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Full Length Article

GI (*GIGANTEA*) Genes from Chia (*Salvia hispanica*): Molecular Characterization, Flowering-Related Expression and Evolutionary Features

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Abstract

Chia (*Salvia hispanica*), originated in Mexico, has outstanding nutritional and health-promoting values, but it is the only ancient American Indian staple crop failed in introduction to the Old Continents. After the rediscovery and revival of chia as a new crop in recent years, the short-day (SD) habit is a crucial limitation for its worldwide cultivation. The circadian oscillator GIGNATEA (GI) is an important transcription factor regulating flowering time through photoperiod-pathway. In this study, we cloned the full-length cDNAs of two *GI* genes from chia, and analyzed the molecular characteristics of the genes and the encoded proteins. Alternative transcription initiation sites, alternative poly A tailing sites, and 5'-UTR intron retention exist in some of their mRNAs. The origin of *GI* gene accompanied with the transition from aquatic to terrestrial habits during plant evolution. *GI* duplication events occurred at order, family and genus levels in angiosperms. *ShGI-1* and *ShGI-2* were similar to each other in organ specificity with peak expression in small buds. In mature leaf, *ShGI-2* is dominant over *ShGI-1* in terms of expression level with highest expression in the afternoon, but on the Autumnal Equinox day *ShGI-1* is dominant over *ShGI-2* with peaks at noon and in the evening. KT, BR, GA_3 and IAA upregulated the expression of *ShGI-1* and *ShGI-2* in long-days (LDs) and inhibited their expression in SDs, with GA_3 being the most effective phytohormone. Under most abiotic stresses, *ShGI* expression fluctuated and returned to near-basal levels. *ShGI* expression was upregulated by low temperature. SA sharply upregulated *ShGI* expression after 24 h of treatment. This is the first report of *GI* genes from the order Lamiales, which will promote the dissection of flowering mechanism of chia and other Lamiales plants, enrich the evolution and expression characteristics of plant *GIs*, and promote the study on interaction between photoperiod and hormone pathways in flowering time control.

Keywords: Abiotic stresses; Chia (*Salvia hispanica*); Evolution; GIGNATEA (GI); Photoperiod; Phytohormones

Introduction

Since the rediscovery and revival of the ancient and sacred oil crop chia (*Salvia hispanica*) in recent years, it has become more and more attractive due to its high content of polyunsaturated fatty acids (PUFAs) especially α-linolenic acid (ALA) (Sreedhar *et al*. 2015). Chia, an oil crop containing the highest level of ALA among the known crops, grows in deserts below 4,000 feet in Mexico and Southwest Americas, and was cultured by ancient Astek and Mayas as one of the important staple crops (Ayerza and Coates 2005). It is also one of the most valuable crops in the Lamales order. Chia was the sacred crop of Aztecs, but the attempts have failed in introducing chia to the world since the discovery of the New World by Christopher Columbus, because it has strict short-day habit, high sensitivity to changes in photoperiods and weak tolerance to cold (Jamboonsri *et al*. 2012). Because of photoperiod sensitivity, the feasible geographic belts for cultivating traditional chia germplasms for grain production is restricted to 22°55'N-25°05'S (Hildebrand *et al*. 2013), and at higher latitudes the probability of the crop reaching maturity is low

(Ayerza and Coates 2005). Nowadays chia is commercially cultivated in several low-latitude agricultural regions in the world, mainly in Bolivia, Paraguay, Argentina, Mexico, Australia, Central America, Peru, Ecuador and Colombia, and the total acreage in 2014 was 370,000 hectares (Sosa 2016; Orona-Tamayo *et al.* 2017). In China, we tested chia cultivation at a 30˚ N site in winter-warm Sichuan Basin, it flowered in October, and less than one-half of the seed could reach full maturation even if we harvested it in late December (Win *et al.* 2018). Analyzing its flowering regulation mechanism is the basic prerequisite for creating precocious varieties and extending its cultivation to middle- and high-latitude agricultural regions.

The floral induction is mainly regulated by five pathways, including photoperiod, autonomic, gibberellin, vernalization and aging pathways (Borner *et al.* 2000; Yuan *et al*. 2016; Ozturk 2017), in which the photoperiod pathway in monocots or dicots 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. It is reported that *GIGANTEA* (*GI*) is one of the important genes involved in normal life activities in plants. It encodes a nucleoprotein that participates in many molecular regulatory responses, such as control of circadian rhythms, transcriptional regulation of flowering, tolerance to stresses, etc. Numerous studies suggest that GI is one of the key factors controlling the plants circadian rhythm and flowering time and positively regulates the expression of downstream genes such as *CO*, *FT* and *SOC1* (Mizoguchi *et al.* 2005; Jung *et al*. 2007; Duan *et al.* 2019; Chen *et al*. 2020).

With the in-depth study of *GI*, it is clear that *GI* gene and protein sequences are quite conservative among plants. However, the structure and function of *GI* have not yet been fully understood (Dalchau *et al.* 2011; Kim *et al.* 2012). GI regulates gibberellin signaling through stabilization of the DELLA proteins in *Arabidopsis* (Nohales and Kay 2019). GI recruits the UBP12 and UBP13 deubiquitylases to regulate accumulation of the ZTL photoreceptor complex (Lee *et al.* 2019). HOS15 associates with a histone deacetylase complex to inhibit transcription of the GI-mediated photoperiodic flowering pathway in *Arabidopsis* (Park *et al*. 2019). Most modulation levels of light and temperature signaling by GI regulate the output and pace of the circadian clock (Nohales *et al*. 2019; Ronald *et al*. 2020; Park *et al*. 2020). Circadian process will establish the daily phasing of the behavioral, developmental, and the proper coordination of physiology and metabolism; AtGI is a co-chaperone and promotes maturation of F-box protein ZEITLUPE, which is a crucial regulator of the circadian clock (Cha *et al*. 2017). In addition to controlling plant flowering time and circadian rhythms, GI also has numerous functions such as stress tolerance. Suárez-López *et al.* (2001) firstly found it as a flowering regulatory factor in *Arabidopsis* to activate *FT* by regulating the transcription factor gene *CO* in long days (LDs), allowing the plant to grow from vegetative stage to reproductive stage. At present, there are many studies about *AtGI* gene, which is known to play a role in drought tolerance, circadian clock control, miRNA processing, chlorophyll accumulation, light signal transmission, cold resistance, salt tolerance and herbicide resistance, besides regulating flowering time (Cao *et al.* 2005; Mishra and Panigrahi 2015; Cha *et al*. 2019). Mutation of *gi* in *Arabidopsis* caused flowering delaying and increased tolerance to H_2O_2-induced oxidative stress (Fowler *et al*. 1999; Thiruvengadam *et al.* 2015), whereas overexpression of *AtG1* caused early flowering (Mizoguchi *et al*. 2005). AtGI interacted with FLAVIN-BINDING, KELCH REPEAT, AND F-BOX1 (FKF1) proteins to form the complex AtGI-AtFKF1, which promotes flowering advancement by degrading CO inhibitors. However, it is strange that overexpression of *OsGI* in rice resulted in postponement of flowering time in LDs or short days (SDs), increased the expression of *Hd1*, and down-regulated the expression of *Hd3a*, which indicates that the regulatory effect of *CO* on *FT* in rice is the opposite of that in *Arabidopsis* (Hayama *et al*. 2003). In addition, another study of short-day plant *Pharbitis nil* found that overexpression of *PnGI* delayed flowering and *PnFT1* was down-regulated (Higuchi *et al*. 2011). Bendix *et al.* (2013) studied the function of *GI* (*GI1*) in maize and found that the mutant *gi1* promoted pre-flowering in LDs but did not show significant difference with wild-type in SDs, suggesting that wild-type *GI1* participates in a pathway that suppresses flowering in LDs. The expression of *GI* in *Brassica oleracea* was the highest at the 8 to 12 h of the light period and lowest at dawn under LD conditions, and down-regulation of *GI* expression in transgenic *B. rapa* enhanced salt tolerance (Thiruvengadam *et al*. 2015; Kim *et al*. 2016b). Li *et al*. (2013) isolated three *GI* genes (*GmGI1*, *GmGI2* and *GmGI3*) from soybean (*Glycine max*), *GmGI1* had two alternative splices (*GmGI1α* and *GmGI1β*), and all GmGIs interacted with FKF1/FKF2 proteins to promote flowering. In summary, *GI* is one of the key genes controlling flowering time, but there are significant differences in function and mechanism among different photoperiod-types of plants. *GI* genes were also cloned and characterized from many other crops such as longan (*Dimocarpus longan*), sweet potato (*Dioscorea esculenta*), chrysanthemum (*Dendranthema morifolium*), soybean and rapeseed (*Brassica napus*) (Li *et al*. 2013; Xie *et al.* 2015; Huang *et al*. 2017; Tang *et al.* 2017).

Chia is a revived crop with worldwide potential importance, but there are a few reports on chia about its flowering regulation mechanism. Our team is engaged in molecular dissection of the fatty acid and flowering traits of chia and the key enzyme loci *FAD2* and *FAD3* of ALA biosynthesis pathway as well as the flowering-related regulatory loci *CRY* and *SOC1* from chia have been reported in our previous studies (Xue *et al.* 2017, 2018; Chen *et al.* 2019, 2020). In this study we cloned two *GI* genes (*ShGI1*

and *ShGI2*) from chia, analyzed their gene and protein structural features, and investigated their expression features as related to organ-specificity, diurnal dynamics, seasonal transition dynamics and responsiveness to phytohormones and abiotic stresses. Furthermore, our phylogenetic analysis also revealed some new features of plant *GI* evolution.

Materials and Methods

Plant materials, treatment and nucleic acid extraction

For cloning and expression study of *GI* genes, chia plants were 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 and 23:28 of the day. They are 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 days old), medium seeds (MS, about 20 days old) and late seeds (LS, about 30 days old) were sampled for detecting the organ-specificity of the cloned genes.

There were treatments with growth-stimulating phytohormones. The methods used to cultivate the seedlings of chia in the artificial climate chambers followed the reference of Xue *et al*. (2017). The 6-leaf stage seedlings were moved to the plant growth chambers for treatments with two styles 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 30ºC 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} gibberellin (GA_3) 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 respectively. Adult leaves were sampled at each time point for characterization of responsiveness of cloned genes to growth-stimulating phytohormones.

There were treatments with growth-inhibiting phytohormones and abiotic stresses. Chia seedlings were cultured in the artificial climate chamber and subjected to high temperature at 38ºC, low temperature at 4ºC, mechanical wounding, 100 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 h, 0.5 h, 3 h, 9 h, 24 h and 48 h time points after treatment, adult leaf samples were taken for characterization of responsiveness of cloned genes to growth-inhibiting phytohormones and abiotic stresses (Xue *et al*. 2017).

In nucleic acids preparation, each study had three biological replicates. Samples were all kept in liquid nitrogen for transportation and stored at -80ºC. Total RNA was extracted using the Biospin Plant Total RNA Extraction Kit (BioFlux), and total gDNA was extracted from adult leaves using a CTAB method (Saghai-Maroof *et al*. 1984). Electrophoresis and spectrophotometric detection were adopted to detect the quality and quantity of the nucleic acids.

Cloning of the conserved region sequences of chia *GI* genes

Since chia does not have whole-genome sequencing database and little EST, TSA, GSS and other tag sequences of chia could be found in GenBank, traditional dark-box strategy should be used to clone its genes. In order to clone the conservative regions of chia *GI* genes, the *Arabidopsis thaliana GI* mRNA (NM_102124.3) was firstly retrieved from NCBI GenBank, and used as an electron probe for the *in silico* cloning of *GI* sequences from the chia-relative species such as sesame (*Sesamum indicum*), *Erythranthe guttatus*, *Salvia pomifera* and *Salvia miltiorrhiza*. All *GI* reference mRNA, TSA, EST and gDNA tag sequences were downloaded and multiple alignments were created. At the conservative sites of *GI* alignments, degenerate primer combination FLGIC + RLGIC was designed (Table 1). One μg of total RNA equal-proportionally mixed from all organs was subjected to gDNA deletion and reverse-transcribed using the PrimeScript Reagent Kit with gDNA Eraser (TaKaRa Dalian, China) to obtain the first strand library of the total cDNAs as a template for conventional Taq-PCR amplification of the conservative regions of chia *GI* genes (Annealing at 58ºC and extension for 2 min). Conventional electrophoresis, gel recovery, recombination with pMD19-T vector 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 and walking primers.

5'-RACE and 3'-RACE of chia *GI* genes

To obtain the sequence information of the 5'-ends (since the transcription initiation site) and 3'-ends (before the poly A tail), we performed rapid-amplification of cDNA ends (RACE) of chia *GI* genes. The sequencing result of conservative region colonies signified one chia *GI* gene, which was named as *ShGI*. Then 5'-RACE and 3'-RACE primers of *ShGI* were designed (Table 1) according to the conservative sites within the conservative region sequence. One μg of total RNA from organ-mixture was used to handle RACE procedures in terms of the usual manual of the SMARTer™ RACE Amplification Kit (Clontech, USA) to obtain the first-strand total cDNA templates of the 5'-RACE and 3'-RACE. Primers FShGI3-1 and FShGI3-2 were used for pairing with the universal primers LUPM and NUP (Table 1) for 3'-RACE primary and nested amplifications of *ShGI*, respectively. The PCR annealing temperature was 64°C and the extension time was 1 min.

Primers RShGI5-1 and RShGI5-2 were matched with the universal primers LUPM and NUP (Table 1) for primary and nested amplifications of 5'-RACE of *ShGI*, respectively. The PCR annealing temperature was 62ºC and the extension time was 1 min. Electrophoresis, gel recovery, TA cloning and sequencing were performed.

Cloning of full-length sequences of chia *GI* genes

Based on the sequencing results of 5'-RACE and 3'-RACE colonies, cDNA ends of two chia *GI* genes, *ShGI-1* and *ShGI-2*, were produced. When they were assembled with the *ShGI* conservative region sequence, correct matching pairs between the 5'-ends and the 3'-ends can be revealed. Then we designed the primer combinations of FShGI-1 + RShGI-1 and FShGI-2 + RShGI-2 (Table 1) for PCR amplification of the full-length sequences of the two chia *GI* genes, using 3'-RACE template, annealed at 62°C, and extended for 5 min. Electrophoresis, gel recovery, TA cloning and sequencing were performed.

qRT-PCR detection of expression profiles of chia *GI* genes

In order to reveal the organ-specificity, photoperiod induction, phytohormone responsiveness and abiotic stress responsiveness, the transcriptional expression of *ShGI-1* and *ShGI-2* was detected by using primer pairs FShGI-1RT + RShGI-1RT and FShGI-2RT + RShGI-2RT, respectively. The *25SrRNA* gene was detected by primer pair F25SRT + R25SRT as internal control (Table 1). The stability of the reference gene *26SRNA*/*25SRNA* in plants was reported by a literature (Singh *et al.* 2004). It is one of the most conserved housekeeping genes among eukaryotes in terms of both sequence and expression. Its feasibility as an internal control in perilla and chia has been proved in our previous studies (Xue *et al.* 2018). The contaminated genomic DNA in the total RNA was eliminated before reverse transcription according to the manual of the PrimeScript Reagent Kit with gDNA Eraser (TaKaRa Dalian, China) with prolonged DNase treatment, and the complete digestion of DNA was ascertained by 50-cycles of PCR amplification of *25SRNA* gene using the treated RNA as template, which did not generate detectable product. qRT-PCR was performed on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, U.S.A.) with thermal cycling parameters of 95°C for 10 min and 45 cycles of amplification (95ºC for 10 sec, 64ºC for 20 sec and 72ºC for 10 sec). When qRT-PCR was completed, the temperature was raised from 65ºC to 95ºC, and the melting curve was detected to confirm the specificity of the amplification. Only those results without distinct dimers and nonspecific products were used for analysis. The results with dimers or nonspecific products were abandoned, and PCRs were redone with optimized annealing temperatures and other PCR conditions until acceptable results were achieved.

Bioinformatics analysis involved in this study

Sequence creation, analysis, annotation, translation, alignments, assemblage and other analysis were mainly performed on Vector NTI Advance 11.5.1 and DNAStar version 7.1.0 softwares. *In silico* cloning, BLAST and CDD assays were performed on NCBI (http://www.ncbi.nlm.nih.gov), and protein analyses were performed on Expasy (http://www.expasy.org), GSDS 2.0 (http://gsds.cbi.pku.edu.cn/), CBS (http://www.cbs.dtu.dk/services/), etc. Based on multi-alignment, gb sequence analysis and oligo analysis on Vector NTI Advance 11.51, candidate primers were manually or automatically designed corresponding to the optimum conservative or divergent sites, and were evaluated on Primer Premier 6 to choose the best ones for practical utilization. In calculating the Tm value of the PCR primer, the Nucleic Concentration in Reaction Conditions on Primer Premier 6 was set to 100 nM. On the French website (http://www.phylogeny.fr/) (Dereeper *et al*. 2008), "A la Carte" mode was selected for phylogenetic tree construction. Number of bootstraps was set to 1000, until the completion of tree-building.

Results

Cloning of full-length cDNAs of *ShGI* genes

Electrophoresis showed that a specific 3.6 kb band was amplified for the conservative region of the chia *GI* genes. Sequencing result of three positive clones produced one member gene, and its NCBI BLASTn analysis showed highest homology to plant *GI* genes and was named as *ShGI*. No significant bands were found in the primary amplifications of 5'-RACE and 3'-RACE of *ShGI*, with smear at the predicted size. The 5'-RACE nested PCR of *ShGI* generated a band of about 400 bp. After TA cloning, all the clones had insert length polymorphisms, and sequencing results of batch clones generated the 5'-ends of two chia *GI* genes, named as *ShGI-1* and *ShGI-2* respectively. The net 5'-end lengths of *ShGI-1* were 423, 390, 375, 366, 345, 303 and 301 bp, while the net 5'-end lengths of *ShGI-2* were 408 and 384 bp. The *ShGI* 3'-RACE nested PCR generated a band of about 0.5 kb. All the clones had polymorphic insert length after TA cloning. Sequencing of batch clones produced 3'-ends of two chia *GI* genes. The net 3'-end lengths of *ShGI-1* were 519, 452 and 441 bp, while 560 and 547 bp for *ShGI-2* (Poly A not included). When assembling the conservative region sequence with the cDNA ends, correct end-to-end pairs of RACE results were obtained and PCR primer pairs were designed to amplify the full-length chia *GI* genes. A band of about 4 kb identical to the expected size was obtained in both amplifications of the full-length cDNAs of *ShGI-1* and *ShGI-2*. We used chia total gDNA as a template to amplify the full-length gDNA of the two genes, which was unsuccessful even if we

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

Primers	Sequence (5'→3')	Application
FLGIC	CTCTCTCTAATCTCTCTCCACCCAAA	Forward primer for chia *GI* conservative regions amplification
RLGIC	CGAACTGTAGCTGGGAGGCGACA	Reverse primer for chia *GI* conservative regions amplification
FShGI3-1	GCTTGAATGGGGAGAGTCAGGA	GSP for *ShGI* 3'-RACE primary amplification
FShGI3-2	GGGGAGAGTCAGGATTAGCAGT	GSP for *ShGI* 3'-RACE nested amplification
RShGI5-1	CATGCAAGGGCCCACTGCTC	GSP for *ShGI* 5'-RACE primary amplification
RShGI5-2	CCATGCTCCGGATGGTGAAGAAC	GSP for *ShGI* 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
FShGI-1	CTAGTTAAAGATCTCTTTCTCTCTCTCTAA	*ShGI-1* full-length forward primer
RShGI-1	CATAGAATAATACTACAATTAATATAAATATATTATACATAC	*ShGI-1* full length reverse primer
FShGI-2	ATTCTCTCTCCCATTTCTCTCTCTAA	*ShGI-2* full-length forward primer
RShGI-2	GAGAGAATGAGTTATCCAAACAATAAGAAC	*ShGI-2* full length reverse primer
F25SRT	GATTTCTGCCCAGTGCTCTGAA	*25SrRNA* qRT-PCR forward primer
R25SRT	TCTGCCAAGCCCGTTCCCTT	*25SrRNA* qRT-PCR reverse primer
FShGI-1RT	TGTCGCCTCTCAGCCACC	*ShGI-1* qRT-PCR forward primer
RShGI-1RT	GTTCACGTCCGGTAGTTTGC	*ShGI-1* qRT-PCR reverse primer
FShGI-2RT	TGTCGCCTCCCAGCCACA	*ShGI-2* qRT-PCR forward primer
RShGI-2RT	GTTCACATCCGGTGGTTTGG	*ShGI-2* qRT-PCR reverse primer

replaced reagents and optimized the amplification cycle parameters, indicating that they either have very long introns or have very complex structures.

Structure and features of *ShGI* genes

ShGI-1 has two versions of mRNA (GenBank Accession Numbers MH107333 and MH107334, poly A not included, Fig. S1). The longest standard mRNA of *ShGI-1* is 3837 bp with 5'-UTR of 178 bp, ORF of 3504 bp and 3'-UTR of 155 bp, while the longest mRNA with 5'-UTR intron retention is 4067 bp with 5'-UTR of 408 bp, ORF of 3504 bp and 3'-UTR of 155 bp. This 5'-UTR intron has non-standard splicing left border (GG…AG). *ShGI-2* has longest mRNA of 3876 bp (GenBank Accession Number MH107335, poly A not included) with 5'-UTR of 163 bp, ORF of 3504 bp and 3'-UTR of 209 bp (Fig. S1). The G+C contents of the 5'-UTR, ORF and 3'-UTR are 38.48/42.94%, 46.89/46.99% and 26.45/35.71% in *ShGI-1/ShGI-2*, respectively. The identity percentages between *ShGI-1* and *ShGI-2* are 90.2% on mRNA level and 93.5% on ORF level. BLASTn analysis shows that *ShGI-1* and *ShGI-2* have high homology to sesame *GIGANTEA*-like LOC105178750 and LOC105158892 mRNAs, *E. guttatus GIGANTEA*-like LOC105959402 mRNA, etc.

Characterization of deduced ShGI proteins

The ShGI-1 and ShGI-2 proteins (Fig. S1) are 1167 and 1174 aa in length, with theoretical MWs of 127.56 and 105.84 kD, pIs of 6.35 and 6.45, respectively, which are weakly acidic. The identity percentage between the two proteins is 95.1% and the positives percentage is 96.2%. BLASTp result shows that ShGI-1 and ShGI-2 have high homology with sesame GIGANTEA-like and *E. guttatus* GIGANTEA-like.

SignalP 4.1 (Petersen *et al*. 2011) prediction indicates that ShGI-1 and ShGI-2 do not contain a signal peptide. BaCelLo (Pierleoni *et al*. 2006), EpiLoc (http://epiloc.cs.queensu.ca/) and Plant-mPLoc (Chou and Shen 2010) predicted the subcellular localization of ShGI-1 and ShGI-2 to be in the nuclear. SLP-Local (Matsuda *et al*. 2005) predicted them to be in cytoplasm or nuclear. YLoc (Hooper *et al*. 2014) predicted them to be in the nucleus. TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and TOPCONS (Tsirigos *et al*. 2015) predicted no transmembrane structures in ShGI-1 and ShGI-2. NetPhos3.1 (http://www.cbs.dtu.dk/services/NetPhos/) predicted 117/102 potential phosphorylation sites in ShGI-1/ShGI-2, including 77/68 S (serine), 28/22 T (threonine) and 12/12 Y (tyrosine) sites. Summarily, both ShGI-1 and ShGI-2 proteins are most probably located in the nucleus and might be regulated by phosphorylation.

In the secondary structure of ShGI-1/ShGI-2 predicted by SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/secpred_sopma.pl), α-helix, β-sheet (extended strand), β-turn and random coil account for 48.93/46.69%, 10.45/10.87%, 6.68/5.69% and 33.93/36.75%, respectively (Fig. 1). The α-helices in their proteins are nearly evenly distributed, but their locations are somewhat different between ShGI-1 and ShGI-2.

In *Arabidopsis* and other plants, *GI* plays an important role in the photoperiodic regulation of flowering (Park *et al*. 2013). *ShGI* may be involved in the regulation of flowering in chia according to ShGI protein structure, key sites in the conserved region, and physico-chemical properties.

Phylogenetic relationships of *GIs* in plants

In order to explore the phylogenetic relationship of plant *GI* genes, we selected some representative species that have complete genome sequence from different taxa of plant kingdom (green algae, ferns, gymnosperms, monocots and dicots). GI protein sequences from Chia and these species were used to construct a phylogenetic tree of plant GI proteins (Fig. 2). The phylogenetic relationships are

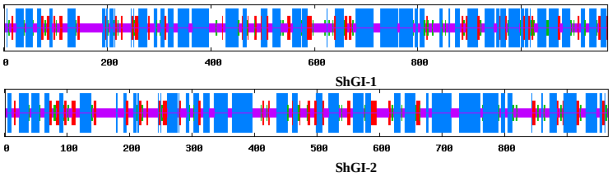

Fig. 1: Predicted secondary structures of ShGI-1 and ShGI-2

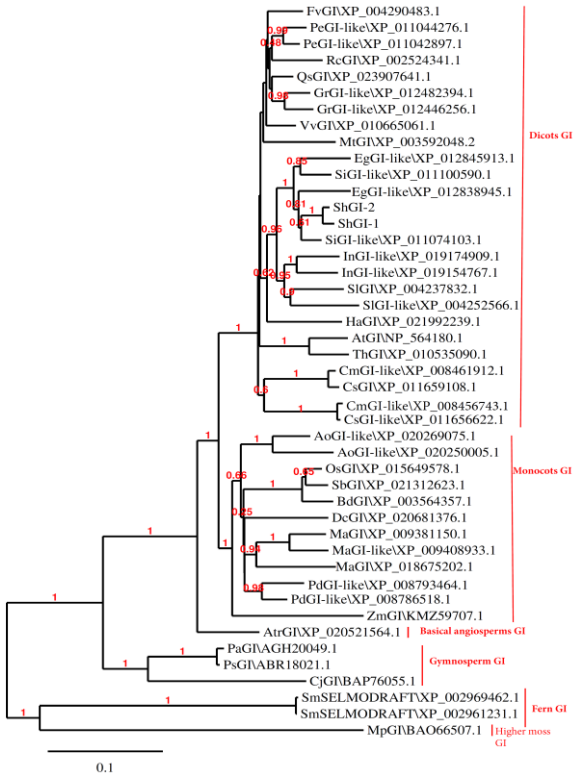

Fig. 2: Phylogenetic relationship of GI proteins from plant kingdom
Ao, *Asparagus officinalis*; At, *Arabidopsis thaliana*; Atr, *Amborella trichopoda*; Bd, *Brachypodium distachyon*; Cs, *Cucumis sativus*; Dc, *Dendrobium catenatum*; Eg, *Erythranthe guttatus*; Fv, *Fragaria vesca*; Gr *Gossypium raimondii*; Ha, *Helianthus annuus*; In, *Ipomoea nil*; Ma, *Musa acuminate*; Mt, *Medicago truncatula*; Pa: *Picea abies*; Pe, *Populus euphratica*; Pd, *Phoenix dactylifera*; Rc, *Ricinus communis*; Sb, *Sorghum bicolor*; Sh, *Salvia hispanica*; Si, *Sesamum indicum*; Sl, *Solanum lycopersicum*; Sm, *Selaginella moellendorffii*; Th, *Tarenaya hassleriana*; Vv, *Vitis vinifera*

consistent with the previous taxonomic research in the academic community, which are divided into several major groups based on the evolutionary relationships of *M. polymorpha*, *Selaginella moellendorffii*, gymnosperms, basal angiosperms, monocots and dicots, but some new evolutionary features of the *GI* genes can be observed.

There is no *GI* gene in aquatic lower plants such as green algae and mosses, but *GI* genes exist in aquatic-to-terrestrial transitional plant *M. polymorpha*, lower fern *S. moellendorffii*, gymnosperms, basal angiosperm *Amborella trichopoda*, monocots and dicots. This means that the origin of the *GI* gene was far earlier than the origin of flowering plants. It is assumed that *GI* originated during the transition of plants from aquatic to terrestrial habits.

There is only one *GI* gene in *Dioscorea paniculata*, *S. moellendorffii*, gymnosperms and basal angiosperm *A. trichopoda*. Although there are two GI protein sequences cloned from *S. moellendorffii*, but they are highly similar to each other, which might be caused by the heterozygosity of the genome sequencing materials, though recent *GI* duplication in *S. moellendorffii* could not be excluded.

Basal angiosperms also have only one *GI* gene, and no uniform duplication of *GI* gene occurred in gymnosperm ancestor, angiosperm ancestor, monocot ancestor and dicot ancestor. However, *GI* gene duplication events occurred at angiosperm order level (*e.g.*, Lamiales in which sesame and *E. guttatus* are located), family level (*e.g.*, Malvaceae in which cotton is located) or genus and lower levels, thus many monocot and dicot species have two or more *GI* genes. As for genus-level *GI* duplication, there should be many events. For example, the well-known genome triplication in the ancestor of tribe *Brassiceae* would certainly lead to *GI* gene triplication, but this is not the focus of this study.

In Lamiales, there are two *GI* genes in *E. guttatus* and sesame; the orthologous genes from different species are clustered together, while the paralogous genes within a species are far apart, implying that an order-level *GI* gene duplication event occurred in Lamiales. However, the two chia *GI* genes cloned in this study correspond to only one *GI* gene of *E. guttatus* and sesame. It is speculated that another *GI* gene might have been lost in chia or in *Salvia* genus and the remaining one experienced a recent duplication event in genus *Salvia*. Whether or not the orthologous gene corresponding to another *GI* gene of sesame and *E. guttatus* has been really deleted in genus *Salvia* needs to be clarified in future research.

Organ-specificity of *ShGI* genes

The results of qRT-PCR (Fig. 3) show that *ShGI-1* is expressed in all organs but with strong organ-specificity. Its expression is very high in small buds, low in roots, stems, leaves, middle buds, big buds and seeds, and very low in stems and functional leaves. The organ-specificity of *ShGI-2* is similar to *ShGI-1*. Overall, *ShGI-1* is higher than *ShGI-2* in expression in all organs. The latter results of this study will show that *ShGI* has circadian rhythm fluctuations. As each organ was detected for only one time point of the day, the organ-specificity of *ShGI* genes reveled here is not the complete profile of gene features.

Circadian rhythms of *ShGI* genes in response to seasonal transition

The qRT-PCR was used to detect the circadian rhythms and the response to the seasonal change of long-short photoperiods of *ShGI-1* and *ShGI-2* in moderately mature functional leaves. The results showed that there were significant differences between *ShGI-1* and *ShGI-2* (Fig. 4).

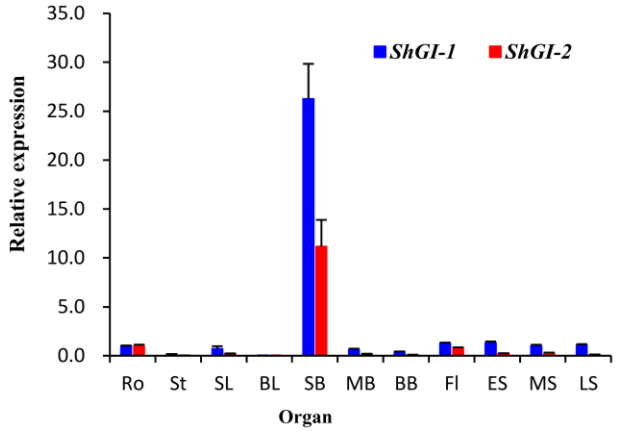

Fig. 3: Relative expression of *ShGI-1* and *ShGI-2* genes in different chia organs
Ro: root; St: stem; SL: small leaf; BL: big leaf; SB: small bud; MB: middle bud; BB: big bud; Fl: flower; ES: early seed; MS: middle seed; LS: late seed.

On August 21–22 (LD, sunny, 28–38ºC), *ShGI-1* expression was low in the whole day but peaked at midnight, whereas *ShGI-2* kept high level from late morning to midnight and low level from midnight to early morning. On September 5-6 (LD, rainy, 20–24ºC), both *ShGI-1* and *ShGI-2* were distinctly expressed from late morning to the evening with a peak in the afternoon, *ShGI-2* was more distinct than *ShGI-1* and from the evening to the early morning their expression was low. On September 20 and 21 (Autumnal equinox, sunny, 20–28ºC), *ShGI-1* was distinctly expressed from the morning to the midnight with two peaks at noon and in the evening respectively, while *ShGI-2* was only slightly upregulated during daytime with relatively low level, and from midnight to the early morning they both were not expressed. On October 5–6 (SDs, cloudy to overcast, 20–29ºC), the expression of *ShGI-1* and *ShGI-2* was similar to that on September 5–6, reaching a peak in the afternoon, but maintaining low levels from midnight to morning. Taken together, the two genes generally have expression peaks from late morning to midnight especially in the afternoon, the expression of *ShGI-2* is higher than that of *ShGI-1* in either LDs or SDs, while *ShGI-1* is dominant over *ShGI-2* on the Autumnal equinox day especially at noon and evening peaks.

Effects of phytohormones on expression patterns of *ShGI* genes in LDs and SDs

In this study, KT, BR, GA_3 and IAA treatments were performed on 6-leaf stage chia seedlings in LDs and SDs, respectively. The expression changes of *ShGI-1* and *ShGI-2* were detected by qRT-PCR (Fig. 5). There were also differences and similarities between/among photoperiods, hormones and genes. In BR treatment, *ShGI-1* and *ShGI-2* were firstly dramatically upregulated in LDs, and then slightly fell back. However, *ShGI-1* and *ShGI-2* were dramatically down-regulated by BR in SDs, then kept at low levels. In GA_3 treatment, *ShGI-1* and *ShGI-2* were significantly upregulated in LDs, but its effect was slower than that of BR; conversely, they were rapidly down-regulated in SDs, and then stayed at low levels. In IAA treatment, *ShGI-1* and *ShGI-2* were gradually upregulated in LDs. However, *ShGI-1* and *ShGI-2* firstly were inhibited slightly by IAA in SDs, and then returned to basal levels with even a little upregulation. In KT treatment, *ShGI-1* and *ShGI-2* were significantly increased to and kept at a certain level in LDs, while in SDs they were down-regulated and restored soon. Taken together, in the chia leaf, *ShGI* genes are promoted to varying degrees in LDs by phytohormones BR, KT, IAA and GA_3, but are inhibited by these phytohormones to varying degrees in SDs, with GA_3 being the most effective phytohormone. LD is opposite to SD in manifesting the effects of phytohormones on *ShGI* expression in chia leaf.

Expression patterns of *ShGI* genes under various abiotic stresses

According to reports, *GI* regulates the circadian rhythm, growth and development of plants, and responses to salt stress, and thus has important basal functions. However, its response to other abiotic stresses is rarely reported. We used 5-week old chia seedlings to perform multiple stress treatments and detected changes in the expression of *ShGI-1* and *ShGI-2* based on qRT-PCR (Fig. 6). The expression of *ShGI-1* and *ShGI-2* was similar to each other under various stresses. After cold treatment at 4ºC, expression of *ShGI-1* and *ShGI-2* slightly fluctuated within 48 h with an overall trend of upregulation, and *ShGI-1* was more sensitive than *ShGI-2*. At 38ºC heat stress, *ShGI-1* and *ShGI-2* were temporarily sharply upregulated and quickly returned to basal levels. After MeJA treatment, *ShGI-1* and *ShGI-2* were upregulated dramatically and then slowly fell back to reach basal levels at 48 h. After mechanical wound, ABA and NaCl treatments, *ShGI-1* and *ShGI-2* were firstly down-regulated, and then fluctuated with recovery or even upregulation, but the overall trends were downregulation. After PEG treatment, *ShGI-1* and *ShGI-2* were relatively stable in expression with a little upregulation. *ShGI-1* and *ShGI-2* responded to SA treatment very slowly, but after 24 h they were significantly upregulated, especially for *ShGI-1*.

Discussion

In this study, we isolated and molecularly characterized the two *GI* genes from chia, which is a recalcitrant short-day crop rediscovered recently. The two *ShGI* genes show typical structural features, and some features of plant *GI* gene origination and evolution are revealed. According to qRT-PCR results, *ShGI-1* and *ShGI-2* both are dominantly expressed in small buds, and are regulated by various internal and external signals with distinct responsiveness patterns especially opposite effects between LD and SD

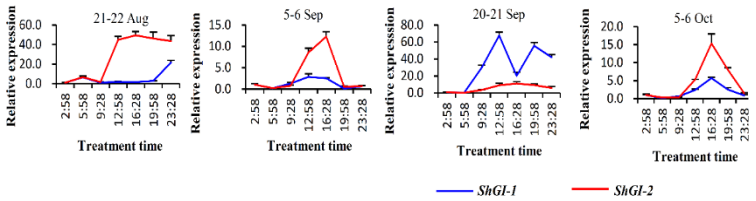

Fig. 4: Circadian rhythm of *ShGI-1* and *ShGI-2* expression, and response to long-short photoperiod seasonal changing

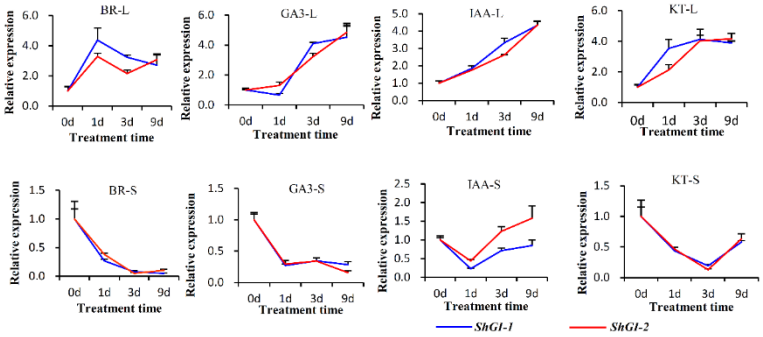

Fig. 5: Influence of important flowering hormones on the expression of *ShGI-1* and *ShGI-2* in long-short period

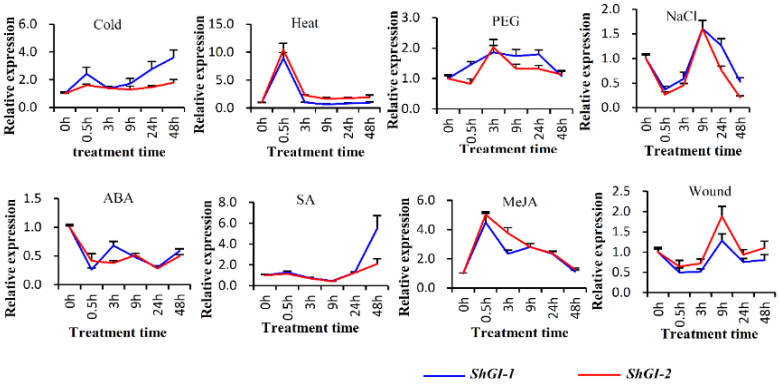

Fig. 6: Influence of abiotic stresses on the expression of *ShGI-1* and *ShGI-2*

photoperiods. Among these results, we mainly discuss the following major aspects.

The effects of phytohormones on expression of *ShGI* depend on the photoperiod condition

A large number of studies have shown that the phytohormones are involved in the regulation of flowering time, especially the gibberellin signaling pathway is one of the five major pathways of flowering induction. The hormonal pathway interacts with the photoperiodic and the vernalization pathway reactions (Seo *et al.* 2011). In tree peony of forcing culture, GA_3-hormone changes promoted *PsSOC1* and *PsSPL9* expression, and repressed *PsSVP* expression, which contributed to the improvement flowering quality (Guan *et al.* 2019). Although *GI* is involved in the regulation of many physiological functions, including flowering time, the report of its response to the phytohormones is not systematic. In view of this, in this study four phytohormones were used to treat chia seedlings in LDs and SDs, respectively. *ShGI-1* and *ShGI-2* were similar to each other in response characteristics. KT, BR, GA_3 and IAA promoted *ShGI-1* and *ShGI-2* in LDs, and inhibited them in SDs. GA_3 has the strongest effect among the four phytohormones.

This study shows that the effects of phytohormones on *GI* expression depend on photoperiod, and the effect in LDs is contrary to that in SDs. This finding will promote the study on the interaction between photoperiod and hormonal pathways. At present, there lacks report on the effects of phytohormones on *GI* expression in both LDs and SDs within a study. In this study, we systematically reveal *ShGI*

expression as influenced by four phytohormones in both LDs and SDs, which provides a reference to other researchers to dive into *GI* regulation mechanisms.

ShGI expression changes in response to seasons and various abiotic stresses

In chia leaf, the expression of *ShGI-2* was higher than that of *ShGI-1* in LDs and SDs, high in the afternoon but low from midnight to the early morning, and was less influenced by rainy. However, from morning to midnight on the Autumnal equinox day, *ShGI-1* expression was dominant over *ShGI-2*. The expression of *AtGI* was high in the afternoon, low in the morning and at night in *Arabidopsis* in LDs or SDs (Mizoguchi *et al*. 2005; David *et al*. 2006; Paltiel *et al*. 2006; Rubio and Deng 2007; Sawa *et al*. 2007; Dalchau *et al*. 2011; Sawa and Kay 2011; Han *et al*. 2013). The *AtGI* gene was transformed into Chinese cabbage with an expression high in the afternoon and low in the morning and at night, regardless of day length (Xie *et al*. 2015; Kim *et al*. 2016a). Both in long-day plants *Annona squamosal*, *Medicago truncatula* and *Populus alba*, and in short-day plants *P. nil* and *Ipomoea batatas*, the expression of *GI* was high in the afternoon and low in the morning and evening in LDs or SDs (Paltiel *et al*. 2006; Ke *et al*. 2017; Tang *et al*. 2017; Barros *et al*. 2017). The *P. nil GI* in the dark continues to retain the same pattern in LDs or SDs, indicating strict biological clock control (Higuchi *et al*. 2011). It can be seen that the circadian rhythmic characteristics of *GI* in the plant kingdom are conserved among species and among gene members and the two chia *ShGI* genes have circadian rhythmic characteristics similar to other plants.

Some abiotic stresses also have an effect on the expression of *ShGI-1* and *ShGI-2*. The response of *ShGI-1* and *ShGI-2* was slow after SA treatment, but they were significantly upregulated after 24 h especially for *ShGI-1*. After MeJA treatment, the expression of *ShGI-1* and *ShGI-2* increased dramatically and gradually returned to the basal levels. After cold treatment at 4°C, *ShGI-1* and *ShGI-2* were slightly upregulated, and *ShGI-1* was more sensitive. In heat treatment at 38ºC, they immediately returned to basal levels after transient upregulation. After mechanical injury, ABA and NaCl treatments, the expression of *ShGI-1* and *ShGI-2* was first downregulated and then fluctuated, with an overall trend of a little downregulation. After PEG treatment, the expression of *ShGI-1* and *ShGI-2* was relatively stable with a little upregulation. Overall, some adversities have a certain influence on the expression of *ShGI*. The expression of *GI* was slightly upregulated when the *P. alba* plants were treated with high concentrations of NaCl, and *Arabidopsis* flowering time was generally delayed after the *PagGI* was transformed into *Arabidopsis* (Ke *et al*. 2017). The expression of *IbGI* in sweetpotato was down-regulated under cold treatment, but upregulated under heat treatment, and both drought and NaCl treatments upregulated *IbGI* (Tang *et al*. 2017). Reducing the expression of *GI* in transgenic rapeseed enhanced plants tolerance to NaCl (Kim *et al*. 2016b). The ABA-dependent signal gene *AtGI* participated in escaping drought in *Arabidopsis* by up-regulating *FT* and advancing flowering (Riboni *et al*. 2016). When sprayed with high or low concentrations of NaCl, *Arabidopsis* plants with *gi* deletion had stronger salt tolerance, while plants with overexpression of *GI* had the weakest salt tolerance (Park *et al*. 2013). Besides regulating plant growth and flowering, GA_3 can also amend antioxidant enzyme and osmotic regulation to improve salt tolerance of okra (Zhu *et al*. 2019). GA_3 and GI might have important mutual interactions to coordinate growth and development with stress tolerance. This study reveals the effects of eight abiotic stresses on the expression of *ShGI* genes in Chia, which is helpful to further study the relationship between adversity and chia flowering and other traits, and also enriches the understanding of the plants *GI* expression characteristics.

Evolutionary characteristics of plant *GI* genes

In this study, we selected representative species that have complete genome sequences from different taxa of plant kingdom, constructed a phylogenetic tree of GI proteins, and revealed some new features of the *GI* evolution in the plant kingdom. This study indicates that green algae and mosses have no *GI*, and *GI* is present in both *M. polymorpha* and higher plants. *GI* originated during the transition process of plants from aquatic habit to terrestrial habit, far earlier than the origin of flowering plants. Though gymnosperms are not considered to have true flowers, recent studies indicate that many of the flowering genes are present in gymnosperms (Mao *et al.* 2019). Obviously, the occurrence of *GI* was not originally to produce flowering traits, but rather to be involved in regulating development and adaptability of terrestrial plants which evolved more complicated traits than aquatic ancestor plants. The function of *GI* is to regulate phyB signaling pathways, biological clocks, flowering time, carbohydrate metabolism, seasonality in growth and cold tolerance (Cao *et al*. 2005; Kim *et al*. 2016a, 2017; Ding *et al*. 2018). It is even possible to discover in the future that *GI* regulates more aspects of growth and adaptability of terrestrial plants. *GI* exists far earlier than the origin of flowering plants, and its function should be far more than regulating flowering. It is speculated that regulating flowering is only a derived function from the original function of *GI* after its origin.

This study found that the duplication events of *GI* genes occurred in the evolutionary process within some orders, families and genera of angiosperms. There is no common duplication of *GI* across the plant kingdom, and thus only one *GI* gene exists in *M. polymorpha*, *S. moellendorffii*, gymnosperms and basal angiosperm *A. trichopoda*. Some monocot or dicot species still have only one *GI* gene, but some other species have two or more *GI*

genes. One *GI* duplication event occurred in the early period of Lamiales evolution, resulting in two *GI* genes in *E. guttatus* and sesame. However, the two *ShGI* genes cloned in this study were the result of a recent duplication in the genus *Salvia*. Whether the ortholog of another *GI* gene of *E. guttatus* and sesame has been lost in Chia and other *Salvia* species needs further cloning study to reach a conclusion. In related previous reports, generally only one *GI* gene for each species was selected (Ke *et al*. 2017; Tang *et al*. 2017), therefore it was not possible to effectively reveal the full-set evolutionary features of plant *GI* genes. For the first time, this study systematically reveals the evolutionary features of *GIs* in plant kingdom.

Studies have shown that *GI* regulates flowering, and activates the flowering gene *FT* by regulating the transcription factor gene *CO* in the flowering pathway of LDs, so that the plant enters the reproductive stage. When the longan *DlGI* gene was transformed into *Arabidopsis*, the flowering time was greatly advanced (Huang *et al*. 2017). When the poplar *PagGI* gene was transformed into *Arabidopsis*, it promoted the expression of *CO* and *FT* genes and caused early flowering (Ke *et al*. 2017), while the absence of *AtGI* caused late flowering of *Arabidopsis* (Tang *et al*. 2017). Since studies have shown that *GI* is a positive regulator of flowering in response to photoperiods by controlling circadian rhythms, and affects plants' resistance to stresses, it is speculated that *ShGI* family may also participate in flowering induction and regulate other physiological functions. Similar to *GI*s from sweetpotato, poplar, chrysanthemum, poplar, *Arabidopsis*, *etc*. (Ke *et al*. 2017; Tang *et al*. 2017), the two chia *GI* genes also have strong organ-specificity with dominant expression in small buds, suggesting that the *GI* gene regulates not only flowering time but also bud primordium differentiation.

GI is a promoting factor for flowering in the long-day plant *A. thaliana*, but in the short-day plant chia it is promoted by four phytohormones in LDs and inhibited by them in SDs. How to link this rule with the mechanism of short-day activation of flowering in Chia, and the mechanism of flowering regulation of typical short-day plants, needs to be studied in depth. In addition, the responses of the chia *GI* family to circadian rhythms, seasonal changes and abiotic stresses also suggest that these environmental factors may affect flowering or other reproductive traits by affecting the expression of *GI*, especially that the associations of chia *GI* genes with SA signaling and cold tolerance deserve special attention in the future.

ShGI-1 and *ShGI-2* are similar to each other in most expression characteristics and in the protein structures, therefore their protein activities and basic physiological functions should also be similar, with redundancy and additive effects. However, they do have distinct differences in circadian rhythm and seasonal changes as well as a little difference in response to phytohormones and abiotic stresses, implying functional divergence in regulating flowering time.

Conclusion

GI originated during the transition from aquatic habit to terrestrial habit of plants, and *GI* duplications occurred only in angiosperm orders, families and genera. Full-length cDNAs of two *GI* genes possibly regulating photoperiod-pathway flowering have been cloned from the revived short-day crop Chia. The 3837-bp *ShGI-1* mRNA and 3876-bp *ShGI-2* mRNA and their encoded proteins have typical structural and molecular features. *ShGI-1* and *ShGI-2* both have dominant expression in small buds, and are regulated by photoperiod, phytohormones and abiotic stresses. In mature leaf, *ShGI-2* is dominant over *ShGI-1* with highest expression in the afternoon, but on the Autumnal Equinox day *ShGI-1* is dominant over *ShGI-2* with peaks at noon and in the evening. KT, BR, GA_3 and IAA upregulate *ShGI-1* and *ShGI-2* in LDs and inhibit them in SDs, with GA_3 being the strongest phytohormone. Low temperature and SA upregulate *ShGI* expression, and other abiotic stresses also exert influences.

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Author Contributions

Conceived and designed the experiments: Bao-Jun Chen and You-Rong Chai. Performed the experiments: Bao-Jun Chen, Yu-Fei Xue, Cheng-Long Yuan, Lin Zhang, Jia-Yi Jiang and Xi-Yue Luo. Analyzed the data: Bao-Jun Chen, Yu-Fei Xue and Xian-Yang Li. Wrote the paper: Bao-Jun Chen and You-Rong Chai.

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