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Transcriptional Regulation of Circadian Rhythm System for Developmental Stages in *Cucumis melo* L.: A Bioinformatic Study

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Highlights:

- The circadian rhythm can also be called the biological clock
- Melon is the most important Cucurbitaceae family members
- The relationship between different developmental processes and circadian rhythm in melon is very important

Keywords:

- Circadian rhythm
- Developmental stage
- Melon
- Bioinformatic

ABSTRACT:

Circadian rhythm is defined biochemical, physiological, metabolic and behavioral responses of all living organisms such as bacteria, mammals, fungi and plants within a 24-hour light and dark photoperiod. In plants, circadian rhythm regulates multiple physiological, metabolic and behavioral processes such as stomatal and leaf movements, stem elongation, enzyme activity and aging. Circadian clock genes are known to play a role in regulating the biosynthesis of phytohormones which are involved in many crucial processes such as seed germination, hypocotyl elongation and photosynthesis. Circadian rhythm regulates and optimizes physiological and molecular mechanisms in a diurnal cycle, anticipating seasonal changes and preparing the plant for different environmental conditions in plant. Circadian rhythm also plays a crucial role in the regulation of plant defense against biotic and abiotic stresses. Melon is a diploid species and one of the most important Cucurbitaceae family members. In this study, we aimed to investigate the relationship between different developmental processes and circadian rhythm in melon. The relationship between circadian rhythm and growth, development and resistance in melon a detailed understanding of the relationship will help to improve future agricultural production and ground for further research on tolerance to environmental stresses will be created.

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INTRODUCTION

In all living organisms, including plants, it is of great importance to understand how the mechanisms regulating biological processes in the day-night cycle are organized within the complex circadian rhythm network to maintain growth and development. In general, the circadian rhythm is defined as the biochemical, physiological, metabolic and behavioral responses of all living organisms such as bacteria, mammals, fungi and plants within a 24-hour light-dark photoperiod. (Srivastava et al., 2019). The circadian rhythm consists of a molecular network of very complex signaling processes. In living organisms, the timing, synchronization and optimization of events related to the internal and external environment and the rhythmic control of signals are carried out by this complex network of interactions (Nimmo et al., 2020).

Plant circadian clocks have many transcriptional and translational feedback mechanisms (Translational/transcriptional feedback loops-TTFLs) that are conserved among plant species (Srivastava, et al., 2019). In plants, circadian rhythm regulates multiple physiological, metabolic and behavioral processes such as stomatal and leaf movements, stem elongation, enzyme activity and aging (Pelvan et al., 2021). In addition, circadian clock genes are known to play a role in regulating the biosynthesis of phytohormones such as gibberellic acid (GA), jasmonic acid (JA), salicylic acid (SA) and abscisic acid (ABA), which are involved in many crucial processes such as seed germination, hypocotyl elongation and photosynthesis (Atamian et al., 2016). It is also predicted to have a very important role in the optimum timing of defense responses against abiotic and biotic stresses in plants (Karapetyan & Dong, 2018).

In the central cycle of the circadian rhythm are *circadian clock associated1* (*CCA1*), *late elongated hypocotyl* (*LHY*), *timing of cab expression1/pseudo-response regulator1* (*TOC1/PRRR1*), which encodes *MYB* (*myeloblastosis*)-transcription factors and belongs to the *pseudo-response regulator* (*PRR*) family (Harmer, 2009). The central cycle combines with the day and night cycle to establish the circadian rhythm in plants (Sharma & Bhatt, 2014). Studies have shown that in *CCA1* and *LHY* mutants, circadian rhythm is disrupted, flowering time is affected, light responses are impaired, hypocotyl elongation is altered, phenotype and daily periods of plants are altered (Inoue et al., 2017). *PRR9* contributes to the regulation of hypocotyl growth under photoperiodic conditions. It has also been reported that *PRR7* and *PPR9* genes are important components of the regulation of the circadian system sensitive to temperature changes (Greenham et al., 2020). It has been suggested that *TOC1* is an important link between environmental signals and circadian rhythm, especially for photomorphogenic outputs, and that *TOC1* contributes to carbon fixation and biomass production in plants by shortening the cycle time and affecting the circadian clock period (Perales & Mas, 2007; Fujiwara et al., 2008). *Early flowering 3* (*ELF3*) encodes a nuclear protein that is rhythmically expressed and interacts with phytochrome B to control plant growth and flowering. *ELF3* is a crucial component of the nuclear circadian clock, independent of light conditions (Liu, 2001). *Gigantea* (*GI*) is one of the most important genes involved in circadian rhythm. Photoperiod-mediated flowering, phytochrome B signaling, circadian clock, carbohydrate metabolism and regulates various processes, including the cold stress response (Srivastava et al., 2019). Genes that play a key role in circadian rhythm such as *CCA1*, *TOC1*, *LHY* and *TIC* are known to have a very important role in plant defense mechanisms against biotic and abiotic stresses (Grundy et al., 2015).

The melon (*Cucumis melo* L.) is a diploid species (2n=24) and one of the most important members of the Cucurbitaceae family. The melon is not only of great economic importance, but also has a remarkable content of substances such as vitamins A and C, β-carotenes, phenolic acids and minerals as well as folic acid, which are very important for nutrition and human health. (Garcia-Mas et al., 2012).

World melon production is estimated at approximately 27.3 million tons from 1.04 million hectares (http://faostat3.fao.org). The melon fruits can be climacteric or nonclimacteric due to their respiration (Périn et al., 2002). The climacteric stage is the last step of physiological stage that signs the starting of fruit ripening. During this stage several changes occur in the fruit (Osorio et al., 2013). In the present study, we aimed to investigate the relationship between different developmental processes and circadian rhythm in melon by bioinformatic tools. The relationship between circadian rhythm and growth and development in melon a detailed understanding of the relationship will help to improve future agricultural production and ground for further research on tolerance to environmental stresses will be created.

MATERIALS AND METHODS

Data Retrieval

RNA-seq transcriptome libraries were downloaded from National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra). The libraries are belonged to fruit climacteric (C), growing (G), post-climacteric (P) and ripening (R) stages (PRJNA543288) (Tian et al., 2019).

Analysis of Circadian Rhythm System gene expressions from RNA-Seq libraries

After sequence quality control, each of the library RNA-Seq reads was mapped to the *C. melo* genome downloaded from Genome ENSEMBL database (https://plants.ensembl.org/Cucumis_melo/Info/Indexusing). To calculate FPKMs (fragments per kilobase of transcript per million mapped) of transcripts Cuffdiff was used (Mortazavi et al., 2008). The transcripts with p value ≤ 0.05 and the log2 fold change with ≥ 1.5 and ≤ - 1.5 were identified as differentially expressed between groups. The annotation of the transcripts performed with ANNOVAR software (Wang et al., 2010).

Gene enrichtment analysis

The analysis of the gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) was performed with the software Blast2GO (Conesa & Götz, 2008). The sequences were uploaded to Blast2GO in FASTA format. The genes with a *p*-value of less than 0.05 were considered significant and assigned to the GO categories cellular components, molecular function, and biological process.

RESULTS AND DISCUSSION

It is known that plants have internal biological clocks that allow them to predict environmental changes over time and adapt to daily or seasonally changing environmental conditions. The circadian clock genes have crucial functions in plants such as metabolism control, hormone signalling growth and development (Farré & Weise, 2012). Hence, as in other organisms, plants circadian clock genes consists of multiple transcriptional/translational mechanisms (Inoue et al., 2018).

Identification of Circadian Rhythm Related DEGs in Different Developmental Stages

In this study, we identified the DEGs and their potential functions existing in circadian rhythm pathway in different developmental stages of *C. melo*. The DEseq package was applied for DEG analysis. The absolute log2 based values were determined.

We compared the growth stages with the maturation stage and identified four DEGs, three of which were downregulated. Among them, *MELO3C010015.2* (*Flowering Locus T*) showed the lowest expression level. The comparison between growth and climacteric stages revealed seven genes, six of which were upregulated. The highest number of DEGs were found in the comparison between growing and post climacteric developmental stages. Among eight genes 50% of them was demonstrated upregulation. From this point of view, we can say that the number of DEGs associated with circadian rhythm denotes increasing from growing stage to post climacteric stage. Also, we performed comparison analysis to find out the DEGs between ripening vs climacteric and ripening vs post climacteric stages. We detected three and two DEGs, respectively. Interestingly no significant DEGs associated with circadian rhythm were found between climacteric and post climacteric stages (Table 1).

Table 1. Differentially expressed circadian rhythm genes at different developmental stages in melon (Fold changes are given in log2-based numbers)

Gene name	**Developmental stages**					**Description**
	GR	**GC**	**GP**	**RC**	**RP**	
MELO3C010015.2	**-6.9**	**12.2**	**-12.2**	**-5.3**	**-5.3**	Protein Flowering Locus T (FT)
MELO3C014767.2	**-3.8**	**4.2**	**-4.5**	//	//	Chalcone synthase 2 (CHS)
MELO3C016660.2	**1.6**	//	**1.7**	//	//	Adagio protein 1/clock-associated PAS protein (ZTL)
MELO3C017961.2	**-1.6**	**2.3**	**-2.5**	//	//	Phytochrome A-associated F-box protein (PHYA)
MELO3C011754.2	//	**2**	//	//	//	Transcription factor TCP7-like (CHE)
MELO3C025894.2	//	**1.5**	**-1.5**	//	//	Phytoclock 1 (PCL1/LUX)
MELO3C030034.2	//	**-2.1**	**2.8**	**2**	**2.6**	Protein GIGANTEA (GI)
MELO3C031303.2	//	**1.5**	//	**-1.6**	//	Phytochrome-Interacting Factor 3 (PIF3)
MELO3C005840.2	//	//	**1.5**	//	//	Protein REVEILLE (RVE)
MELO3C020611.2	//	//	**1.8**	//	//	Phytoclock 1 (PCL1/LUX)

(GR: Growing vs Ripening; GC: Growing vs Climacteric; GP: Growing vs Post-climacteric; RC: Ripening vs Climacteric; RP: Ripening vs Post-climacteric)

Among all the detected DEGs, *MELO3C010015.2* (*FLOWERING LOCUS T*) was found to be exist in all comparisons. It showed a remarkable difference in expression between developmental stages. Namely, its expression decreased in all comparisons except growing vs climacteric stages. It is known that the flowering integrator gene, *FT*, activates the "florigen" that activates flowering genes and enables the transition to the flowering stage (Kazan & Lyons, 2016). It functions in both the vernalization and photoperiod pathways (Seymour et al., 1993; Takada & Goto, 2003). Some studies have shown that this gene might have a role in fruit ripening. In apple, which is also a climacteric plant, *FT* might be involved in fruit development (Mimida et al., 2011). The *PHYA* gene is expressed rhythmically to control plant growth and flowering, and in a study in grapes it was reported to be prominent during the climacteric period (Chervin et al., 2004).

In addition, some of the genes were unique within the comparisons. *MELO3C011754.2* was detected only in growing vs climacteric whereas *MELO3C005840.2* and *MELO3C020611.2* in growing vs post climacteric. All three genes displayed decreased expression patterns according to growing stage. It can be thought that these genes suppression may have a role in circadian rhythm regulation.

In total we have found 24 DEGs within comparisons. The number of up-regulated transcripts was 13 while down-regulated ones was 11. Figure 1a shows circadian rhythm responsive DEGs that were up-regulated among comparisons. Six of the transcripts existed in growing vs climacteric. *MELO3C030034.2* (*GIGANTEA-GI*) was determined in three comparisons. *GI* expression is functionally diverse in plant development; it has been observed to be involved in physiological processes; regulation of flowering time, light signalling, hypocotyl elongation, circadian rhythm control, sucrose signalling, starch accumulation, chlorophyll accumulation, transpiration, biotic and abiotic stress herbicide tolerance (Mishra & Panigrahi, 2015). The expression pattern analysis of down-regulated DEGs were shown in Figure 1b. 36% of the DEGs were detected in growing vs post climacteric.

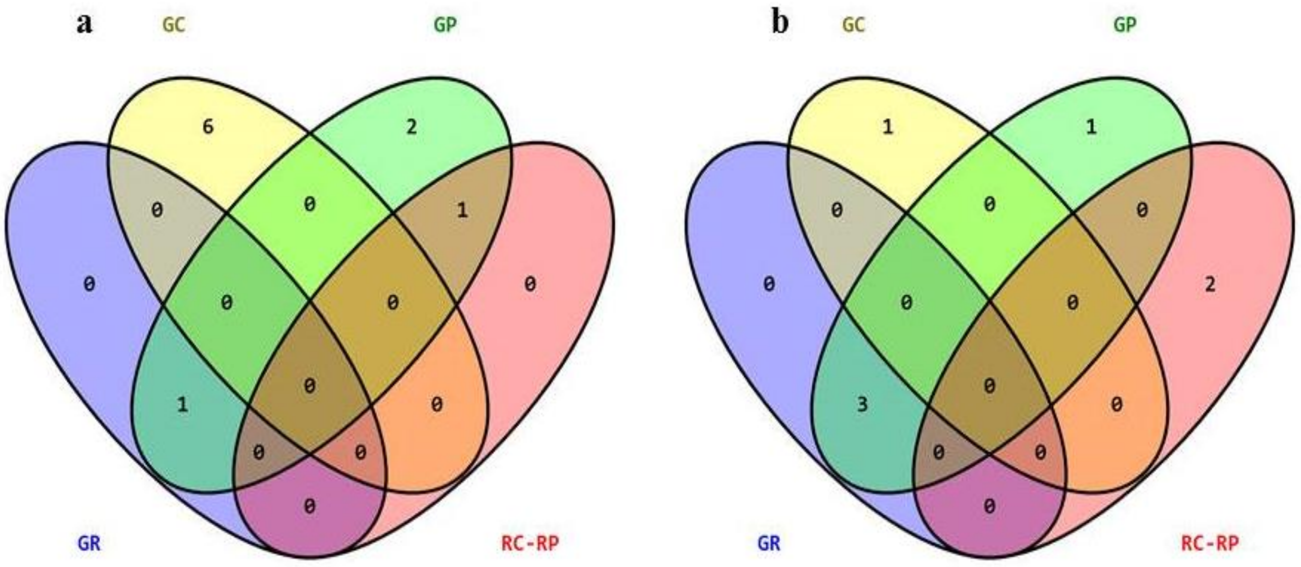

Figure 1. Venn diagrams showing the number of differentially expressed transcripts among developmental stages for up-regulated (a) and Venn diagrams showing the number of differentially expressed transcripts among developmental stages for down-regulated (b)

GO Enrichment Analysis of DEGs

To elucidate the potential role of DEGs at different developmental stages, we performed GO enrichment analysis and classified them as cellular component, molecular function, and biological process. As expected, the highest number of GO term was determined in growing vs post climacteric stages since it consist of the largest number of DEGs. Following the analysis of DEGs, we investigated that in cellular component these genes could mainly be in membrane-bounded organelle, organelle and intracellular (Figure 2). Transferase complex and vacuole terms were not existed in ripening vs climacteric or ripening vs post climacteric stages. Following the GO enrichment analysis in the molecular function category we investigated that the most of DEGs were involved in binding, DNA binding and transferase activity (Figure 3). Functional prediction of biological processes revealed that majority of the DEGs could be involved in biological regulation, metabolic process, response to stimulus and development (Figure 4). Moreover, analysis pointed out that GO terms varied at different stages of development. Except ripening vs post climacteric stages, in all comparisons Biological Regulation and Metabolic Process displayed the highest number, respectively.

The metabolic pathways in which DEG transcripts function were investigated using the KEGG database, the most comprehensive metabolic pathway database available. KEGG pathway enrichment analyses suggest that the transcripts especially developmental genes such as *FT*, *PIF3*, *PHY*, *GI*, *CHS*, *CHE* were involved in circadian rhythm (Figure 5).

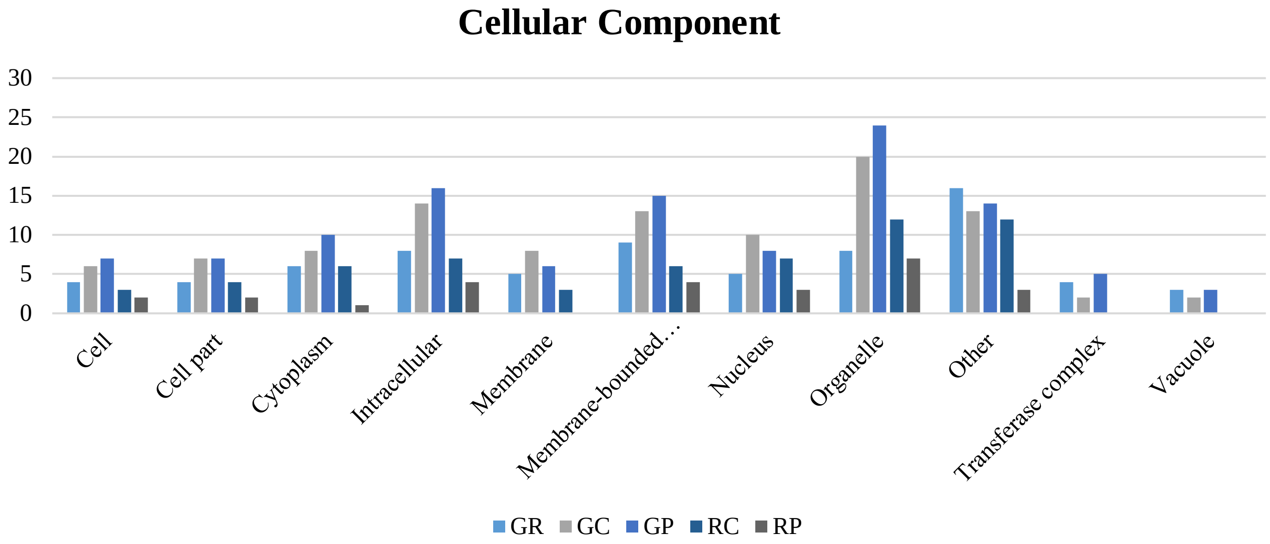

Figure 2. Gene ontology (GO) categories of annotated differentially expressed transcripts for a cellular components

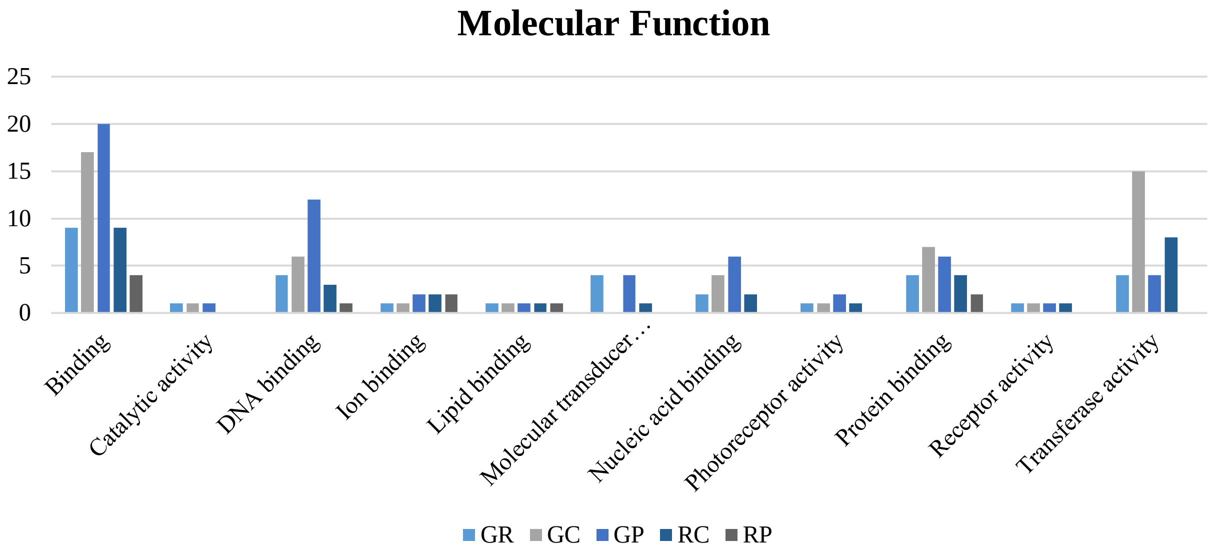

Figure 3. Gene ontology (GO) categories of annotated differentially expressed transcripts for a molecular function

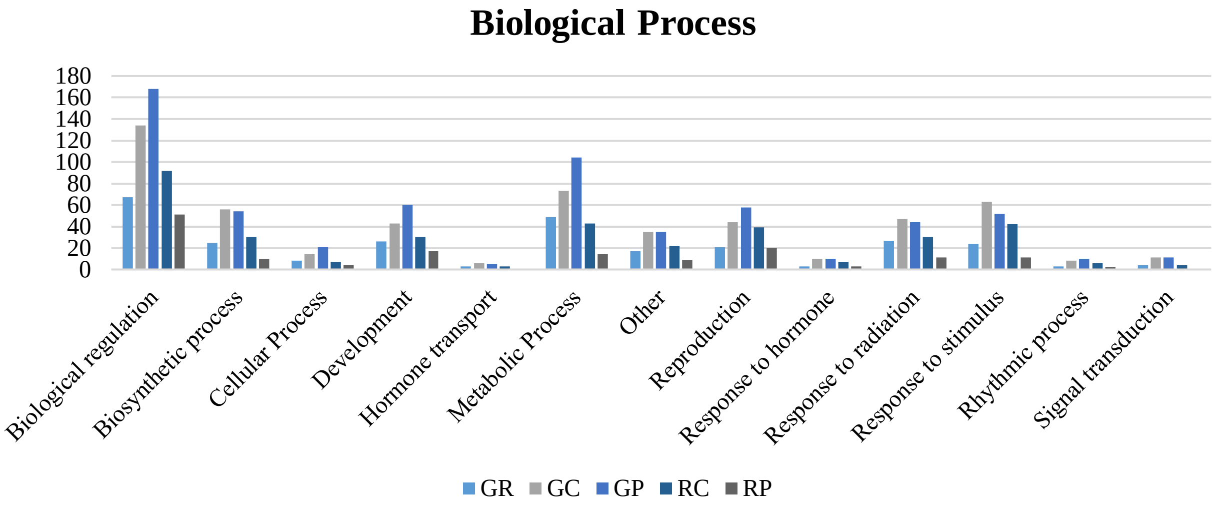

Figure 4. Gene ontology (GO) categories of annotated differentially expressed transcripts for a biological process

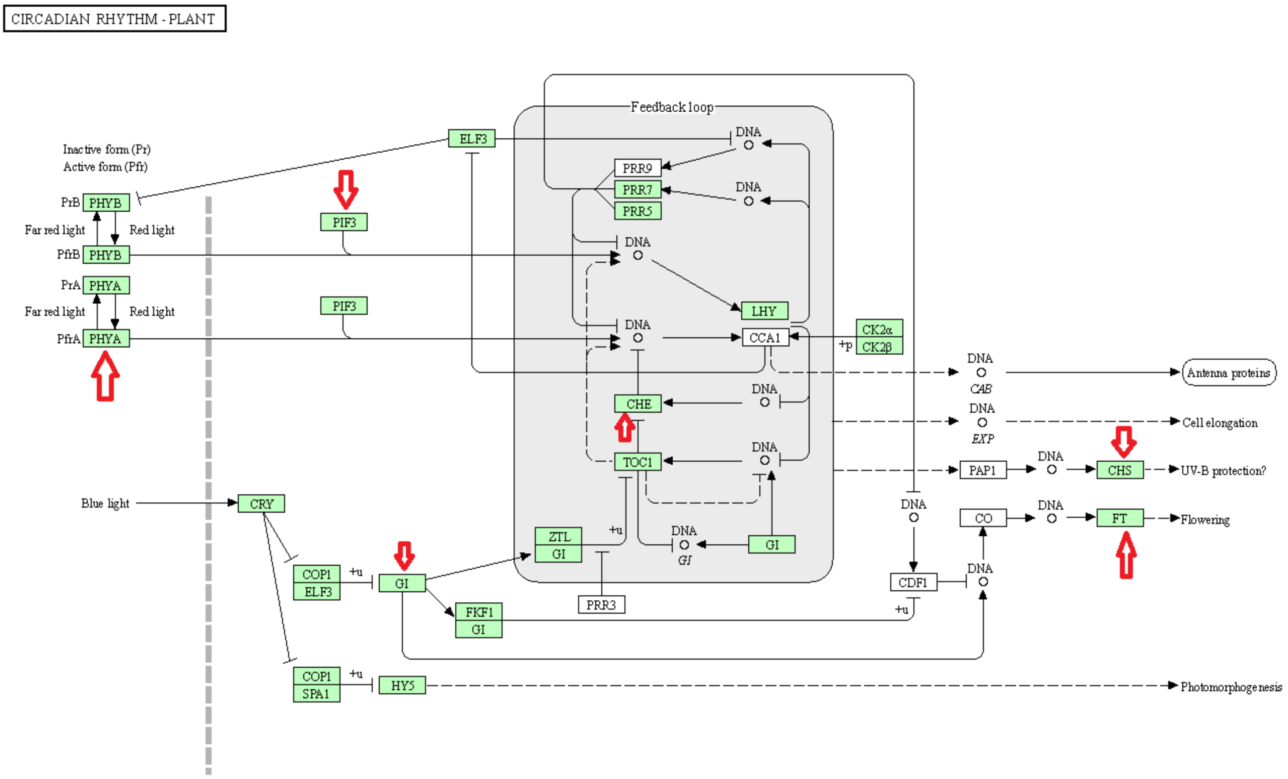

Figure 5. Circadian rhythm KEGG map (Differentially expressed circadian rhythm genes are marked with a red arrow)

CONCLUSION

Cells and organisms respond to the times of the day according to their circadian rhythm, which enables cell regeneration and the secretion of hormones, as well as control over events such as photosynthesis and flowering in plants. In this study, we investigated the relationship between different developmental processes and circadian rhythm in melon using bioinformatics tools. A detailed understanding of the relationship between circadian rhythm and growth and development in melon will help improve future agricultural production and lay the groundwork for further research on tolerance to environmental stresses.

Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author’s Contributions

The authors declare that they have contributed equally to the article.

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