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Full Length Article

Cloning and Expression Features of *Cryptochrome* Genes, *ShCRY1* and *ShCRY2*, from Chia (*Salvia hispanica*)

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Abstract

Chia (*Salvia hispanica* L.) has outstanding nutritional and health-promoting values. After its rediscovery, this crop is being expanded worldwide, but the short-day flowering habit is a crucial limiting factor. Cryptochomes (CRYs) are blue-light receptors regulating flowering time through photoperiod-pathway. Full-length cDNAs of two chia *CRY* genes, *ShCRY1* and *ShCRY2*, were cloned and investigated, both of which had alternative transcription initiation and poly-A tailing sites. They have DNA-photolyase and FAD-binding 7 domains, and ShCRY1 also has a Cryptochrome_C domain. Evolutionary study showed that plant-type *CRY* originated in green algae, accompanying the emergence of green plants. Since *Selaginella moellendorffii*, *CRY* in all vascular plants has undergone diverse duplications; especially the event generating higher plant *CRY1* and *CRY2* accompanied the origin of seed plants. *ShCRY1* had significant expression in large leaves, buds and flowers, with the highest in small buds. *ShCRY2* had a higher expression in middle buds, big buds and flowers, but extremely lower in small buds. In sunny days, both *ShCRY1* and *ShCRY2* showed higher expression during night than in the daytime, with a sharp peak at midnight. In cloudy and rainy days, their expression was low, with a small peak at noon, but no peak at midnight. Expression of *ShCRY1* and *ShCRY2* was upregulated by exogenous KT, BR, GA3 and IAA in long-days, while downregulated in short-days, with GA3 being the strongest phytohormone and *ShCRY2* being more sensitive than *ShCRY1*. After treatments with diverse abiotic stresses, the expression of *ShCRY1* and *ShCRY2* fluctuated, while the trends of inhibition by high temperature and ABA and mild promotion by MeJA were still distinct. This is the first report of *CRYs* from the order Lamiales, which will promote the dissection of flowering mechanism of chia and other short-day plants, and enrich the evolution and expression knowledge of plant *CRYs*.

Keywords: Abiotic stresses; Chia; Cryptochrome; Evolution; Expression; Photoperiod; Phytohormones

Introduction

After a period of vegetative growth, the plant begins to enter reproductive growth, and its sign event is flowering. During this period, the apical meristem begins to grow flower buds. The timing of flower induction is determined by a complex regulatory network monitoring the environmental changes to ensure that the flowering environment is the easiest to achieve reproductive success and maximize seed production (Fornara *et al.*, 2010). In this process, a large number of genes are involved in flower induction. The floral induction is mainly regulated by five pathways: photoperiod, autonomic, gibberellin, vernalization and the newly discovered aging pathway (Moon *et al.*, 2003; Liu *et al.*, 2008a; Yuan *et al.*, 2016; Ozturk, 2017; Wei *et al.*, 2018), in which the photoperiod pathway in monocot and dicot plants is the most conserved flowering response pathway (Yanovsky and Kay, 2003). The length of day and night is perceived by photoreceptors, and the endogenous biological clock synchronizes with the environment.

The *Cryptochromes* (*CRYs*) genes were firstly cloned from *Arabidopsis thaliana* and encode soluble flavoproteins that belong to the DNA photolyase/flavoprotein family and are photoperiod pathway photoreceptors (Cashmore *et al.*, 1999). This family genes contain a large number of structurally similar but functionally diverse proteins, including light repair of DNA damage, resetting biological clocks, control of circadian rhythms, transcriptional regulation of flowering and other traits, visual perception of light, biological detection of Earth's magnetic field, etc. (Ozturk, 2017). The plant-type *CRY* genes regulate flowering time in higher plants (Tagua *et al.*, 2015), which

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are among upstream regulators of the photoperiod pathway, and respond to changes in blue light intensity. They interact with other signal proteins to control the expression of downstream genes *CO*, *FT* and *SOC1* and affect flowering time (Liu *et al.*, 2008b, c; Xu *et al.*, 2009; Ma *et al.*, 2016). The *CRYs* of higher plants are classified into two types: *CRY1* and *CRY2*. These proteins have two conserved domains: the DNA_photolyase and FAD-binding_7 domains. At present, they have been cloned from ferns, tomato, pea, rice, peony and other plants (Kanegae and Wada, 1998; Perrotta *et al.*, 2000; Platten *et al*., 2005; Hirose *et al.*, 2006; Ren *et al.*, 2016). *CRY1* regulates photomorphogenesis in tomato, especially hypocotyl elongation and anthocyanin biosynthesis under blue light, and internode elongation under light-limited conditions, whereas *CRY2* controls flowering time in *Arabidopsis* (Perrotta *et al.*, 2000). In *Arabidopsis CRY2* but not *CRY1* exhibits blue light and photoperiod-dependent circadian rhythms (Ahmad *et al.*, 1998). The stability of CRY1 in *Arabidopsis* is not affected by light, whereas CRY2 is rapidly degraded under blue light (Lin *et al.*, 1998; Gyula *et al.*, 2003), and blue light activates CRY independently of GI pathway. CRY2 can stabilize CO by inhibiting COP1 (Nefissi *et al.*, 2011). In *Arabidopsis*, phytochrome A (phyA), phyB and CRY2 are the main photoreceptors of flowering regulators, and these photoreceptors perceive the light stimulation of the leaves to regulate flowering (Endo and Nagatani, 2008). The *CRYs* in *Arabidopsis* have a key function to promote flowering in long-days (LDs) rather than in short-days (SDs), but in the *tfl1* background *CRYs* also promote early flowering under SDs (Buchovsky *et al.*, 2008). In the salicylic acid (SA), high salt, polyethylene glycol, abscisic acid (ABA) and gibberellin (GA3) treatments of *Arabidopsis*, *CRY2* is upregulated and can be assigned a role of resistance (Xu *et al.*, 2009; Wu and Yang, 2010).

Chia is an annual herbaceous crop that belongs to the family Lamiaceae as are perilla (*Perilla frutescens*) and mint (*Mentha haplocalyx*) etc. Chia belongs to genus *Salvia* which contains the common medicinal plant *Salvia miltiorrhiza*. Chia is native to Mexico and Mesoamerica, and grows mainly in the tropics and subtropics, which is the most sacred economic crop among the five major crops of the ancient Indian Aztec Kingdom (Ayerza and Coates, 2005; Sreedhar *et al*., 2015). Chia seeds contain 28–36% of oil, which harbors 64.5–66.8% of alpha-linolenic acid (ALA). This is even higher than the traditional ALA-rich sources perilla seed oil (ALA accounts for 55–65%) and flax seed oil (57.5% ALA), far higher than seed oil (34.3–38.2% ALA) of the new ALA oil crop *Camelina sativa*. Therefore, chia is the real ALA king in cultivated oil crops (Ayerza and Coates, 2005; Azimova and Glushenkova, 2012; Sreedhar *et al*., 2015). However, chia has failed many times in introduction to the world since the discovery of the New World by Christopher Columbus. This is because it is a strict short-day plant and is highly sensitive to changes in photoperiods (Jamboonsri *et al.*, 2012), and has low cold tolerance.

Our research group has taken the lead in studying chia in China. After planting in the Chongqing open field, it was found that chia could bloom in mid-October after entering SDs. When most of the seeds developed to mid-stage, the temperature became cold, and then most seeds could not reach full mature. Only the lower part of the ear can bear some near-mature seeds. Therefore, cloning the key regulatory genes determining the flowering time of chia and dissect its flowering mechanism, will promote the introduction, extension, and cultivation of chia, provide the chance in breaking its strict short-day habits through genetic improvement, and breed early-flowering cultivars suitable for mid- and high-latitudes. At present, there is no report on functional gene and molecular mechanism study of flowering time in chia. In this study, two *CRY* genes from chia, *ShCRY1* and *ShCRY2*, were cloned. The basic characteristics of genes and proteins were analyzed, and some new features of plant-type *CRY* evolution were revealed. Besides, their transcriptional organ-specificity and responsiveness to multiple hormones under long/short photoperiods, circadian rhythms, seasonal transitions and abiotic stresses were also investigated.

Materials and Methods

Plant Materials, Treatment, and Nucleic Acid Extraction

Chia was grown in Hechuan Farm, Southwest University, sown on May 24, 2016. On August 21–22, September 5–6, September 20–21 and October 5–6, adult leaves were sampled at 2:58, 5:58, 9:28, 12:58, 16:28, 19:58, 23:28 h of the day. They were used for gene cloning and to detect diurnal styles of gene expression. Root (Ro), stem (St), small leaves (SL), big leaves (BL), small buds (SB: about 5 days old), medium buds (MB: about 10 days old), big buds (BB: about 15 days old), flowers (Fl), early seeds (ES: about 10 d old), medium seeds (MS: about 20 d old), and late seeds (LS: about 30 d old) were sampled for detecting the organ-specificity of the cloned genes.

The methods used to cultivate the seedlings of chia in the artificial climate chamber referred Xue *et al.* (2017). At 6-leaf stage, the seedlings were moved to plant growth chamber for treatment with two durations of photoperiods. The LD treatment was 16 h-day and 8 h-night, and the SD treatment was 12 h-day and 12 h-night, with constant temperature of 30°C and relative humidity of 56%. Each photoperiod treatment lasted for one week. Four hormone treatments were carried out, *i.e.*, 80 μmol L^{-1} kinetin (KT), 2 μmol L^{-1} brassinolide (BR), 200 μmol L^{-1} GA3 and 250 μmol L^{-1} indole acetic acid (IAA) (Naeem *et al.*, 2004). Each hormone was treated for 0 d (control/CK, basal level), 1 d, 3 d and 9 d. Adult leaves were sampled at each time point for characterization of phytohormone responsiveness of cloned genes.

Chia seedlings were cultured in the artificial climate chamber and subjected to high temperature at 38°C, low temperature at 4°C, mechanical wounding, 100 μmol μmol L^{-1} MeJA, 100 μmol L^{-1} ABA, 1 mmol L^{-1} SA, 300 mmol L^{-1} sodium chloride (NaCl) and 10% polyethylene glycol 6000 (PEG6000). At 0, 0.5, 3, 9, 24 and 48 h time points after treatment, adult leaf samples were taken for characterization of stress responsiveness of cloned genes (Xue *et al.*, 2017).

Each study had three biological replicates. Samples were all kept in liquid nitrogen for transportation and stored at -80°C. Total RNA was extracted using the Biospin Plant Total RNA Extraction Kit (BioFlux), and total gDNA was extracted from adult leaves using general CTAB method. Electrophoresis and spectrophotometric detection were adopted to detect the quality and quantity of the nucleic acids.

Cloning of Conservative Regions of Chia *CRY* Genes

In order to clone the conservative regions of chia *CRY* genes, the *A. thaliana CRY1* mRNA (NM_116961.4) and *CRY2* mRNA (NM_100320.4) were retrieved from NCBI GenBank, and used as electron probes for the electronic cloning of orthologous sequences from chia-relative species *Sesamum indicum*, *Erythranthe guttatu*, *Salvia pomifera* and *S. miltiorrhiza*, since there was no chia *CRY* sequence in the GenBank. All *CRY* reference mRNAs and TSA, EST, GSS, WGS tag sequences were downloaded and a multiple alignment was created. At the conservative sites of *CRY* alignments, degenerate primers combination FLCRYC + RLCRYC was designed (Table 1). One μg total RNA mixed from all organs was subjected to gDNA deletion and reverse-transcription using the PrimeScript Reagent Kit with gDNA Eraser to obtain the first-strand total cDNA. Then it was used as a template for amplification of the conservative regions of chia *CRY* genes using conventional *Taq* PCR (Annealed at 58°C and extended for 2 min). Conventional electrophoresis, gel recovery, pMD19-T vector recombination and *Escherichia coli* DH5α transformation were performed. After PCR test for positive clones, batches of clones corresponding to insert length polymorphism were sent to Shanghai Lifei Information & Technology Company for sequencing using M13F/M13R primers.

5'-RACE and 3'-RACE

The results of sequencing showed that the conservative regions of 2 chia *CRY* genes were obtained and named as *ShCRY1* and *ShCRY2*. Then 5'-RACE and 3'-RACE primers of *ShCRY1* and *ShCRY2* were designed (Table 1), according to the conservative region sequences. One μg of total RNA of organ-mixture was used as start material for RACE handling using the SMARTer ™ RACE Amplification Kit (Clontech, USA) to obtain the first-strand total cDNA template of the 5'-RACE and 3'-RACE. Primers FShCRY13-1/FCRY13-2 and FShCRY23-1/FCRY23-2 were used for pairing with the universal primer LUPM and NUP (Table 1) for 3'-RACE primary and nested amplifications of *ShCRY1* and *ShCRY2*, respectively. The PCR annealing temperature was 64°C and the extension time was 1 min. Primers RShCRY15-1/RCRY15-2 and RShCRY25-1/RCRY25-2 were matched with the universal primers LUPM and NUP (Table 1) for primary and nested amplifications of 5'-RACE of *ShCRY1* and *ShCRY2*, respectively. The PCR annealing temperature was 62°C and the extension time was 1 min. Electrophoresis, gel recovery, TA cloning and sequencing were performed.

Cloning of Full-length Sequences of Chia *CRY* Genes

Based on the conservative regions and 5'-RACE and 3'-RACE results, we can obtain the full-length cDNAs of *ShCRY1* and *ShCRY2* using Vector NTI assemblage function. Based on this, we designed the primer combinations FShCRY1 + RShCRY1 and FShCRY2 + RShCRY2 (Table 1). Two full-length cDNAs were amplified by PCR using 3'-RACE template, annealed at 52°C and extended for 3 min. Electrophoresis, gel recovery, TA cloning and sequencing were performed.

Bioinformatics Analysis

Assemblage, sequence creation, annotation, translation, alignment and other analysis were mainly performed on Vector NTI Advance 11.5.1 and DNAStar version 7.1.0. Electronic cloning, BLAST, CDD assays were performed at NCBI (http://www.ncbi.nlm.nih.gov) and protein analyzes were performed on Expasy (http://www.expasy.org), GSDS2.0 (http://gsds.cbi.pku.edu.cn/), CBS (http://www.cbs.dtu.dk/services/) etc. Plant CRY-DASH (now renamed as ssDNA PHR) and plant (6–4) PHR do not belong to CRYs and are much more distant from plant-type CRYs (Kavakli *et al.*, 2017; Ozturk, 2017). Therefore, we used plant-type CRY sequences from representative species of green plants with wholes genomes being sequenced for phylogenetic analysis. In the French website (http://www.phylogeny.fr/), "A la Carte" mode was selected for phylogenetic tree construction. The number of bootstrap replications was set to 1000.

qRT-PCR

The transcriptional expression of *ShCRY1* and *ShCRY2* was detected by using primer pairs FShCRY1RT + RShCRY1RT and FShCRY2RT + RShCRY2RT, respectively. The *25SrRNA* was detected by F25SRT + R25SRT as internal control (Table 1). qRT-PCR was performed on a CFX Connect™ Real-time PCR Detection System (Bio-Rad, USA) with a program of 95°C for 10 min, and 45 cycles of amplification (95°C for 10 sec, 61°C for 20 sec, 72°C for 10

Table 1: Primers used in cloning and qRT-PCR detection of *CRY* genes from chia

Primers	Sequence (5'→3')	Application
FLCRYC	GGAGAGATYTGAGGRTKGAAGAYAATCC	Forward primer for Chia *CRY* conservative regions amplification
RLCRYC	GGHGCATYCCATGGATGATGKATCCA	Reverse primer for Chia *CRY* conservative regions amplification
FShCRY13-1	TCACTGGAACACTTCCAGATGGTCGA	GSP for *ShCRY1* 3'-RACE primary amplification
FShCRY13-2	ATTGAGGGTTACAAGTTCGATCCAAAT	GSP for *ShCRY1* 3'-RACE nested amplification
FShCRY23-1	TTTCAGGGAGTTTGCCCGATGGTCAT	GSP for *ShCRY2* 3'-RACE primary amplification
FShCRY23-2	GCAGTGGCTGCCTGAATTAGCAAGGA	GSP for *ShCRY2* 3'-RACE nested amplification
RShCRY15-1	CACTATCAGTGGATCTCTTAGTGAGGAGG	GSP for *ShCRY1* 5'-RACE primary amplification
RShCRY15-2	CGCCCAAACAAACACAGCCACCA	GSP for *ShCRY1* 5'-RACE nested amplification
RShCRY25-1	GAGTGGTCTCAGCTTTGATGACGACGAGT	GSP for *ShCRY2* 5'-RACE primary amplification
RShCRY25-2	ATAAGGGCCTTCCTCCTTAGGGCACCATA	GSP for *ShCRY2* 5'-RACE nested amplification
LUPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	Anchor primer for 5'-and 3'-RACE primary amplification
NUP	AAGCAGTGGTATCAACGCAGAGT	Anchor primer for 5'-and 3'-RACE nested amplification
FShCRY1	ACTGCGCCTTGCCCTGCTTT	*ShCRY1* full-length forward primer
RShCRY1	GAGACAAAGATCTAAATTGTATATATACTAAAG	*ShCRY1* full length reverse primer
FShCRY2	AGAATACTTTAGTACTCCATTTATTTTTCTCC	*ShCRY2* full-length forward primer
RShCRY2	ATATGATCAAGCAAACTAGTTCCGT	*ShCRY2* full length reverse primer
F25SRT	GATTTCTGCCCAGTGCTCTGAA	*25SrRNA* qRT-PCR forward primer
R25SRT	TCTGCCAAGCCCGTTCCCTT	*25SrRNA* qRT-PCR reverse primer
FShCRY1RT	TTGGTGGATGCTGGTATGAGG	*ShCRY1* qRT-PCR forward primer
RShCRY1RT	CTGTAGGTTATCAAGGCGGTCAA	*ShCRY1* qRT-PCR reverse primer
FShCRY2RT	TGCAGAACTCGTCGTCATCAA	*ShCRY2* qRT-PCR forward primer
RShCRY2RT	CTTCCCACGGCTCAAACAATAAC	*ShCRY2* qRT-PCR reverse primer

sec). When qRT-PCR was completed, the temperature was raised from 65°C to 95°C, and the melting curve was detected to confirm the specificity of the amplification.

Results

Cloning of Full-length cDNAs of *ShCRY1* and *ShCRY2*

Electrophoresis showed that 2 specific 1.5-kb bands (Fig. 1) were amplified from the conservative regions of chia *CRY* genes. Sequencing of 16 positive clones produced two member genes and NCBI BLASTn showed highest homology to plants *CRYs*, and they were named as *ShCRY1* and *ShCRY2*. No significant band was found in the primary amplifications of 5'-RACE and 3'-RACE of *ShCRY1* and *ShCRY2*, with only smear at the predicted size. The 5'-RACE nested PCRs of *ShCRY1* and *ShCRY2* each generated a band of about 350 bp (Fig. 1). After TA cloning, 5'-RACE clones had insert length polymorphisms. The net length of *ShCRY1* clones after batch sequencing was 300, 338, 368 bp. The net length of *ShCRY2* clones was 244, 303, 325, 349, 352, 387 bp. The 3'-RACE of *ShCRY1* and *ShCRY2* nested PCRs generated specific bands of about 1.2 and 1.0 kb respectively (Fig. 1). All the 3'-RACE clones had polymorphic insert length after TA cloning, with net length of 1285, 1217, 1181, 1165, 1138 bp for *ShCRY1* clones, and 878, 896, 936, 937, 939 bp for *ShCRY2* clones (Poly-A not included). Bands of about 2.5 kb (Fig. 1) with expected size were amplified for the full-length cDNA of *ShCRY1* and *ShCRY2* using end-to-end primer combinations designed according to RACE results. The sequences corresponded to the assembled ones. We failed in amplifying full-length gDNAs of *ShCRY1* and *ShCRY2* even under optimized reagents and cycle parameters, indicating that they either had very long introns or had very complex structures.

Structural Features of *ShCRY1* and *ShCRY2* Genes

ShCRY1 and *ShCRY2* had longest mRNA of 2706 and 2473 bp (GenBank Accession Numbers MF033091 and MF033092, without poly-A), respectively (Fig. 2). They had 244 and 249 bp of 5'-UTR, 2028 and 1986 bp of ORF, 434 and 238 bp of 3'-UTR, respectively. The G+C contents of the 5'-UTR, ORF and 3'-UTR were 39.59/38.00%, 48.30/45.74%, and 34.79/32.77%, respectively. There were 3 and 6 transcription initiation sites for *ShCRY1* and *ShCRY2* respectively, and both had 5 poly-A tailing sites. The identity percentages between *ShCRY1* and *ShCRY2* on mRNA and ORF levels were 54.9 and 56.6%, respectively. The phylogenetic tree of the coding region indicated that *ShCRY1* and *ShCRY2* were orthologous to the higher-plant *CRY1* and *CRY2*, respectively.

Characterization of Deduced ShCRY1 and ShCRY2 Proteins

The ShCRY1 and ShCRY2 proteins were 675 and 661 aa in length, with theoretical MWs of 76.46 and 74.95 kD, pIs of 5.48 and 5.99, respectively. The identity percentage between the two proteins was 47.3%, and the positives percentage was 60.1%. NCBI CDD analysis showed that ShCRY1 and ShCRY2 had DNA_photolyase and FAD_binding_7 domains, but ShCRY1 had an additional Cryptochrome_C domain, and their carboxy-terminal regions were poorly conserved. SignalP 4.1 predicted that ShCRY1 and ShCRY2 did not contain a signal peptide. Plant-mPLoc predicted that ShCRY1 was localized in the chloroplast and ShCRY2 was located in the chloroplast or nucleus. YLoc predicted that ShCRY1 was located in the chloroplast and ShCRY2 was located in the nucleus. Protcom (http://www.softberry.com/cgi-

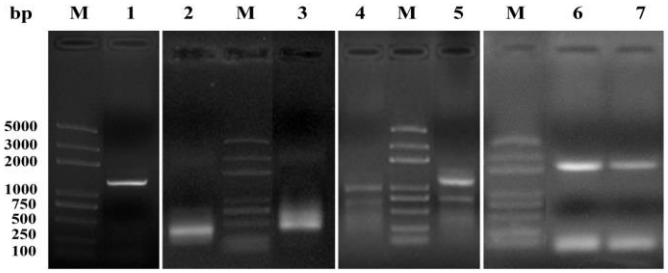

Fig. 1: Electrophoresis results in cloning *ShCRY1* and *ShCRY2*
1: Amplification of conservative regions; 2 and 3: 5'-RACE nested amplification; 4 and 5: 3'-RACE nested amplification; 6 and 7: full-length cDNA amplification; M: Marker

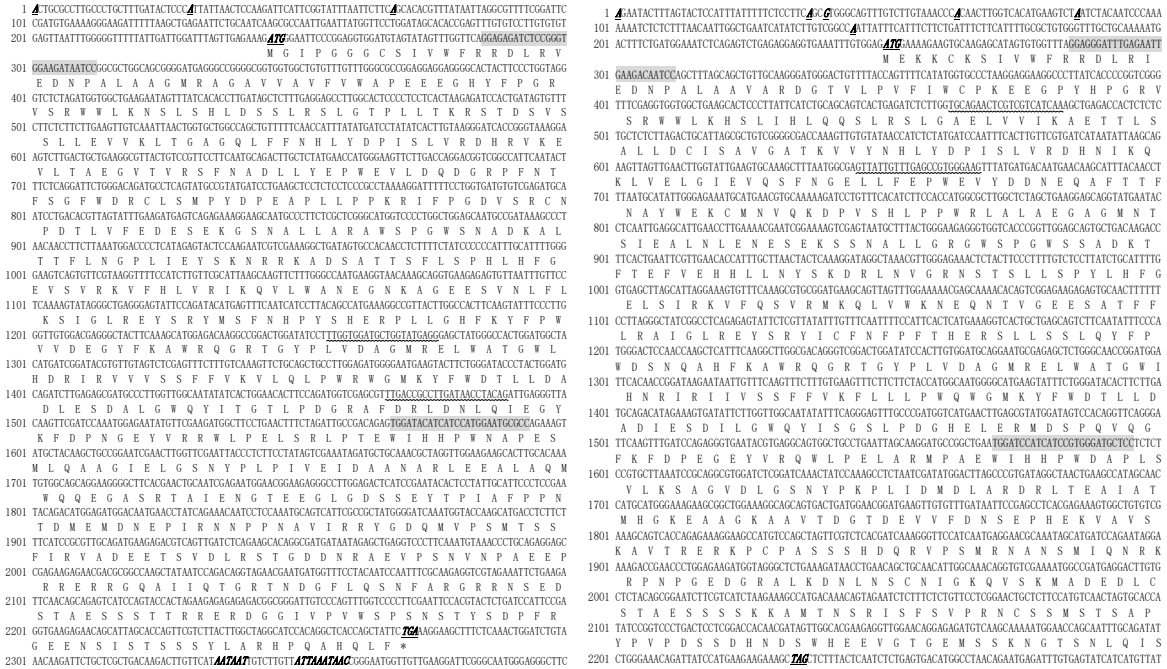

Fig. 2: mRNA and encoded amino acid sequences of *ShCRY1* and *ShCRY2* genes
The start codon ATG and the stop codon TGA/TAG are in underlined bold face. Transcription start sites and polyadenylation sites are in underlined and italic bold face. The possible polyadenylation signals are in italic bold face. The regions corresponding to the primers for conservative region amplification and qRT-PCR are labeled with grey background and wave underlined respectively

bin/programs/proloc/protcomppl.pl), EpiLoc (http://epiloc.cs.queensu.ca/) and BaCelLo predictions indicated that they both were located in the nucleus. TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM/) predictions indicated that ShCRY1 and ShCRY2 had no transmembrane domains. NetPhos 2.0 (http:// www.cbs.dtu.dk /services/NetPhos/) predicted that ShCRY1 had 72 potential phosphorylation sites, including 43 S (serine), 22 T (threonine), 7 Y (tyrosine), and ShCRY2 had 71 potential phosphorylation sites, with 51 S, 15 T and 5Y.

In the secondary structures predicted by SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/secpred_sopma.pl), α-helix, β-sheet (extended strand), β-turn and random coil of ShCRY1/ShCRY2 accounted for 29.19/41.45, 17.63/15.28, 10.52/8.77 and 42.67/34.49%, respectively (Fig. 3). ShCRY2 had significantly higher ratio and more even distribution of α-helix than ShCRY1, especially the C-terminus of ShCRY1 lacked α-helix. The tertiary structures of ShCRY1 and ShCRY2 predicted by SWISS-MODEL were similar to each other (Fig. 4). More than 10 large α-helices surrounded at different directions to form a rhombogen-like or spheroid-like shape, with a medium-sized α-helix protruding outside. The number of β-sheets was limited but they were located at one side of the core.

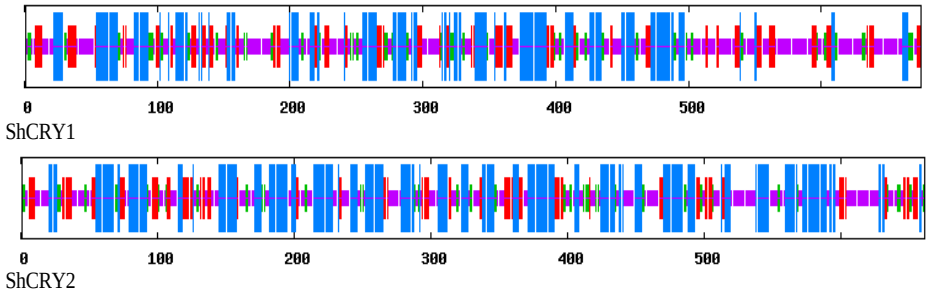

Fig. 3: Predicted secondary structures of ShCRY1 and ShCRY2

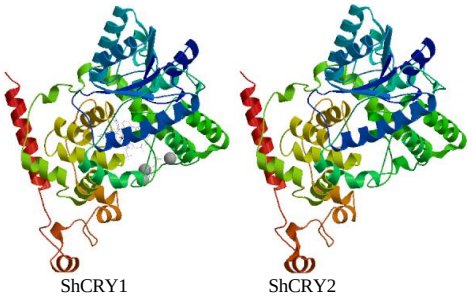

Fig. 4: Predicted tertiary structures of ShCRY1 and ShCRY2

Homology of Chia CRY Proteins and the Evolutionary Features of Plant CRYs

In order to explore the phylogenetic relationships of plant-type *CRY* genes, we selected representative species from different taxa in green plants (Viridiplantae, including green algae, mosses, ferns, gymnosperms, monocots and dicots) with whole genomes being sequenced to carry out phylogenetic analysis (Fig. 5). Some new phenomena of CRY evolution could be observed. There was only one plant-type *CRY* gene in non-vascular plants, including aquatic green algae *Chlamydomonas reinhardtii*, *Volvox carteri* and *Monoraphidium neglectum*, and terrestrial lower plants *Klebsormidium nitens*, *Physcomitrella patens* and *Marchantia polymorpha*. Old and new gene duplication events of *CRY* were common in vascular plants. There were 3 *CRY* genes in *Selaginella moellendorffii* and the duplication time was very old. In fern *Adiantum capillus-veneris*, the 5 *CRY* genes were formed by 3 ancient duplications and 1 recent duplication. However, the CRY family members of ferns did not correspond to the two types of CRY (CRY1 and CRY2) of seed plants. Notably, although 6 CRY proteins were identified in *S. moellendorffii* genomes, they belonged to 3 pairs of possible allelic genes due to their high pairwise similarity (SmCRY1-1/SmCRY1-2, SmCRY2-1/SmCRY2-2, and SmCRY3-1/SmCRY3-2). This might be caused by heterogeneity of the genome sequencing material, so only SmCRY1-1, SmCRY2-1, and SmCRY3-1 were used for phylogenetic analysis.

The gene duplication event that caused *CRY1* and *CRY2* in seed plants took place during the process of gymnosperms origin from a fern ancestor, because dicots, monocots and gymnosperms all had CRY1 as a group and CRY2 as another group. In most seed plants, there was a typical CRY1 and a typical CRY2. ShCRY1 and ShCRY2 of chia showed homology to CRY1 and CRY2 first from Lamiales species *S. indicum* and *E. guttata*, then from other dicots, monocots, and lower plants, respectively. In some seed plants, such as *Cryptomeria japonica*, *Dendrobium catenatum*, *Musa acuminata*, and rice, there had been recent duplications of *CRY*. However, the monocot perennial seaweed herb *Zostera marina* only contained CRY1, whereas CRY2 was missing. Furthermore, aquatic green algae (typically unicellular) and terrestrial green algae (typically filamentous) were distinctly different in CRY sequence and phylogeny, with the former being primitive and the latter being close to land plants CRYs.

Organ Specificity of *ShCRY1* and *ShCRY2* Expression

The results of qRT-PCR showed that *ShCRY1* and *ShCRY2* were expressed in all organs but were significantly different (Fig. 6). The expression of *ShCRY1* was low in roots, stems, and young leaves, but was high in seeds, big leaves, buds, flowers, especially in small buds. *ShCRY2* had low expression in the all organs, especially in small bud and seeds, but was relatively high in middle buds, big buds, and flowers. *ShCRY1* was generally higher than that of *ShCRY2* in all organs, and the peak of *ShCRY1* appeared earlier than that of *ShCRY2* in the whole growth process of chia.

Circadian Rhythm of *ShCRY1* and *ShCRY2* Expression and its Response to the Long-short Photoperiod Seasonal Shift

The qRT-PCR was used to examine the circadian rhythms of *ShCRY1* and *ShCRY2* in adult leaves and the response to the long-short photoperiods seasonal shift (Fig. 7). The results showed that *ShCRY1* and *ShCRY2* were basically similar to each other in circadian rhythm and response to seasonal

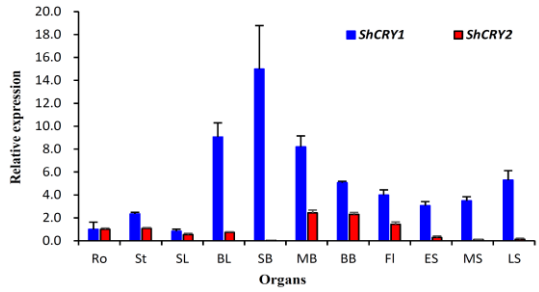

Fig. 6: Relative expression of *ShCRY1* and *ShCRY2* genes in different chia organs

Ro: root; St: stem; SL: small leaf; BL: big leaf; SB: small bud; MB: middle bud; BB: big bud; Fl: flower; ES: early seed; MS: middle seed; LS: late seed

change. On August 21–22 (LD, sunny, 28–38°C), *ShCRY1* and *ShCRY2* were lower during daytime than at night, and *ShCRY1* had a prominent peak at midnight, while *ShCRY2* was more steady. On September 5–6 (LD, rainy, 20–24°C), affected by the rainy weather, their expression was low, with less fluctuation within a whole day, and a small peak at noon, but no peak at midnight. Expression style on September 20–21 (Autumnal equinox, sunny, 20–28°C) was similar to August 21–22, and the two genes were also similar, low in daytime and high at night with a prominent peak at midnight. On October 5–6 (SD, cloudy to overcast, 20–29°C), two genes were significantly lower than that on September 20–21 and August 21–22, while significantly higher than on September 5–6, and the circadian rhythm was also in-between. It was low during daytime than at night, like on September 20–21 and August 21–22, but it was similar to September 5–6 with a small peak at noon and a high level from midnight to early morning.

Effects of Phytohormones on the Expression of *ShCRY1* and *ShCRY2* under Different Photoperiods

In the present study, KT, BR, GA3, and IAA treatments were performed on 6-leaf stage chia seedlings at the long and short photoperiods respectively, and expression of *ShCRY1* and *ShCRY2* was detected by qRT-PCR (Fig. 8). Firstly, *ShCRY1* and *ShCRY2* showed a certain response to the four hormones, indicating that phytohormones affected the expression of *ShCRY* genes. Secondly, the expression was related to the photoperiods, and the trends were opposite under SDs and LDs. After treatments with four exogenous hormones under LDs for 9 d, the overall trend of *ShCRY* genes was up-regulation, though certain fluctuations or even a slight down-regulation existed. In GA3 treatment for 1 d the expression was slightly downregulated, but was then continuously up-regulated thereafter. Expression of *ShCRY* genes was significantly upregulated after 1 d by KT, BR and IAA treatments, but later on the increase was limited with slight fluctuation. Under SDs, the overall trend of *ShCRY* expression under treatments with four hormones

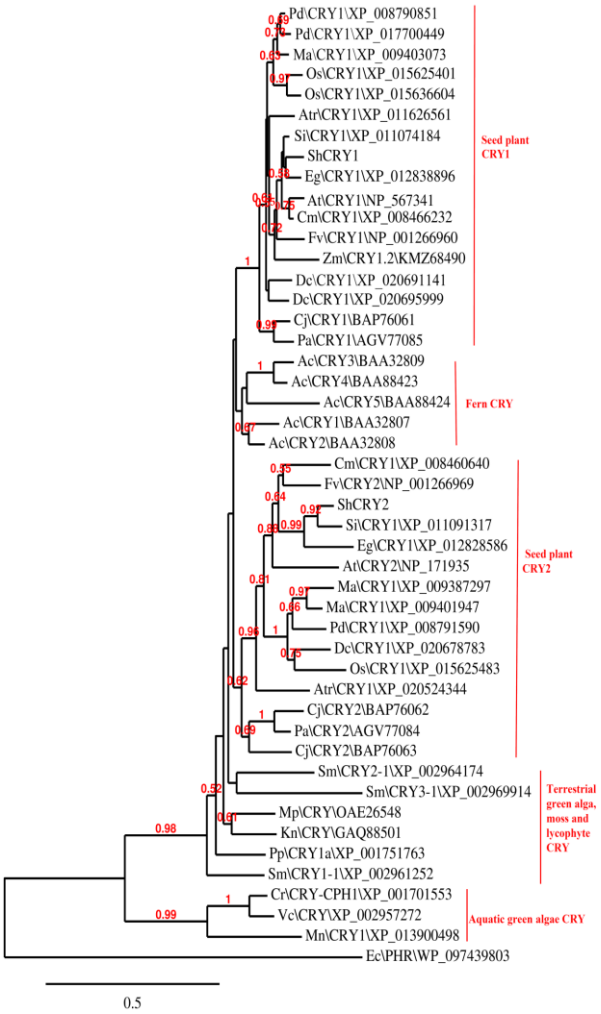

Fig. 5: Phylogenetic relationship of CRY proteins from green plants

Ac, *Adiantum capillus-veneris*; At, *Arabidopsis thaliana*; Atr, *Amborella trichopoda*; Cj, *Cryptomeria japonica*; Cm, *Cucumis melo*; Cr, *Chlamydomonas reinhardtii*; Dc, *Dendrobium catenatum*; Ec, *Escherichia coli*; Eg, *Erythranthe guttata*; Fv, *Fragaria vesca*; Kn, *Klebsormidium nitens*; Ma, *Musa acuminata*; Mn, *Monoraphidium neglectum*; Mp, *Marchantia polymorpha*; Os, *Oryza sativa*; Pa, *Picea abies*; Pd, *Phoenix dactylifera*; Pp, *Physcomitrella patens*; Sh, *Salvia hispanica*; Si, *Sesamum indicum*; Sm, *Selaginella moellendorffii*; Vc, *Volvox carteri*; Zm, *Zostera marina*; *EcPHR* serves as the outgroup

was down-regulation, with slight down-regulation on 1 d and more significant downregulation on 3 d and 9 d. Thirdly, *ShCRY1* and *ShCRY2* generally showed similar trends in response to the four hormones, but difference still existed. The two genes were very similar in LDs, but *ShCRY1* was significantly smaller in response than *ShCRY2* under SDs, indicating that *ShCRY2* was more sensitive to hormones than *ShCRY1* under SDs. There was more obvious difference with GA3 treatment under SDs, showing that *ShCRY1* was not down-regulated and *ShCRY2* was obviously down-regulated.

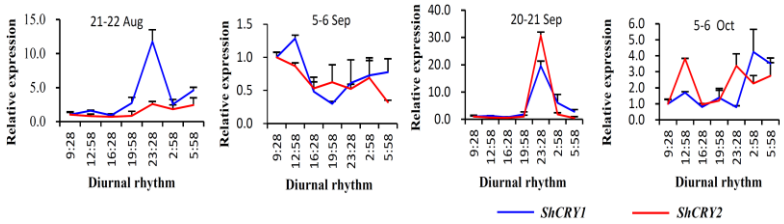

Fig. 7: Circadian rhythm of *ShCRY1* and *ShCRY2* expression, and response to long-short photoperiod seasonal shift

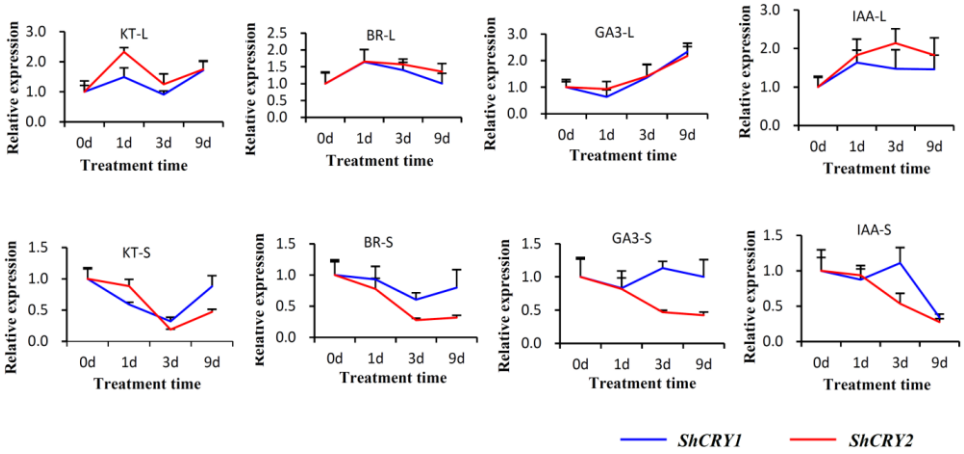

Fig. 8: Influence of KT, BR, GA3 and IAA on the expression of *ShCRY1* and *ShCRY2* under long (-L) and short (-S) photoperiod conditions, respectively

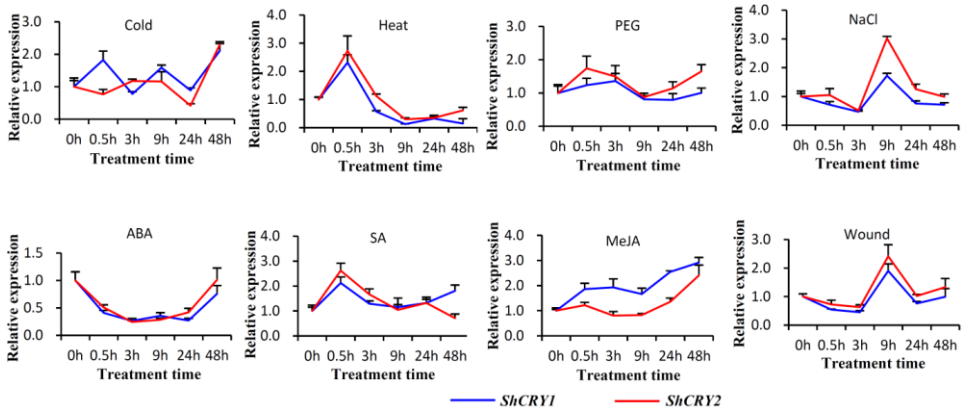

Fig. 9: Influence of abiotic stresses on the expression of *ShCRY1* and *ShCRY2*

Effect of Abiotic Stresses on the Expression of *ShCRY1* and *ShCRY2*

CRYs regulate the circadian rhythm and growth of plants, and have important basic functions. However, it is rarely reported whether various abiotic stresses affect their expression. We used 5-week old chia seedlings for treatments with a variety of abiotic stresses and detected changes in the expression of *ShCRY1* and *ShCRY2* based on qRT-PCR (Fig. 9). There was a temporary up-regulation of *ShCRY1* and *ShCRY2* at 0.5 h under 38°C heat stress, but then they were continuously inhibited. After cold treatment at 4°C, the expression of *ShCRY1* and *ShCRY2* fluctuated up and down within 48 h. After MeJA treatment, *ShCRY1* was up-regulated but the amplitude was within 3 fold, while *ShCRY2* did not change much at first but was slightly up-regulated after 24 h. After PEG and SA treatments, *ShCRY1* and *ShCRY2* did not change significantly and were basically steady. After mechanical injury, ABA and NaCl treatments, *ShCRY1* and *ShCRY2* were down-regulated, and gradually recovered to basal levels in ABA treatment as the stress signals died down, or returned to basal levels after a

transient upregulation in wound and NaCl treatments.

Discussion

A large number of studies have shown that the phytohormones are involved in regulating flowering time, especially the gibberellin signaling pathway is one of the five major pathways of flowering induction, and the hormone pathways are intertwined with the photoperiodic and vernalization reactions (Simpson *et al.*, 1999; Borner *et al.*, 2000; Nelson *et al.*, 2000; Seo *et al.*, 2011). Although *CRYs* are involved in the regulation of many physiological functions, including flowering, as a biological clock, the report of its response to the phytohormones is not systematic. In view of this, treatments with four phytohormones were performed on chia under LDs and SDs. The results showed that both *ShCRY1* and *ShCRY2* responded to these four hormones, promoted under LDs, while inhibited under SDs. This study showed that the effect of phytohormones on *CRYs* depends on photoperiod style, and LDs and SDs have contrary effects. This finding will promote the study on the interaction between photoperiods and hormones to reveal the comprehensive effects on the flowering mechanism. *CRY1* and *CRY2* were upregulated after treatment with GA3 under LDs in tomato (Facella *et al.*, 2012), and *CRY1* and *CRY2* were upregulated after treatment with IAA, BR and GA3 under LDs in *Arabidopsis* (de Wit *et al.*, 2016), which are consistent with chia. It seems that whether it is high-temperature short-day plants or low-temperature long-day plants, the phytohormones can promote the expression of *CRY* under LDs. At present, there is no report on the effect of phytohormones on *CRY* under SDs. This study systematically revealed the response characteristics of *CRY* to four phytohormones under LDs and SDs.

In short-day plant chia, *ShCRY1* and *ShCRY2* were characterized by higher expression during night than in daytime and a peak at midnight. On cloudy and rainy days, their expression was low, with less fluctuation within a whole day, and a small peak at noon, but no peak at midnight. During the conversion from high temperature long days in summer to low temperature short days in autumn, their expression firstly rised and then decreased, and the circadian rhythm had certain changes. The expression of *CRY1* and *CRY2* was high in the daytime and low at night in the long-day plant *Arabidopsis* (Tóth *et al.*, 2001), which was contrary to chia. In the long-day plant pea, the 3 *CRY* genes were expressed lower in both continuous white and blue lights than that in dark, but *CRY1* and *CRY2a* did not greatly fluctuate during a whole day, while *CRY2b* showed peak at the end of late daytime (Platten *et al.*, 2005). The flowering habit of *Orobanche minor*, which was also from Lamiales, did not belong to SDs at all, but was consistent with the long-day type host plant *Trifolium pratense* (Holdsworth and Nutman, 1947). However, the natural diurnal variation of *OmCRY1* expression was also low in daytime and high at night (Okazawa *et al.*, 2005), similar to chia. The peaks of *CRY1a* and *CRY2* from neutral daylight plant tomato appeared in daytime, while the peak of *CRY1b* appeared at the end of the evening (Facella *et al.*, 2008). It seems that *CRYs* are not conserved in diurnal rhythms among species genes, and may be affected by various factors such as the types of plant photoperiod, inter-plant kinship, and inter-plant interactions.

ShCRY1 and *ShCRY2* were rapidly upregulated by high temperature at 37°C, but then were successively inhibited. After mechanical injury, ABA, and NaCl treatments, *ShCRY1* and *ShCRY2* were firstly down-regulated, then recovered to basal level in ABA treatment or recovered to basal level after a transient upregulation in wound and NaCl treatments. After cold treatment at 4°C and PEG treatment, *ShCRY1* and *ShCRY2* did not show significant change but some fluctuations. MeJA caused slow and continuous upregulation of *ShCRY1* and *ShCRY2*. *CRYs* as rhythmic genes responded to the photoperiods, and their expression characteristics also fluctuated under abiotic stresses, and were particularly inhibited by high temperature and ABA, and mildly promoted by MeJA. With the weakening of stress signals, expression of *CRY* tended to return to the basal level. The expression of *CRY1* in *Arabidopsis* treated with SA was slightly upregulated in LDs (Wu and Yang, 2010). ABA inhibited the expression of *CRY2* in *Arabidopsis* (Kim *et al.*, 2014). This study systematically revealed the effects of eight abiotic stresses on expression of *CRY* genes in chia.

To reveal evolutionary feature of plant-type *CRY* genes, we selected representative species from typical taxonomic units, constructed a phylogenetic tree of the CRY proteins from green plants, and revealed some new features of the *CRY* gene evolution. There was only one *CRY* gene in algae, mosses, and *M. polymorpha*, but old and new duplications of *CRY* genes in vascular plants were common, especially the duplication event that resulted in the origin of *CRY1* and *CRY2* in seed plants occurred during the transition from the fern ancestor to gymnosperms. This was earlier than previous speculation that this event occurred at the time node of the common ancestor of the monocot and dicot plants (Mei and Dvornyk, 2015; Kavakli *et al.*, 2017). The reason for difference was not the methodology, but the previous studies focused on whole CRY/PHR family of the entire biosphere. In these reports, when the phylogenetic trees were built, too few plant species were selected and the representative plant taxonomy units were not rich enough. Another reason was that there were less complete genome sequencing data of gymnosperms and ferns in the previous GenBank. As the plant genome database was improved, our study selected abundant species and dedicated to the establishment of plant-type CRY phylogenetic tree, thus it could reveal more accurate and detailed features of plant *CRY* evolution. We used alternative methods and parameters to make the tree, and the results were similar and did not affect the basic conclusions of plant *CRY* evolution here.

CRYs are functionally conserved positive regulators

of flowering and other developments in response to photoperiods in both dicots and monocots (Guo *et al.*, 1998; Hirose *et al.*, 2006). Because *ShCRY1* and *ShCRY2* are typical angiosperms *CRY*s, they might also participate in the induction of flower formation and regulate other developments in chia. Many studies show that *CRY1* and *CRY2* regulate flowering in *Arabidopsis* and other plants, and that *AtCRY2* is the dominant flowering time gene in *Arabidopsis*, which resulted in early flowering when transformed into barley (El-Assal *et al.*, 2011). When *Arabidopsis CRY1* and *CRY2* were overexpressed, they promoted the expression of *FT* and caused early flowering, while the deletion of *Arabidopsis CRY1* and *CRY2* caused late flowering (Liu *et al.*, 2008b, 2013; Yuan *et al.*, 2016). However, given that the origin of *CRY1* and *CRY2* occurred before the seed plants, it is speculated that there should be significant functional divergence between *CRY1* and *CRY2* in the seed plants. Similar to peony, *Brassica napus*, *Arabidopsis*, sorghum, rice, tea and sunflower (Chatterjee *et al.*, 2006; Ren *et al.*, 2016), organ specificity of two *CRY* genes in chia was significantly different from each other. The expression of *ShCRY1* was generally higher in all organs than that of *ShCRY2* during the whole growth period, especially before and after the occurrence of small buds. *ShCRY2* was not only lower than *ShCRY1* in the major organs during the whole growth period, but its expression declined to very low level in small buds. *ShCRY2* was more sensitive to the four hormones in SDs, and the inhibition was greater, while *ShCRY1* was more insensitive. In addition, there were some differences in the response of the two genes to circadian rhythms, seasonal changes, and abiotic stresses. Conclusively, *CRY1* and *CRY2* in chia have diverged in function. Perhaps *ShCRY1* is coupled with the season, and *ShCRY2* is coupled with intrinsic hormones, which needs to be revealed by gene function studies.

The water has strong resistance to red light, far-red light and ultraviolet rays, while the blue-green light has a weaker barrier effect. In the long-term submerged growth and evolution of *Z. marina*, it lost a complete set of stomatal genes, terpene pathway genes, ethylene signal genes, UV protection and the red-far-red receptor phytochrome genes (Olsen *et al.*, 2016). In this study, we further revealed that *Z. marina* retained only *CRY1* and lost *CYR2*, which are of great value for further study of the functional divergence between *CRY1* and *CRY2*. There are more than one assumptions. Firstly, *Z. marina* might require only one *CRY* gene in the dark environment for submerged growth, thus *CRY1* is a basic/high-efficiency/low-light type, and *CRY2* is non-basic/low-efficiency/high-light type. Secondly, because of functional divergence, *CRY1* is kept for flowering and seeding, and *CRY2* is deleted for non-necessary stomata function and phytochrome related function. Thirdly, plant-type *CRY1* and *CRY2* diverged in photo-spectrum, thus *CRY1* senses some blue light that is not easily blocked by water, and *CRY2* senses other blue light that is easily blocked by water.

Conclusion

In this study two *CRY* genes, *ShCRY1* and *ShCRY2*, from chia were isolated and characterized. They had typical structural, molecular and expressional features, while distinct organ-specificity and responses to diverse physiological and environmental factors indicated their functional divergence. The effect of phytohormones on chia *CRY* expression varied depending on the photoperiod. Chia *CRY* expression also changed in response to circadian rhythms, climate and seasons, and was affected by a variety of abiotic stresses. This study also revealed some new evolutionary features, especially the origin and duplication, of plant-type *CRY* genes. This is the first *CRY* report in the order Lamiales. It will promote the dissection of flowering mechanism of chia and other short-day plants, and also enrich the evolution and expression characteristics of *CRY*, promote the study on interaction between photoperiod pathway and hormone pathways in flowering time control.

Acknowledgements

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References

Ahmad, M., J.A. Jarillo and A.R. Cashmore, 1998. Chimeric proteins between cry1 and cry2 *Arabidopsis* blue light photoreceptors indicate overlapping functions and varying protein stability. *Plant Cell*, 10: 197–207

Ayerza, R. and W. Coates, 2005. *Chia: Rediscovering a Forgotten Crop of the Aztecs*, pp: 1–197. The University of Arizona Press, Tucson, Arizona, USA

Azimova, S. and A. Glushenkova, 2012. *Lipids, Lipophilic Components and Essential Oils from Plant Sources*, pp: 1–484. London: Springer Science+Business Media, LLC, Dordrecht, The Netherlands

Borner, R., G. Kampmann, J. Chandler, R. Gleiner, E. Wisman, K. Apel and S. Melzer, 2000. A MADS domain gene involved in the transition to flowering in *Arabidopsis*. *Plant J.*, 24: 591–599

Buchovsky, A.S., B. Strasser, P.D. Cerdán and J.J. Casal, 2008. Suppression of pleiotropic effects of functional *CRYPTOCHROME* genes by *TERMINAL FLOWER 1*. *Genetics*, 180: 1467–1474

Cashmore, A.R., J.A. Jarillo, Y.J. Wu and D. Liu, 1999. Cryptochromes: blue light receptors for plants and animals. *Science*, 284: 760–765

Chatterjee, M., P. Sharma and J.P. Khurana, 2006. *Cryptochrome 1* from *Brassica napus* is up-regulated by blue light and controls hypocotyl/stem growth and anthocyanin accumulation. *Plant Physiol.*, 141: 61–74

de Wit, M., D.H. Keuskamp, F.J. Bongers, P. Hornitschek, C.M.M. Gommers, E. Reinen, C. Martínez-Cerón, C. Fankhauser and R. Pierik, 2016. Integration of phytochrome and cryptochrome signals determines plant growth during competition for light. *Curr. Biol.*, 26: 3320–332

El-Assal, S.E.D., S.M. Abd-alla, A.A. El-Tarras and M.A. El-Awady, 2011. Production of early flowering transgenic barley expressing the early flowering allele of the *Cryptochrome2* gene. *GM Crops*, 2: 50–57

Endo, M. and A. Nagatani, 2008. Flowering regulation by tissue specific functions of photoreceptors. *Plant Signal. Behav.*, 3: 47–48

Facella, P., L. Daddiego and G. Perrotta, 2012. CRY1a influences the diurnal transcription of photoreceptor genes in tomato plants after gibberellin treatment. *Plant Signal. Behav*., 7: 1034–1036

Facella, P., L. Lopez, F. Carbone, D.W. Galbraith, G. Giuliano and G. Perrotta, 2008. Diurnal and circadian rhythms in the tomato transcriptome and their modulation by cryptochrome photoreceptors. *PLoS One*, 3: e2798

Fornara, F., A. de Montaigu and G. Coupland, 2010. Snapshot: control of flowering in *Arabidopsis*. *Cell*, 141: 550–550

Guo, H.W., H.Y. Yang, T.C. Mockler and C.T. Lin, 1998. Regulation of flowering time by *Arabidopsis* photoreceptors. *Science*, 279: 1360–1363

Gyula, P., E. Schäfer and F. Nagy, 2003. Light perception and signalling in higher plants. *Curr. Opin. Plant Biol*., 6: 446–452

Hirose, F., T. Shinomura, T. Tanabata, H. Shimada and M. Takano, 2006. Involvement of rice cryptochromes in de-etiolation responses and flowering. *Plant Cell Physiol.*, 47: 915–925

Holdsworth, M. and P.S. Nutman, 1947. Flowering responses in a strain of *Orobanche minor*. *Nature*, 160: 223–224

Jamboonsri, W., T.D. Phillips, R.L. Geneve, J.P. Cahill and D.F. Hildebrand, 2012. Extending the range of an ancient crop, *Salvia hispanica*, L.—a new ω3 source. *Genet. Resour. Crop Evol.*, 59: 171–178

Kanegae, T. and M. Wada, 1998. Isolation and characterization of homologues of plant blue-light photoreceptor (*cryptochrome*) genes from the fern *Adiantum capillusveneris*. *Mol. Gen. Genet.*, 259: 345–353

Kavakli, I.H., I. Barış, M. Tardu, Ş. Gül, H. Öner, S. Çal, S. Bulut, D. Yarparvar, Ç. Berkel, P. Ustaoğlu and C. Aydın, 2017. The photolyase/cryptochrome family of proteins as DNA repair enzymes and transcriptional repressors. *Photochem. Photobiol.*, 93: 93–103

Kim, S.I., S.I. Song and H.S. Seo, 2014. *Cryptochrome* 2 negatively regulates ABA-dependent seed germination in *Arabidopsis*. *Life Sci. J*., 11: 880–884

Lin, C.T., H.Y. Yang, H.W. Guo, T. Mockler, J. Chen and A.R. Cashmore, 1998. Enhancement of blue-light sensitivity of *Arabidopsis* seedlings by a blue light receptor *cryptochrome 2*. *Proc. Natl. Acad. Sci. USA*, 95: 2686–2690

Liu, C., H. Chen, H.L. Er, H.M. Soo, P.P. Kumar, J.H. Han, Y.C. Liou and H. Yu, 2008a. Direct interaction of AGL24 and SOC1 integrates flowering signals in *Arabidopsis*. *Development*, 135: 1481–1491

Liu, H.T., X.H. Yu, K.W. Li, J. Klejnot, H.Y. Yang, D. Lisiero and C.T. Lin, 2008b. Photoexcited CRY2 interacts with CIB1 to regulate transcription and floral initiation in *Arabidopsis*. *Science*, 322: 1535–1539

Liu, L.J., Y.C. Zhang, Q.H. Li, Y. Sang, J. Mao, H.L. Lian, L. Wang and H.Q. Yang, 2008c. COP1-mediated ubiquitination of *CONSTANS* is implicated in cryptochrome regulation of flowering in *Arabidopsis*. *Plant Cell*, 20: 292–306

Liu, Y.W., X. Li, K.W. Li, H.T. Liu and C.T. Lin, 2013. Multiple bHLH proteins form heterodimers to mediate *CRY2*-dependent regulation of flowering-time in *Arabidopsis*. *PLoS Genet.*, 9: e1003861

Ma, D.B., X. Li, Y.X. Guo, J.F. Chu, S. Fang, C.Y. Yan, J.P. Noel and H.T. Liu, 2016. Cryptochrome 1 interacts with PIF4 to regulate high temperature-mediated hypocotyl elongation in response to blue light. *Proc. Natl. Acad. Sci. USA*, 113: 224–229

Mei, Q.M. and V. Dvornyk, 2015. Evolutionary history of the photolyase/cryptochrome superfamily in eukaryotes. *PLoS One*, 10: e0135940

Moon, J., S.S. Suh, H. Lee, K.R. Choi, C.B. Hong, N.C. Paek, S.G. Kim and I. Lee, 2003. The *SOC1* MADS-box gene integrates vernalization and gibberellin signals for flowering in *Arabidopsis*. *Plant J.*, 35: 613–623

Naeem, M., I. Bhatti, R.H. Ahmad and A.M.Y. Ashraf, 2004. Effect of some growth hormones (GA3, IAA and kinetin) on themorphology and early or delayed initiation of bud of lentil (*Lens culinaris* Medik). *Pak. J. Bot.*, 36: 801–809

Nefissi, R., Y. Natsui, K. Miyata, A. Oda, Y. Hase, M. Nakagawa, A. Ghorbel and T. Mizoguchi, 2011. Double loss-of-function mutation in *EARLY FLOWERING 3* and *CRYPTOCHROME 2* genes delays flowering under continuous light but accelerates it under long days and short days: an important role for *Arabidopsis CRY2* to accelerate flowering time in continuous light. *J. Exp. Bot.*, 62: 2731–2744

Nelson, D.C., G. Lasswell, L.E. Rogg, M.A. Cohen and B. Bartel, 2000. *FKF1*, a clock-controlled gene that regulates the transition to flowering in *Arabidopsis*. *Cell*, 101: 331–340

Okazawa, A., C. Trakulnaleamsai, H. Hiramatsu, E.I. Fukusaki, K. Yoneyama, Y. Takeuchi and A. Kobayashi, 2005. Cloning of a *cryptochrome* homologue from the holoparasitic plant *Orobanche minor* Sm. *Plant Physiol. Biochem.*, 43: 499–502

Olsen, J.L., P. Rouzé, B. Verhelst, Y.C. Lin, T. Bayer, J. Collen, E. Dattolo, E. De Paoli, S. Dittami, F. Maumus, G. Michel, A. Kersting, C. Lauritano, R. Lohaus, M. Töpel, T. Tonon, K. Vanneste, M. Amirebrahimi, J. Brakel, C. Boström, M. Chovatia, J. Grimwood, J.W. Jenkins, A. Jueterbock, A. Mraz, W.T. Stam, H. Tice, E. Bornberg-Bauer, P.J. Green, G.A. Pearson, G. Procaccini, C.M. Duarte, J. Schmutz, T.B. Reusch and Y. van de Peer, 2016. The genome of the seagrass *Zostera marina* reveals angiosperm adaptation to the sea. *Nature*, 530: 331–335

Ozturk,N.,2017. Phylogenetic and functional classification of the photolyase/cryptochrome family. *Photochem. Photobiol.*, 93: 104–111

Perrotta, G., L. Ninu, F. Flamma, J.L. Weller, R.E. Kendrick, E. Nebuloso and G. Giuliano, 2000. Tomato contains homologues of *Arabidopsis cryptochromes 1* and *2*. *Plant Mol. Biol.*, 42: 765–773

Platten, J.D., E. Foo, F. Foucher, V. Hecht, J.B. Reid and J.L. Weller, 2005. The *cryptochrome* gene family in pea includes two differentially expressed *CRY2* genes. *Plant Mol. Biol.*, 59: 683–696

Ren, X.X., S.L. Wang, J.Q. Xue, F.Y. Zhu, C.J. Liu and X.X. Zhang, 2016. Molecular cloning and expression analysis of cryptochrome gene *PsCRY2* in tree peony. *Hortic. Plant J*., 2: 357–362

Seo, E., J. Yu, K.H. Ryu, M.M. Lee and I Lee, 2011. WEREWOLF, a regulator of root hair pattern formation, controls flowering time through the regulation of *FT* mRNA stability. *Plant Physiol.*, 156: 1867–1877

Simpson, G.G., A.R. Gendall and C. Dean, 1999. When to switch to flowering. *Annu. Rev. Cell Dev. Biol.*, 99: 519–950

Sreedhar, R.V., P. Kumari, S.D. Rupwate, R. Rajasekharan and M. Srinivasan, 2015. Exploring triacylglycerol biosynthetic pathway in developing seeds of chia (*Salvia hispanica* L.): A transcriptomic approach. *PLoS One*, 10: e0123580

Tagua, V.G., M. Pausch, M. Eckel, G. Gutierrez, A. Miralles-Duran, C. Sanz, A.P. Eslava, R. Pokorny, L.M. Corrochano and A. Batschauer, 2015. Fungal cryptochrome with DNA repair activity reveals an early stage in cryptochrome evolution. *Proc. Natl. Acad. Sci. USA*, 112: 15130–15135

Tóth, R., E. Kevei, A. Hall, A.J. Millar, F. Nagy and L. Kozma-Bognár, 2001. Circadian clock-regulated expression of *phytochrome* and *cryptochrome* genes in *Arabidopsis*. *Plant Physiol.*, 127: 1607–1616

Wei, X., X. Zhang, J. Mao, G. Liang and H. Tong, 2018. Different light qualities modify morphology and expression of *CsLHY* in *in vitro* plantlets of *Camillia sinensis*. *Intl. J. Agric. Biol*., 20: 676–682

Wu, L. and H.Q. Yang, 2010. *CRYPTOCHROME* 1 is implicated in promoting R protein-mediated plant resistance to *Pseudomonas syringae* in *Arabidopsis*. *Mol. Plant*, 3: 539–548

Xu, P., Y. Xiang, H.L. Zhu, H.B. Xu, Z.Z. Zhang, C.Q. Zhang, L.X. Zhang and Z.Q. Ma, 2009. Wheat cryptochromes: subcellular localization and involvement in photomorphogenesis and osmotic stress responses. *Plant Physiol.*, 149: 760–774

Xue, Y.F., N.W. Yin, B.J. Chen, F.F. Liao, A.N. Win, J.Y. Jiang, R. Wang, X.Y. Jin, N. Lin and Y.R. Chai, 2017. Molecular cloning and expression analysis of two *FAD2* genes from chia (*Salvia hispanica*). *Acta Physiol. Plantarum*, 39: 95

Yanovsky, M.J. and S.A. Kay, 2003. Living by the calendar: how plants know when to flower. *Nat. Rev. Mol. Cell Biol*., 4: 265–276

Yuan, S., Z.W. Zhang, C. Zheng, Z.Y. Zhao, Y. Wang, L.Y. Feng, G.Q. Niu, C.Q. Wang, J.H. Wang, H. Feng, F. Xu, F. Bao, Y. Hu, Y. Cao, L.G. Ma, H.Y. Wang, D.D. Kong, W. Xiao, H.H. Lin and Y.K. He, 2016. *Arabidopsis* cryptochrome 1 functions in nitrogen regulation of flowering. *Proc. Natl. Acad. Sci. USA*, 113: 7661–7666

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