Characterization and expression analysis of circadian clock genes in the diploid woodland strawberry *Fragaria vesca*

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Abstract

Strawberry is an economically important fruit crop worldwide. Circadian clock genes are endogenous timers that regulate a wide range of metabolic processes and consequently plant development. However, little is known about the circadian clock genes in strawberry. In the present work, we identified 12 primary circadian clock genes from the diploid woodland strawberry (*Fragaria vesca* L.) genome. Phylogenetic, conserved motif, and gene structure analyses revealed the evolutionary relationships of strawberry circadian clock genes with homologous genes from other species. Promoter analysis revealed different regulatory elements responding to abiotic and biotic stresses and phytohormones. We characterized the transcript patterns of strawberry circadian clock genes over a 48-h period. The expression patterns of seven circadian clock genes displayed circadian rhythms. We also examined the expression patterns of these genes in response to low-temperature stress and six of them showed an upregulated expression. Interestingly, most of these upregulated genes were highly expressed during the day. Our study reveals the characteristics of primary circadian clock components in diploid woodland strawberry and their responses to low-temperature stress and lays a foundation for future functional studies of these circadian clock genes during the growth and development of diploid woodland strawberry.

Additional key words: *Arabidopsis thaliana*, cold stress, gene expression, phylogenetic analysis.

Introduction

Circadian rhythms are found in a wide variety of organisms ranging from cyanobacteria to mammals (Dong and Golden 2008, Wang *et al.* 2017). The periodicity of circadian rhythms is approximately 24 h, which is consistent with the light-dark cycle present on Earth (Greenham and McClung 2015). Circadian rhythms are driven by an endogenous circadian clock (Matsushika *et al.* 2000). The circadian clock of *Arabidopsis* is composed of multiple interlocking transcription-translation feedback loops (Dong *et al.* 2011, Maibam *et al.* 2013, Hsu and Harmer 2014). Different circadian clock proteins function at certain times during the day and night to maintain circadian rhythms (Hsu and Harmer 2014). Two morning-phased components circadian clock-associated 1 (CCA1) and late elongated hypocotyl (LHY), along with one evening-phased component timing of

CAB 1 (TOC 1), comprise the core circadian feedback loop in *Arabidopsis* (Dong *et al.* 2011). CCA1 and LHY are MYB transcription factors that have partially redundant functions (Hsu and Harmer 2014). The expression of CCA1 and LHY peaks at dawn, whereas the expression of TOC1 is greatest in the early evening (Dong *et al.* 2011). TOC1 (also known as pseudo-response regulator 1 (PRR1)), together with PRR9, PRR7, PRR5, and PRR3, plays a key role in the circadian clock (Matsushika *et al.* 2000). The expression of TOC1 is repressed by CCA1 and LHY, while TOC1 is required for the induction of CCA1 and LHY (Alabadi *et al.* 2002, Dong *et al.* 2011). PRR9, PRR7, and PRR5 play redundant roles in repressing the expression of CCA1 and LHY (Nakamichi *et al.* 2010). CCA1 and LHY belong to the small REVEILLE subfamily, in which several

Submitted 7 August 2017, last revision 22 November 2017, accepted 11 December 2017.

Abbreviations: BOA - brother of lux arrhythmo; CCA1 - circadian clock-associated 1; CHE - CCA1 hiking expedition; ELF - early flowering; LHY - late elongated hypocotyl; LUX - lux arrhythmo; PRR - pseudo-response regulator; qPCR - quantitative polymerase chain reaction; RVE - REVEILLE; TOC 1 - timing of CAB 1; ZTL - ZEITLUPE.

Acknowledgements: This work was supported by the Jiangsu Planned Projects for Postdoctoral Research Funds (No. 1601103B), the Jiangsu Provincial Natural Science Foundation of China (No. BK20130722), and the National Natural Science Foundation of China (No. 31301764).

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homologues of *CCA1/LHY*, i.e., *REVEILLE 8 (RVE8)*, *RVE4*, and *RVE6* play important roles in regulating the expression of many evening-phase genes (Rawat *et al.* 2011, Hsu and Harmer 2014). In the circadian clock system, evening-phase components include *PRR5*, *TOC1*, *CHE* (*CCA1 hiking expedition*), *LUX* (*LUX arrhythm*), *BOA* (*brother of lux arrhythm*), *ELF3* (*early flowering 3*), *ELF4*, and *ZTL* (*ZEITLUPE*) (Hsu and Harmer 2014). *CHE* has a function in repressing the expression of *CCA1* (Pruneda-Paz *et al.* 2009). *LUX*, *ELF3*, and *ELF4* form the “evening complex”, which negatively regulates the expression of *PRR9* (Nusinow *et al.* 2011, Herrero *et al.* 2012, Hsu and Harmer 2014). *ZTL* is an F-box protein that targets *TOC1* and *PRR5* for ubiquitination (Mas *et al.* 2003, Hsu and Harmer 2014).

Many circadian clock components take part in regulating different biological processes, such as the diurnal control of hypocotyl elongation, photosynthesis, and saccharide metabolism, as well as abiotic and biotic stress responses (Hsu and Harmer 2014, Greenham and McClung 2015). The *CCA1* and evening complex proteins (*ELF3*, *ELF4*, and *LUX*) are involved in regulating hypocotyl elongation (Nusinow *et al.* 2011, Campoli *et al.* 2012). In addition, *CCA1* and *LHY* have been reported to regulate carbon partitioning towards starch (Greenham and McClung 2015). In *Arabidopsis*, *PRR9*, *PRR7*, and *PRR5* negatively regulate the biosynthetic pathways of chlorophyll, carotenoids, abscisic acid, and α -tocopherol (Fukushima *et al.* 2009). Several components of the circadian clock system (*CCA1*, *LHY*, *PRR9*, 7, and 5) have been implicated in regulating low-temperature stress (Fukushima *et al.* 2009, Dong *et al.*

al. 2011, Hsu and Harmer 2014).

The circadian clock has been well studied in *Arabidopsis*, and its importance in controlling key agricultural traits is becoming clear (Hsu and Harmer 2014, Bendix *et al.* 2015). A number of circadian clock system proteins have been identified and analysed in many crops, such as *Castanea sativa* (Ibanez *et al.* 2008), *Oryza sativa* (Yang *et al.* 2013), *Brassica rapa* (Lou *et al.* 2012), and *Glycine max* (Marcolino-Gomes *et al.* 2014). Until recently, circadian clock system genes have not been well characterized in the strawberry genome. To date, only a few circadian clock components and their potential roles in regulating flowering time in strawberry have been reported (Mouhu *et al.* 2009).

The cultivated strawberry (*Fragaria* × *ananassa* Duch.) is an important horticultural crop throughout the world (Wei *et al.* 2016). *F.* × *ananassa* is an octoploid strawberry, harbouring eight sets of chromosomes ($2n = 8x = 56$), which complicates genetic studies (Shulaev *et al.* 2011). By comparison, the diploid woodland strawberry (*Fragaria vesca*) has a small and sequenced genome ($2n = 14$, 240 Mb) that is highly congenic with octoploid cultivated strawberry (Shulaev *et al.* 2011), making it a good platform to study circadian clock genes in strawberry. The aim of the present work was to isolate and characterize 12 primary circadian clock genes from *F. vesca* and to conduct a broad bioinformatics analysis, including chromosome position, evolutionary relationships, protein motifs, gene structure, and *cis*-acting elements. We further investigated the expression profiles of these genes at different times as well as in response to cold stress.

Materials and methods

Plants, growth conditions and treatments: A seventh-generation inbred line Rugen F7-4 of *Fragaria vesca* L. (gift from Dr. Gu; for more information about this line see Gu *et al.* 2016) was used in this study. Strawberry seedlings were grown at a temperature of 24 °C, a relative humidity of 60 %, a 10-h photoperiod, and an irradiance of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Fully expanded leaves from 8-week-old seedlings were collected for real-time qPCR analysis at 4-h intervals from Zeitgeber time 0 (ZT0) to ZT48. For cold treatments, strawberry seedlings were transferred to 4 °C for 24 h. Control plants were maintained at 24 °C. In addition, leaves were collected at 4-h intervals starting at ZT0 for 48 h. All samples were immediately put into liquid N₂ and stored at -80 °C before RNA extraction.

Identification of strawberry circadian clock genes: To identify strawberry circadian clock genes, we downloaded the amino acid sequences of *Arabidopsis* circadian clock proteins from *TAIR* (<http://www.arabidopsis.org/>; Lamesch *et al.* 2012) and used them as query sequences to conduct *BLASTP* searches in the *F. vesca* genome (fvesca_v1.0_genemark_hybrid) database at the genome database for *Rosaceae* (*GDR*,

<http://www.rosaceae.org/>; Jung *et al.* 2014). The top hit sequences were selected on the basis of whether they had an E-value close to 0, using a cutoff E-value of $1e-30$. The circadian clock protein sequences from peach (*Prunus persica* v.2.1), tomato (*Solanum lycopersicum* iTAG2.4), rice (*Oryza sativa* v7_JGI), and maize (*Zea mays* Ensembl-18) were obtained from *Phytozome* (<https://phytozome.jgi.doe.gov/pz/portal.html>) using the same *BLASTP* method (details listed in Table 1 Suppl.). The identified strawberry circadian clock genes were mapped to chromosomes using *MapInspect* software (<http://www.files32.com/MapInspect-i366074.asp>).

Phylogenetic analysis and gene structure: Phylogenetic trees of circadian clock proteins were generated using Molecular evolutionary genetic analysis (*MEGA*) 5.0 software (Tamura *et al.* 2011). The neighbour-joining method was applied to construct circadian clock trees with the p-distance model, pairwise deletion of gaps, and bootstrapping (1000 replicates; random seed). Conserved protein motif analysis was performed by online Multiple expectation-maximization for motif elicitation (*MEME*) v. 4.10.2; <http://meme-suite.org/tools/meme>) (Bailey *et al.*

2009). The parameters were as follows: minimum length of the motif, 6; maximum length of the motif, 100; and maximum number of motifs, 10. The genomic and coding sequences of strawberry circadian clock genes were retrieved from *GDR*. Then, these genomic and coding sequences were analysed using the online program gene structure display server (*GSDS*) 2.0 (Hu *et al.* 2015) to infer the exon-intron distribution of strawberry circadian clock genes. The whole genomic sequences of strawberry circadian clock genes were retrieved from National Center for Biotechnology Information (*NCBI*, <http://www.ncbi.nlm.nih.gov/>). The 1.5 kb upstream regions of the translation start sites of the strawberry circadian clock genes were used for regulatory elements analysis using *PlantCARE* (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) (Lescot *et al.* 2002).

RNA extraction and gene expression analysis: Total RNA was extracted from frozen leaves using the *PD* kit (*Pudishengwu*, Shanghai, China). The gDNA *ERASER* in the kit was used to remove gDNA from RNA samples. Then, RNAs were reverse-transcribed into cDNA using

BioTeke (Beijing, China) *SupermoIII RT* kit. Each real-time qPCR was performed with a total volume of 20 mm³ consisting of 10 mm³ of *SYBR Green Premix Ex Taq* (*TaKaRa*, Tokyo, Japan), 0.8 mm³ of 1.0 μM of each primer, and 7.4 mm³ of sterile distilled H₂O using the 7500 real-time PCR system (*Applied Biosystems*, New York, USA). The amplification conditions were as follows: 95 °C for 10 min; 39 cycles of 95 °C for 15 s and 60 °C for 40 s; then, the temperature was gradually increased by 0.5 °C every 10 s to perform a melting curve analysis. The real-time qPCR results are presented as 2^{-ΔCT} values (Schmittgen and Livak 2008), which were normalized to *GAPDH* (Gu *et al.* 2016) using the ΔCT method (Schmittgen and Livak 2008). The expression data were analysed using *Microsoft Excel 2010*. Student's *t*-test was applied to compare statistical significances between the low-temperature treated and the control samples. The primers used in this study are listed in Table 2 Suppl. The real-time qPCR results were confirmed by three biological repetitions. Each biological repetition was performed with three technical repetitions.

Results

A total of 16 *Arabidopsis* primary circadian clock genes *CCA1*, *LHY*, *PRR9*, *PRR7*, *RVE8*, *RVE4*, *RVE6*, *PRR5*, *PRR3*, *TOC1*, *CHE*, *LUX*, *BOA*, *ELF3*, *ELF4*, and *ZTL* (Hsu and Harmer 2014), were used for a *BLASTP* search in *GDR* to identify primary circadian clock genes in the *F. vesca* genome (*fvesca_v1.0_genemark_hybrid*). As a result, we identified 12 circadian clock genes in strawberry (Table 1). Detailed information (including gene location, genomic length, and number of amino acids) regarding each gene is listed in Table 1. These

genes were distributed over six chromosomes. Four circadian clock genes were mapped to chromosome 7, and two circadian clock genes were found on chromosome 6. Chromosomes 2, 3, and 4 contained one circadian clock gene each (Fig. 1).

CCA1/LHY and *TOC1 (PRR1)* comprise the core circadian feedback loop (Dong *et al.* 2011). To gain a better understanding of the potential function and evolutionary relationships of strawberry *CCA1/LHY* and *TOC1 (PRR1)*, we constructed phylogenetic trees of

Table 1. Characteristics of primary circadian clock genes in *Arabidopsis* and *Fragaria vesca*. ^a - Hsu and Harmer (2014); ^b - IDs, gene length, and the number of amino acids (*Fragaria vesca* whole genome v. 1.0. build 8 assembly and annotation) are available at genome database for *Rosaceae* (<http://www.rosaceae.org>).

AGI (<i>Arabidopsis</i>)	Name (<i>Arabidopsis</i>)	Time of protein activity (<i>Arabidopsis</i>) ^a	Gene Id (<i>F. vesca</i>) ^b	Name (<i>F. vesca</i>)	Gene location	Length [bp] ^b	Number of aa ^b
AT2G46830	<i>AtCCA1</i>	morning	--				
AT1G01060	<i>AtLHY</i>	morning	gene18601	<i>FvLHY</i>	LG7:2710468 to 2713025	2558	712
AT2G46790	<i>AtPRR9</i>	after dawn	gene18611	<i>FvPRR9</i>	LG7:2760853 to 2764189	3337	658
AT5G02810	<i>AtPRR7</i>	after dawn	gene18151	<i>FvPRR7</i>	LG6:10027241 to 10031868	4628	765
AT3G09600	<i>AtRVE8</i>	afternoon	gene18104	<i>FvRVE8</i>	LG6:10090024 to 10092841	2818	276
AT5G02840	<i>AtRVE4</i>	presumably afternoon	--				
AT5G52660	<i>AtRVE6</i>	presumably afternoon	gene13188	<i>FvREV6</i>	LG7:22153843 to 22157234	3392	348
AT5G24470	<i>AtPRR5</i>	afternoon	gene12454	<i>FvPRR5</i>	LG7:20753894 to 20757515	3622	672
AT5G60100	<i>AtPRR3</i>	evening	--				
AT5G61380	<i>AtTOC1</i>	evening	gene26055	<i>FvTOC1</i>	LG5:8058964 to 8063051	4088	536
AT5G08330	<i>AtCHE</i>	evening	gene12294	<i>FvCHE</i>	LG5:13952649 to 13953503	855	284
AT3G46640	<i>AtLUX</i>	evening	gene30204	<i>FvLUX</i>	LG3:19000151 to 19001107	957	318
AT5G59570	<i>AtBOA</i>	evening	--				
AT2G25930	<i>AtELF3</i>	evening	gene02656	<i>FvELF3</i>	LG2:20296037 to 20299458	3422	769
AT2G40080	<i>AtELF4</i>	evening	gene20968	<i>FvELF4</i>	LG7:18198444 to 18201502	3059	304
AT5G57360	<i>AtZTL</i>	evening	gene16999	<i>FvZTL</i>	LG4:3187275 to 3188789	1515	504

CCA1/LHY and *PRR* family genes by comparing their protein sequences in *Arabidopsis*, peach, tomato, maize, rice, and strawberry. Meanwhile, the conserved motifs and exon/intron organization of these proteins were analysed to obtain further insight into the relationships between these orthologues. The phylogenetic results showed that *FvLHY* and *PpLHY* had a close evolutionary relationship. The protein motif analysis showed that motifs 2, 3, 4, 5, 6, 9, and 10 were found in all 9 *CCA1/LHY* proteins. Motifs 8 and 9 were identified in *OsCCA1* and *ZmCCA1/ZmLHY* (Fig. 2). The

exon/intron distribution results showed that *FvLHY* and *ZmLHY* contained three phase 0 introns, while the other *CCA1/LHY* genes usually had 5 - 6 introns (Fig. 1 Suppl.). As illustrated by Fig. 3, strawberry *PRR* proteins had a close evolutionary relationship with those of peach. Motif 2 and motif 4 were observed in all *PRR* proteins. In addition, the majority of *PRR* proteins contained motif 1 and motif 3, except for *SIPRR3* and *ZmPRR5*. These conserved motifs may contribute to the common functions of *PRR* family proteins. The gene structure analysis of *PRR* family genes is shown in Fig. 2 Suppl.

Fig. 1. Chromosomal locations of circadian clock genes on the seven chromosomes of *Fragaria vesca*. The scale on the left is in megabases.

Table 2. Hormone- or stress-related and tissue- or organ-specific *cis*-elements in the promoter regions of circadian clock genes in *F. vesca*. ^a - The different *cis*-elements are represented by numbers: 1 - fungal elicitor, 2 - elicitor, 3 - anaerobic induction, 4 - irradiance, 5 - heat stress, 6 - low temperature, 7 - defense and stress, 8 - wounding, 9 - ABA, 10 - SA, 11 - MeJA, 12 - ethylene, 13 - GA, 14 - auxin, 15 - meristem, 16 - endosperm, 17 - shoot, 18 - cell cycle, 19 - seed, 20 - metabolism, 21 - MYB binding site, 22 - circadian control. ^b - numbers in columns indicate the number of the *cis*-elements.

	1 ^a	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
<i>FvCCA1</i>	2 ^b		2	10			2		2	1					1	3						1
<i>FvPRR9</i>			3	37	4		1		8		4	1			1	4					2	4
<i>FvPRR7</i>			6	22			1		2	1	6		1	1	1	4					4	
<i>FvRVE8</i>	1		2	18	1			1	2	2	6	1	2	1		9	1			1	1	1
<i>FvREV6</i>	1		1	27			1				4			1	2	2				1	3	2
<i>FvPRR5</i>				31	1		2		5	1	4		3	1		3		2		1	1	2
<i>FvTOC1</i>	1		2	16	1	2	1			1	2	1	1		1	5						3
<i>FvCHE</i>				22	2	1		1			2	3		1		1			1		1	3
<i>FvLUX</i>	2	1	2	14	2		4		2		6				2	4				1	1	3
<i>FvELF3</i>	2		1	17		1	1		4	2	4			1		1				1		3
<i>FvELF4</i>	2			17		2			1	1	2		3		1				1			2
<i>FvZTL</i>	1			21	4					1	4		1		2	2					1	3

To understand the potential regulatory mechanisms of circadian clock genes in *F. vesca*, the 1.5 kb sequences upstream of the translation start sites of the strawberry circadian clock genes were analysed using *PlantCARE*. A set of *cis*-regulatory elements involved in abiotic and biotic stress responses, phytohormone responses, and plant growth and development were identified (Table 2).

For instance, fungal elicitor responsive elements were found in eight circadian clock genes. All strawberry primary circadian clock genes had light-responsive elements. Heat-stress- and low-temperature-responsive elements were identified in 7 and 4 circadian clock genes, respectively. In addition, the promoter regions of 8 circadian clock genes contained defence and stress-

responsive elements. Among phytohormone-related regulatory elements, elements responsive to abscisic acid (ABA), salicylic acid (SA), and methyl jasmonate were identified in the promoter regions of 8, 8, and 11 strawberry circadian clock genes, respectively. The elements responsive to ethylene, gibberellin (GA), and auxin were observed in 4, 6, and 6 strawberry circadian clock genes, respectively (Table 2). Many hormone-responsive elements were identified in the promoter

regions of strawberry circadian clock genes, suggesting that phytohormones might have a high degree of crosstalk with circadian clock genes. Additionally, many circadian clock genes contained meristem-, endosperm- and seed-responsive elements, indicating that strawberry circadian clock genes might have a role in regulating strawberry growth and development. Notably, the elements responsive to circadian-control were found in 11 strawberry circadian clock genes (Table 2).

Fig. 2. Phylogenetic and motif analyses of LHY and CCA1 proteins. A phylogenetic tree was constructed using the full-length protein sequences of 9 LHY and CCA1 proteins retrieved from *Arabidopsis thaliana* (At), *Prunus persica* (Pp), *Solanum lycopersicum* (Sl), *Zea mays* (Zm), *Oryza sativa* (Os), and *Fragaria vesca* (Fv) databases. The protein motifs of the strawberry LHY and CCA1 proteins are shown below the graph and denoted by rectangles with different colours. To distinguish motif 3 and motif 6, motif 3 is indicated by the number 3. The sequences of these motifs are shown in Fig. 3 Suppl.

To test whether the expression of strawberry circadian clock genes exhibited circadian rhythms, we investigated their expression using real-time qPCR. The expression profiles of seven strawberry circadian clock genes displayed circadian rhythms (Fig. 4). For example, the mRNA abundance of *FvLHY* and *FvRVE8* was high during the morning, declined gradually during the day, and was lowest during the afternoon and early night. The expression of *FvPRR9*, *FvPRR7*, and *FvPRR5* peaked during the afternoon. Two evening-phased components (*FvTOC1* and *FvLUX*) were expressed highly during the first half of the night (Fig. 4). On the other hand, the expression profiles of *FvRVE6*, *FvCHE*, *FvELF3*, and *FvZTL* showed no obvious circadian rhythms in strawberry (Fig. 4).

Plant circadian clock genes play important roles in regulating low-temperature stress response (Eriksson and

Webb 2011). To investigate the potential roles of strawberry circadian clock genes during plant responses to low-temperature stress, we analysed the expression profiles of 11 strawberry circadian clock genes in the leaves of *F. vesca* plantlets exposed to low-temperature stress (Fig. 5). In general, low-temperature stress led to the upregulation of morning-phase and day-phase components. For instance, the expression of *FvLHY* and *FvRVE8* was induced by cold stress from ZT4 to ZT16, while *FvPRR9*, *FvPRR7*, *FvPRR5*, and *FvRVE6* were upregulated by cold treatment for 24 h (Fig. 5). On the other hand, low-temperature stress inhibited the induction of *FvTOC1*, *FvLUX*, *FvELF3*, and *FvZTL* during the night (Fig. 5). These data suggest different circadian clock genes might have different functions in response to low-temperature stress.

Fig. 3. Phylogenetic and motif analyses of PRR proteins. An unrooted tree was constructed using full-length PRR protein sequences from six different species (for their names and abbreviations see Fig. 2). The protein motifs (numbered 1 - 10) are shown below the graph, and the sequences of these motifs are shown in Fig. 4 Suppl.

Discussion

Circadian rhythms are driven and regulated by a number of circadian clock components. These circadian clock components function at different times of the day to regulate many biological processes (Bendix *et al.* 2015). Although the overall architecture of the circadian clock is generally conserved, the key genes involved often vary among organisms (Linde *et al.* 2017). In the present work, a total of 12 primary circadian clock genes were identified in the *F. vesca* genome (accession Hawaii-4). It

is noteworthy that the homologues of *AtCCA1*, *AtRVE4*, *AtPRR3*, and *AtBOA* were absent in the *F. vesca* genome. CCA1 and LHY play important roles in regulating the morning-phase circadian rhythm in *Arabidopsis* (Mizoguchi *et al.* 2002, Hsu and Harmer 2014). Most plant species have only one of CCA1 and LHY, usually LHY (Calixto *et al.* 2015), and strawberry contains only *FvLHY*. The expression data also demonstrated that *FvLHY* was expressed highly at the beginning of the day (Fig. 4).

Fig. 4. Expression patterns of circadian clock genes in *Fragaria vesca*. Eleven circadian clock gene expression profiles were measured by real-time qPCR. Means $\pm$ SDs were obtained from three biological replicates and three technical replicates. Leaves were collected from the beginning of the light period (ZT0) at 4-h intervals until ZT48. White and black bars on the x-axis indicate day and night periods, respectively.

Fig. 5. Expression patterns of circadian clock genes in *Fragaria vesca* in response to low-temperature stress generated by real-time qPCR. Means $\pm$ SDs from three biological replicates and three technical replicates. *, **, ***, and **** indicate significant differences between the treated (grey lines) and control plants (black lines) at $P \leq 0.05$, 0.01, 0.001, and 0.0001, respectively. Leaves were collected from ZT0 at 4-h intervals until ZT24. White and black bars on the x-axis indicate day and night period, respectively.

These results suggest that *FvLHY* is necessary for the maintenance of morning-phase circadian rhythm in strawberry. The *TOC1* genes (also known as *PRR1*), together with *PRR9*, *PRR7*, *PRR5*, and *PRR3*, belong to the PRR gene family (Matsushika *et al.* 2000). The expression of these genes is regulated by the circadian clock, although their transcripts peak at different times of the day: *PRR9* at dawn, *PRR7* in the morning, *PRR5* around noon, and *TOC1* and *PRR3* in the evening (Matsushika *et al.* 2000, Nakamichi *et al.* 2010). In *F. vesca*, the expression of *FvPRR9*, *FvPRR7* and *FvPRR5* was greatest around ZT8 (noon). These data indicate that the regulation of afternoon-phased circadian rhythms might be mainly regulated by these three PRR genes.

PRR5, *TOC1*, *CHE*, *LUX*, *BOA*, *ELF3*, *ELF4*, and *ZTL* are involved in regulating the circadian clock during the evening in *Arabidopsis* (Hsu and Harmer 2014). However, we failed to identify a homologue of *AtBOA* in the diploid *F. vesca* genome. The expressions of *AtCHE* (Pruneda-Paz *et al.* 2009) and *AtELF3* (Lu *et al.* 2012) are clock-regulated, but their strawberry homologues did not show any obvious circadian rhythms. Only two evening-phase genes (*FvTOC1* and *FvLUX*) showed periodic expression patterns. These results suggest that *FvTOC1* and *FvLUX* might play pivotal roles in regulation of the circadian clock during the evening.

The plant circadian clock system influences the low-temperature stress response (Eriksson and Webb 2011, Hsu and Harmer 2014). The best-characterized low-temperature stress response pathway is the C-repeat (CRT)-binding factor/dehydration-responsive element (DRE) binding factor (CBF/DREB)-mediated transcriptional regulatory pathway (Chinnusamy *et al.* 2007, Shi *et al.* 2015). Three members of the CBF/DREB1 family, *CBF1*, *CBF2*, and *CBF3* (or *DREB1b*, *DREB1c*, and *DREB1a*, respectively) are rapidly induced by low temperatures (Fowler and Thomashow 2002, Medina *et al.* 2011). In addition, the induction of these CBF genes was directly activated by

CCA1 and *LHY* in *Arabidopsis* (Dong *et al.* 2011) and might be negatively regulated by *PRR5*, *PRR7*, and *PRR9* (Nakamichi *et al.* 2009). Our current data show that the expression of morning-phase components (*FvLHY*) and day-phase components (*FvPRR5*, *FvPRR7*, and *FvPRR9*) were induced by cold treatment, whereas evening-phase components were slightly downregulated by low-temperature stress. These results suggest that morning-phase and day-phase circadian clock genes (*FvLHY*, *FvPRR5*, *FvPRR7*, and *FvPRR9*) might play pivotal roles in coordinating circadian clock regulation under low-temperature stress in strawberry. As the expression of CBF genes was gated by morning-phase components (*CCA1/LHY*) in *Arabidopsis* and the expression of *FvLHY* was induced by cold treatment, we speculate that the induction of *FvCBF* genes might also be regulated by *FvLHY*. However, the molecular function of the circadian clock genes in regulating low-temperature response is still poorly understood in strawberry. Further detailed work on the regulation and function of these circadian clock genes in strawberry will greatly increase our knowledge of how strawberry circadian genes are involved in the regulation of low-temperature stress.

In conclusion, we performed a broad study of circadian clock genes in *F. vesca* by investigating their evolutionary relationships, protein motif distributions, gene structure, and *cis*-regulatory elements. Among these genes, we found that seven strawberry circadian clock genes (*FvLHY*, *FvPRR9*, *FvPRR7*, *FvRVE8*, *FvPRR5*, *FvTOC1*, and *FvLUX*) had typical circadian expression patterns, which suggest that these genes might play central roles in regulating circadian rhythms in strawberry. In addition, we examined the expression profiles of these circadian clock genes under low-temperature stress, which provided additional insights into their functions. Taken together, our research lays the foundation for future work to reveal the functions of circadian clock genes in strawberry.

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