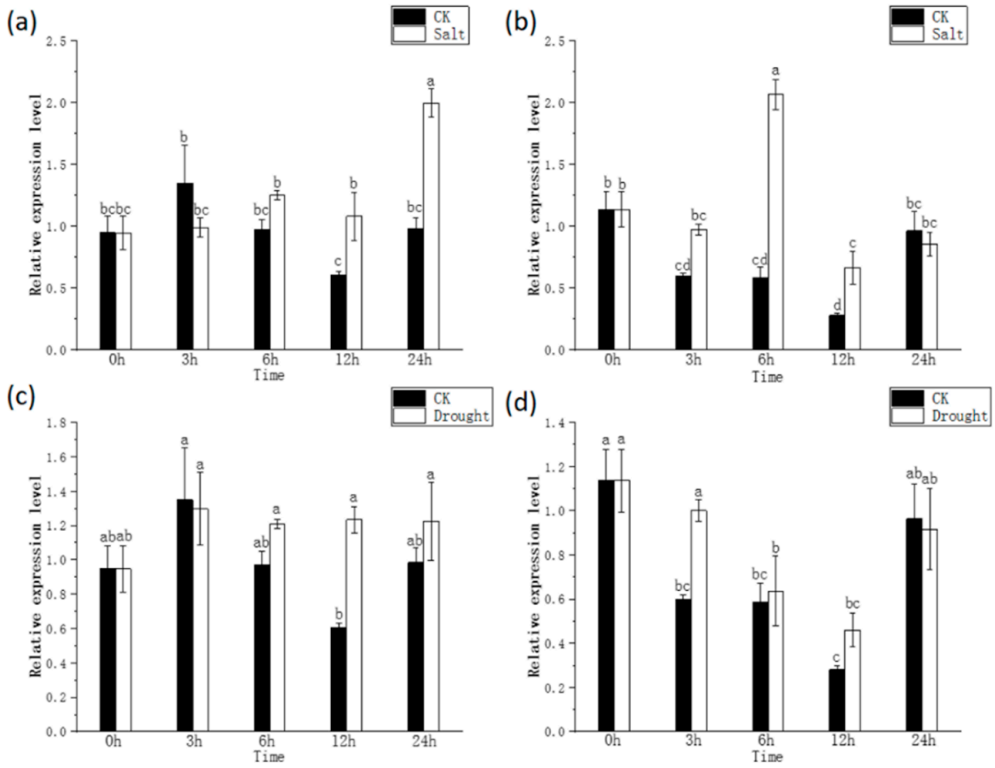

Figure 4. Relative expression level of *CRY1* under osmotic stress (**a**) Relative expression level of *CRY1* in strawberry root under salt stress. (**b**) Relative expression level of *CRY1* in strawberry leaf under salt stress. (**c**) Relative expression level of *CRY1* in strawberry root under drought stress. (**d**) Relative expression level of *CRY1* in strawberry leaf under drought stress. The significance was annotated by letters. Different letters represent significant differences. $p < 0.05$.

3.5. Subcellular Localization of FaCRY1

The GFP fluorescence signals of FaCRY1::GFP fusion proteins were predominantly located in both nucleus and cytoplasm (Figure 5), which indicated that the FaCRY1 was nucleus and cytoplasm localized protein.

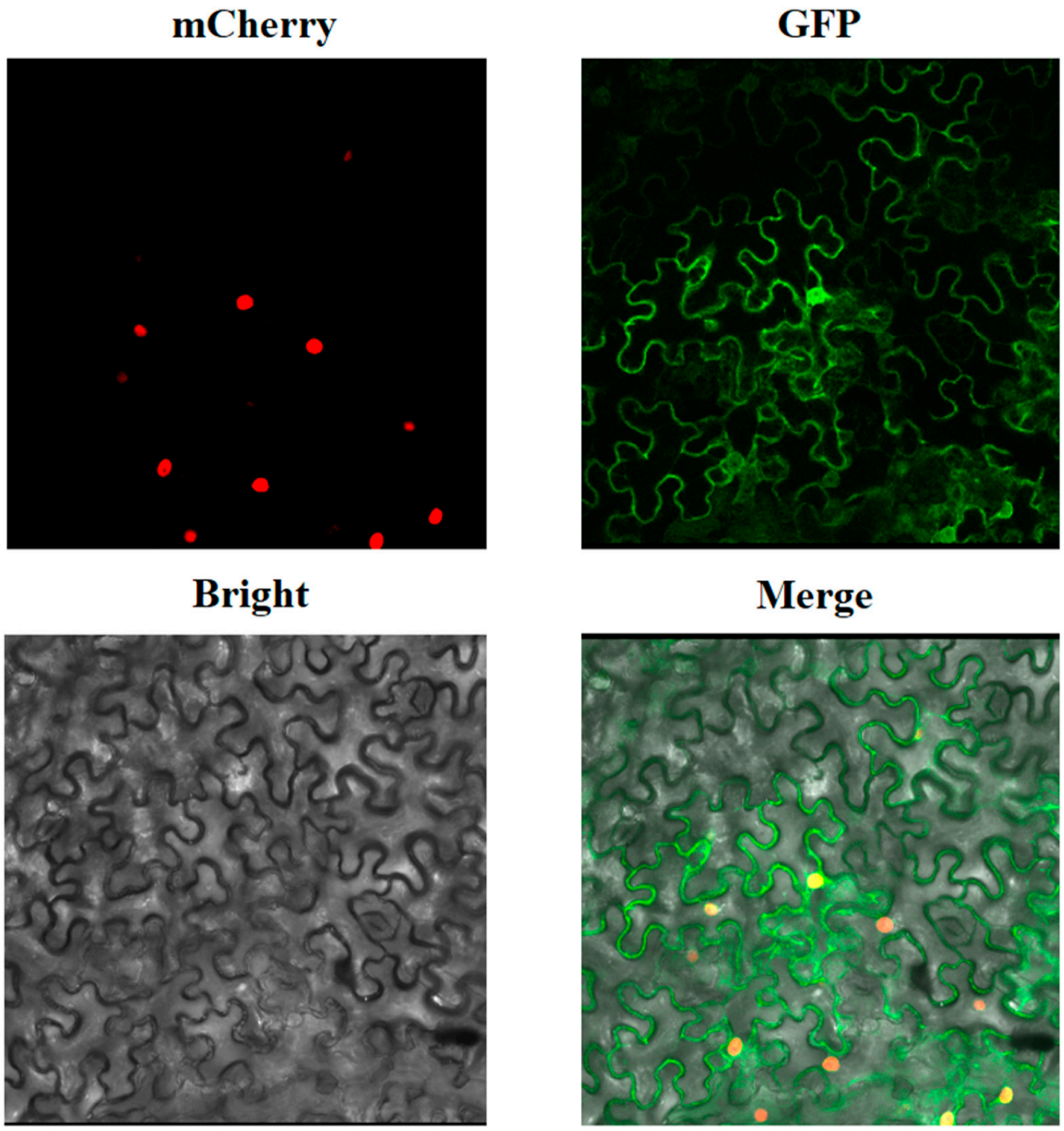

Figure 5. Subcellular localization of FaCRY1 proteins. The FaCRY1:GFP fusion protein was observed in tobacco epidermal cells.

3.6. Yeast Two-Hybrid Library Screening Assay

Auto-activation assay showed that full-length sequence of *FaCRY1* (amino acids 1 to 646) could not be used as a bait as a result of strong auto-activation. Only pGBKT7-CRY1-BD-4 had no transcriptional activation and could be used for yeast two-hybrid screening assay.

A total of 260 monoclonal yeast and 68 gene fragment sequences were obtained. Ten genes were cloned and inserted into pGADT7 vector for the second rotary experiments.

The final yeast two-hybrid validation results showed that only C1-4 (CSN5a-like, COP9 signalosome complex subunit 5a-like), C1-64 (JAZ5, Aromas JASMONATE ZIM-domain protein 5), C1-111 (NDUFB9, NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 9), C1-152 (eIF3G, eukaryotic translation initiation factor 3 subunit G-like), and C1-229 (NF-YC9, nuclear transcription factor Y subunit C-9) could interact with FaCRY1 (Figure 6).

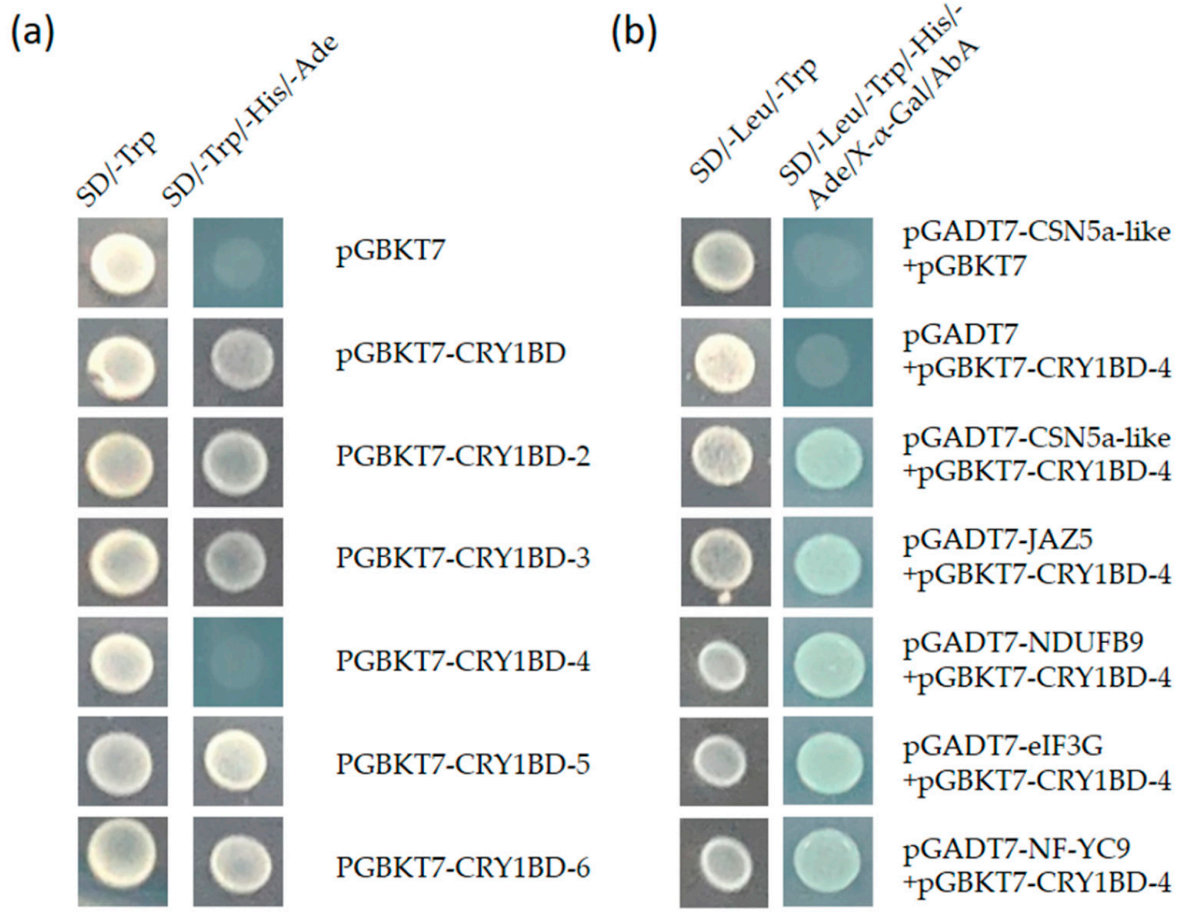

Figure 6. Yeast two-hybrid screening assay. (**a**) Bait plasmid transcriptional activation and toxicity test. (**b**) Yeast two-hybrid interaction assay.

3.7. Heterologous Overexpression of Strawberry CRY1 in Tobacco

The validation of transgenic tobacco using GUS staining analysis showed that three independent lines were obtained. All three transgenic plants in the tissue culture bottle already gave flower (Figure 7). In contrast, the control plants were still at the vegetative growing stage. We selected OE-8 and OE-35 lines that had higher expression level of *GUS* gene for further analysis.

To confirm the potential function of FaCRY1 involving flowering time regulation, we performed qRT-PCR to examine some genes in the downstream photoperiodic flowering time pathway, such as *COP1*, *HY5*, and *CO* [7,11,12,41–43]. The qRT-PCR results showed that the expression level of *COP1* did not change significantly, but the important downstream transcription factor *HY5* was significantly up-regulated. The *CO* gene, a key factor involved flowering, was also up-regulated, indicating that *FaCRY1* may affect strawberry flowering time regulation.

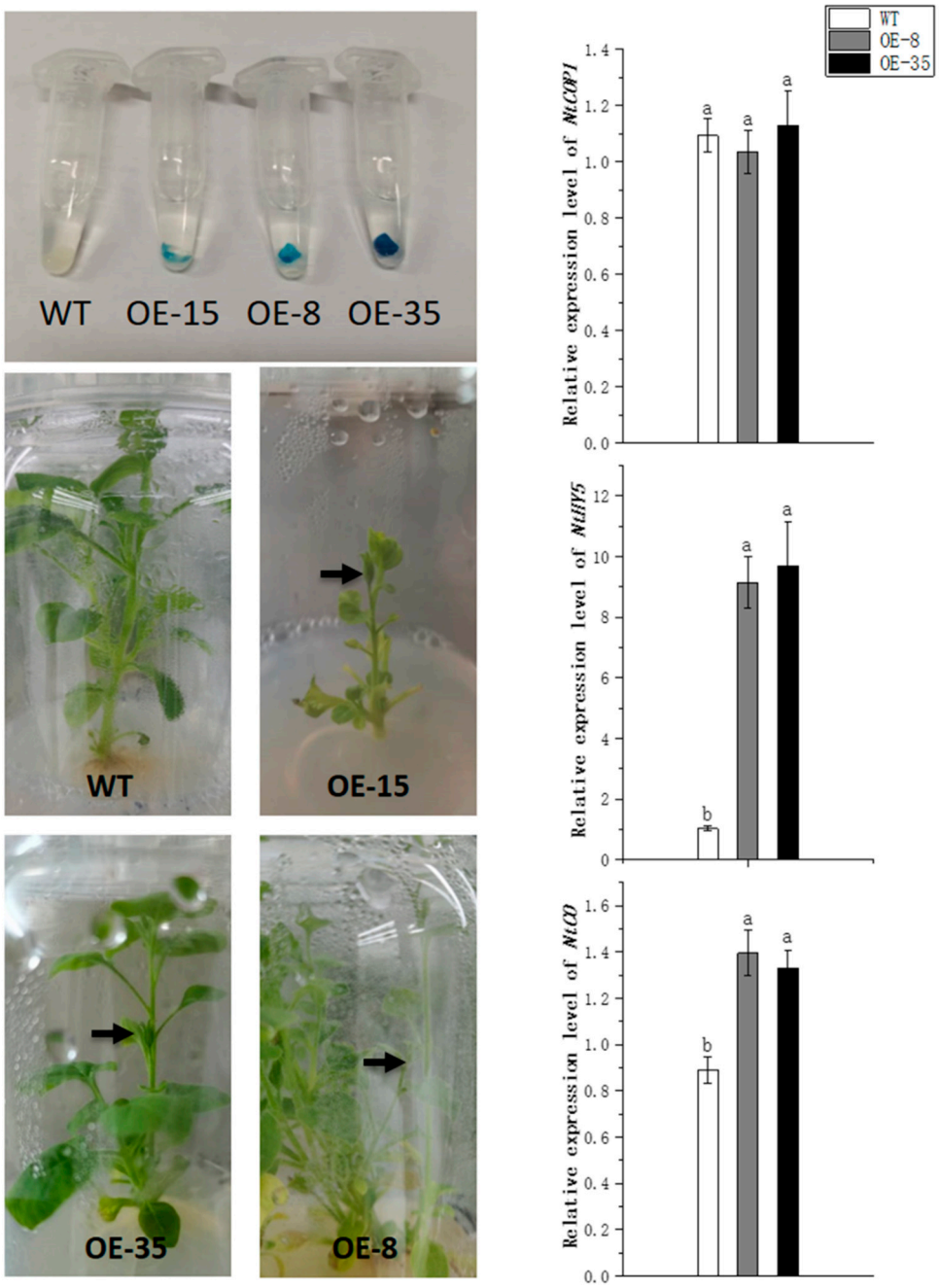

Figure 7. Genotype and phenotype of transgenic tobacco. Note: The arrow in the Figure pointed to was the flower bud of tobacco. Different letters represent significant differences. $p < 0.05$.

4. Discussion

The *CRY1* genes were involved in various biological processes, which have been widely identified in various plants during the past 20 years. In our study, FaCRY1 had a very close relationship with FvCRY1 and other Rosaceae plants, which suggested a common ancient origin of the CRY1 proteins from these species (Figure 1); But also showed great variation compared with *Arabidopsis* or tomato (Figure 1). The promoter region was critical for gene expression regulation [7]. Cis-regulatory element analyses showed that *FaCRY1* promoter distributed many elements related to the light, hormone, and stress response (Figure 2), which indicated that FaCRY1 participate in these signaling pathways as several previous studies have been reported [13,16,17,19,21,22,44–49]. In this study, the expression peaks of *FaCRY1* were observed in the leaf (Figure 2a) and fruit (Figure 2b). It has been

demonstrated that CRY1 plays key role in the regulation of light signaling and fruit quality in *Arabidopsis* and tomato [21,50–52]. However, CRY1 was also involved in the regulation of flowering time, which has functional redundancy with CRY2 [42,53,54]. These results indicated the potential function of CRY1 gene in strawberry growth and development.

Previous studies had demonstrated CRY1 also plays an important role in plant response to low temperature stress. Impaired response of *cry1* mutants reflected participation of photoreceptor in acquiring freezing tolerance [15]. In this study, the qRT-PCR showed that the transcription of *CRY1* in strawberry leaf was significantly induced from 3 h (Figure 3), which suggested that low temperature could up-regulate the expression of *CRY1* in a short time. In addition to the fact that drought and salt stress can share a common signaling pathway mainly due to their osmotic effects [55]. The OE-CRY1 mutants had larger stomatal opening, which showed a more sensitive phenotype to drought, while *cry1* mutants were more resistant to drought stress in *Arabidopsis* [17,20]. Transgenic lines of *Arabidopsis* overexpressing *TaCRY1a* and *TaCRY2* (from *T. aestivum*) were more sensitive to high salt stress (120 mM NaCl) when compared with the watered control [19]. Strawberries were very sensitive to osmotic stress [56]. Interestingly, the expression of *CRY1* in strawberry did not decrease to adapt to stress when strawberry was treated with osmotic stress (Figure 4). In this study, the expression of *CRY1* in strawberry roots (Figure 4c) and leaves (Figure 4d) was significantly up-regulated only at 12 h and 3 h under drought stress. Salt stress forced the expression of *CRY1* in strawberry leaves to be significantly up-regulated for a long time from 6 h to 12 h (Figure 4b). While in roots, *CRY1* was significantly induced from 12 h and still in a rising trend at 24 h (Figure 4a). Xu et al. [19]. found a remarkable increase in *TaCRY2* transcripts in *Arabidopsis* roots treated with salt stress (250 mM NaCl) after 12 h, and transcription of *TaCRY1a* was induced by salt stress after 24 h of exposure to the stressor agent, which was similar to our results (Figure 4). These results showed that *CRY1* in strawberry leaves responded more rapid to stress than in roots (Figure 4). Meanwhile, the *CRY1* expression level was more amenable to salt stress than to drought stress (Figure 4). In addition, the relative expression of *CRY1* up-regulated to transmit stress signals in a short term, and then may regulated by a negative feedback mechanism similar to CRY2-COP1-HY5-BICs in *Arabidopsis* [57].

In this study, FaCRY1 protein was located in both cytoplasm and nucleus (Figure 5). It displays a light-responsive nucleocytoplasmic shuttling pattern such as that of *Ta*CRY1a (*Triticum aestivum*), LfCRY1 (*Lilium formolongi*) and AtCRY1 (*Arabidopsis thaliana*) [19,53,58]. which suggest FaCRY1 may participate in signal transmission and can complete its post-translational life cycle inside the nucleus [59]. In addition, nucleocytoplasmic shuttling was used by the cell to control gene expression programs in response to the environment [19,60], such as osmotic stress.

Yeast two hybrid library screening assay was a technology to obtain new interacting proteins, which had been widely used to find new target proteins interacting with bait proteins nowadays. In this study, we identified five interacting proteins of CRY1: CSN5a-like, JAZ5, NDUFB9, eIF3G, and NF-YC9 (Figure 6b). The yeast auto-activation assay showed that the area near the MTHF region of the PHR domain led to the activity in the FaCRY1 protein (Figure 6a). In *Arabidopsis*, the COOH-terminus of AtCRY (CCT1, amino acids 490 to 681) [35] and CRY1N493 (amino acids 1–493) [34] also has self-activation property in yeast. The pGBKT7-CRY1BD-4 retains part of the N-terminal PHR domains (responsible for sensing light) and a complete C-terminal structure (CCE), which plays a key role in the function of CRY. As a region of protein interaction, CCE domain transmits various signals of CRY to downstream factors [61]. In *Arabidopsis*, the loss of function of any member of CSN will cause constitutive photomorphogenetic, which was characterized by hypocotyl elongation, closed and undeveloped cotyledons, unable to yellowing, and deep purple seed coat [62]. Two conserved genes named CSN5A and CSN5B encode two isoforms of CSN Subunit 5 [63], of which CSN5A was considered to play the largest role in the role of CSN5 [64]. These results suggests that CRY1 may participate in the light morphogenesis through the interaction with CSN5a-like in strawberry. In this study, the

two CRY1 interacting proteins played important roles in the stress response mechanism: JAZ5 and eIF3G (Figure 6b). Jaz family was a transcription inhibitor that plays a role in jasmonic acid signaling pathway [65], in which JAZ5, as an inhibitory protein of jasmonic acid signaling [66,67], can interact with MYB21/24 to participate in jasmonic acid mediated stamen development [68]. It can also bind to WRKY57 and WRKY33 to participate in jasmonic acid mediated resistance to Botrytis cinereal [69]. EIF3G was very important for the translation initiation pathway [70] and plays an important role in plant resistance to stress. *TaeIF3G* transgenic expression enhanced the tolerance of the *TaeIF3G* overexpressing parental yeast cells and *Arabidopsis* plants under different abiotic stress conditions [71]. In addition, eIF3G can also help plants resist virus infection in biological stress [72–74]. In this study, we found that *CRY1* can be significantly induced by low temperature, drought and salt stress in strawberry (Figures 3 and 4), which suggested that CRY1 may participate in stress response through interaction with these proteins. NF-YC9 was mainly involved in flowering time regulation [75,76], ABA response transcription [77], actively regulates the speed of petal expansion, and mediates crosstalk between ethylene and GA [78]. These results suggested that CRY1 may be involved in flowering time regulation and hormone signal response in strawberry. We noticed that yeast two hybrid analysis was in vivo validation, and these potential interacting proteins of CRY1 need to be further verified by in vitro protein analysis before further analysis.

The qRT-PCR results showed that *HY5* and *CO* gene was significantly up-regulated in the young leaves of tobacco tissue culture seedlings after heterologous overexpressing *FaCRY1* (Figure 7), which suggested that CRY1 may regulate the expression of *CO* through *HY5* [12]. In tomato, both OE-CRY1a and OE-CRY2 showed a phenotype of late flowering [51,79], however, *cry1a*, *cry2* and *cry1acry2* double mutants showed no significant difference in flowering time [52]. This evidence suggested that the role of CRYs in regulating flowering was divergent from different species. In tomato, the expression of *COP1* in OE-CRY1a showed no changed; But significantly up-regulation in *cry1a* mutant; The expression of *HY5* was significantly up-regulated in OE-CRY1a and down-regulated in *cry1a* mutant [80]. However, *HY5* was significantly up-regulated and *COP1* did not change significantly in strawberry (Figure 7). These results suggested that CRY1 mainly inhibited the degradation of HY5 by COP1 protein and did not affect the transcription level of *COP1* to transmit signal in strawberry, which was similar to *Arabidopsis* [11]. Blue light may regulate *CO*, *SOC1* gene expression and mediate flowering through BBX28c1 in our previous study [7]. We noticed that NF-YC9 plays an important role in the regulation of flowering time, NF-YC9 could form a complex with CO and drive the transcription of downstream genes, such *FT* and *SOC1* [81,82]. These results implied a new flowering mechanism may exist in strawberry: CRY1-NF-YC9-CO, which needs to be further studied.

5. Conclusions

In this study, we found that *CRY1* was mainly expressed in functional leaves and fruits of strawberry; With the highest expression level in fruits at the initial red stage; Low temperature, drought and salt stress could all significantly induce the expression of *CRY1* in strawberry. The CRY1 protein was localized in cytoplasm and nucleus. Five interacting proteins (CSN5a-like, JAZ5, NDUFB9, eIF3G, NF-YC9) of CRY1 were identified through yeast two-hybrid library screening. Three independent tobacco mutants with heterologous overexpression of strawberry *CRY1* were obtained. The qRT-PCR of mutants showed that *CRY1* can promote the expression of downstream transcription factor *HY5* and flowering gene *CO*. These results will help to further improve the mechanism of CRY1 in horticultural crops.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8050460/s1. Table S1: Primers used in this study; Table S2: Coding region sequences of *FaCRY1*. Figure S1: Detection the Kanamycin gene using PCR to validate insertion of the T-DNA region.

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