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RESEARCH ARTICLE

Studies on the expression patterns of the circadian rhythm regulated genes in mango

Sumersing I. Patil[1] · Sayali N. Vyavahare[1] · Bal Krishna [1] · Prafullachandra V. Sane[1]

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Abstract Mango, an important fruit crop of the tropical and subtropical regions shows alternate bearing in most varieties causing a financial loss to the farmer. Genetic reasons for this undesirable trait have not been studied so far. In our attempts to investigate the genetic reasons for alternate bearing we have initiated studies on genes associated with the induction, repression and regulation of flowering in mango. We have previously identified and characterized *FLOWERING LOCUS T* (*FT*) genes that induce flowering and two *TERMINAL FLOWER1* (*TFL1*) genes that repress flowering. In this communication, we have explored the association of *GI-FKF1-CDF1-CO* module with the regulation of flowering in mango. The role of this module in regulating flowering has been well documented in photoperiod sensitive plants. We have characterized these genes and their expressions during flowering in Ratna variety as also their diurnal fluctuations and tissue specific expressions. The data taken together suggest that *GI-FKF1-CDF1-CO* module may also be employed by mango in regulating its flowering. Further, we suggest that the temperature dependent flowering in mango is probably associated with the presence of temperature sensitive elements present in the promoter region of one of the *GIGANTEA* genes that have been shown to be closely associated with floral induction.

Keywords Mango · Flowering genes · Circadian clock · Expression · *GI-FKF1-CDF1-CO*

Introduction

Flowering induction in plants is an important phase change and has to be at a time which is most appropriate for the plant's successful reproductive activity. Light period is the most important and robust factor to determine the seasonal change as it is highly predictable year after year. During evolution, photoperiod has been employed to trigger some of the most important events in plant's life. The light period, light quality and its intensity coupled with the variation in temperature have been responsible for inducing various important changes during plant's life cycle. This is achieved through an intricate network of genes that finally delivers the signal to induce expression of the key protein that brings about desired changes.

In recent years, the model plant *Arabidopsis thaliana* has been extensively studied for understanding the flowering mechanism from the genetic and molecular biology perspective. These studies and those on other plant species have revealed at least five flowering pathways, namely photoperiod, autonomous, vernalization, circadian clock, and gibberellic acid (GA) pathway that are involved in this important process. Several floral integrator genes, such as *GIGANTEA* (*GI*), *CONSTANS* (*CO*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *APETALA1* (*AP1*), *LEAFY* (*LFY*), *FLOWERING LOCUS T* (*FT*), *TERMINALFLOWER1*(*TFL*) and *FLOWERING LOCUS D* (*FD*) have been demonstrated to be the key transcription factors in the flowering process (Weigel et al. 1992; Bowman et al. 1993; Fowler et al. 1999; Gocal et al.

✉ Bal Krishna
balkrishna.yadav@jains.com; balkrishna000@gmail.com

[1] Plant Molecular Biology Lab, Jain R&D Lab, Jain Irrigation Systems Ltd., Agri Park, Jain Hills, Shirsoli Road, Jalgaon 425001, India

2001; Robson et al. 2001; Jakoby et al. 2002; Moon et al. 2005; Wickland and Hanzawa 2015).

Among the several flowering associated genes/proteins, one of the key protein is CONSTANS whose expression is dependent in many plants on the light and dark cycle (Valverde et al. 2004). Several studies, particularly using the model plant Arabidopsis whose flowering is photoperiod dependent; have demonstrated that *CONSTANS* expression occurs in leaves under long day conditions (Zeevaart 2008; Xiao et al. 2018; Zhang et al. 2019; Liu et al. 2020). This expression is controlled by the upstream genes which are circadian clock regulated namely *GI*, *FKF1, CDF1* and *ZEITLUPE* (*ZTL*) (Sawa et al. 2007; Fornara et al. 2009; Sawa and Kay 2011; Song et al. 2014). In long day plants *CO* accumulates almost 10 h after the dawn. Once *CO* accumulates in the leaf tissue it induces expression of *FT*, the most important member of the florigen complex, in the leaf vasculature. The FT is translocated to the apical meristem with the help of two proteins. The FT-INTERACTING PROTEIN 1 (FTIP1), an endoplasmic reticulum membrane protein, is involved in uploading FT from companion cells to the sieve elements (Liu et al. 2012). The other protein SODIUM POTASSIUM ROOT DEFECTIVE1 (NaKR1), is needed to transport FT through phloem (Zhu et al. 2016).Once FT reaches apical meristem it complexes with *FD*, a bZIP transcription factor which results in induction of downstream genes such as *LEAFY*, *AP1* etc. (Abe et al. 2005; Wigge et al. 2005).

GI expression is circadian clock controlled and expresses in several tissues and is also involved in many plant processes. Besides, its crucial role in flowering time regulation the *GI* is also involved in miRNA processing, drought tolerance, starch accumulation, light signaling, cold tolerance, herbicide resistance and chlorophyll accumulation (Mishra and Panigrahi 2015). In the plant species whose flowering is regulated by photoperiod, the role of *GI* has been well understood. Its precise function in day neutral plants such as tropical tree species has still not been properly investigated. In photoperiodic plants the transcription, translation and stabilization of CO protein is regulated by the clock controlled genes *GI, FKF1, ZTL and CDF1* genes that function upstream of *CO*. GI forms a complex with FKF1 protein that has three domains namely the LOV which has a blue light receptor which is responsible for its interaction with GI protein (Sawa et al. 2007). The GI-FKF1 complex degrades the CDFs that are responsible for repressing the transcription of not only the *CO* but also the *FT* gene. The Kelch repeat domain of *FKF1* is responsible for identifying the CDFs and the GI-FKF1 complex degrades the CDFs through its ubiquitinase activity (Nelson et al. 2000; Zhang et al. 2015). In long day plants, induction and accumulation of CO in late afternoon is associated with the loss of CDF protein in the afternoon and removal of suppression of expression of CO. This can be achieved by FKF1. FKF1 through its LOV domain binds to CO protein and stabilizes it (Song et al. 2012). It has been demonstrated that FKF1 forms a complex with GI and binds on the *CO* promoter that induces *CO* expression (Sawa et al. 2007). Sawa and Kay (2011) showed through immune precipitation studies that GI binds on the promoter of *FT* and can activate its expression. They showed that GI protein can induce *FT* expression without affecting the *CO* expression under short-day conditions. Thus GI and FKF1 and their complex have various roles in the floral induction through *CO* expression and stabilization as also through activating the expression of *FT* directly.

Another protein involved in *CO* expression and stabilization is ZEITLUPE (ZTL) and Song et al. (2014) showed that ZTL can physically interact with CO protein and negatively control the CO stability. GI also stabilizes ZTL protein and Song et al. (2014) suggested that relative changes in the GI, FKF1 and ZTL proteins regulate the stability of CO protein and expression of *FT*. The mechanism for expression of *CO* under long day conditions is therefore well understood.

Mango (*Mangifera indica* L.) belongs to the family '*Anacardiaceae*'. Mango being a perennial tree and grown in varied climates from tropics to subtropics, the irregular flowering habits of most of the mango cultivars result in poor income to the farmers unless they manipulates it horticulturally with additional expenditure. Till date molecular and genetic factors associated with regulation of flowering in mango have not been studied well. In terms of genes associated with regulation of flowering in mango, three *MiFTs,* two *MiTFL1s* (Nakagawa et al. 2012; Krishna et al. 2017; Vyavahare et al. 2017; Fan et al. 2020), two *AP1s* (Yu et al. 2020), one *SOC1* (Wei et al. 2016) and one *CO* (Liu et al. 2020) have been reported. Besides these reports, a few transcriptome studies (Yadav et al. 2019; Sharma et al. 2020) have also been reported in mango in relation to flowering.

The regulation of flowering in day neutral plants is not well characterized. *DAY NEUTRAL FLOWERING* (*DNF*) gene has been identified as a negative regulator of *CO* expression. This protein is an E3 ligase and represses expression of *CO* between four to seven hours after dawn (Morris and Jackson 2010) ensuring that flowering in Arabidopsis does not occur in short days. The flowering in mango is known to be temperature dependent (Núñez-Elisea and Davenport 1994). Studies by Núñez-Elisea and Davenport (1994) showed that floral buds get initiated when mean minimum temperatures are around 15 °C under Florida conditions that are similar to those in the tropics in India. Davenport (2007) provided details of how temperatures and other environmental factors affect induction of

flowering in mango. Sukhvibul et al. (2000) working with two mono embryonics namely 'Irwin' and 'Sensation' and two poly-embryonics namely 'Nam Dok Mai' and 'Kensington' cultivars of mango observed that inflorescence development occurred when these were grown under controlled conditions of 20/10, 25/15 and 30/20 °C. Rajatiya (2018) concluded that a combination of day temperature around 30 °C with a low temperature of less than 19 °C at night induce flowering in mango in Gujarat region of India. It is not known whether in the day neutral mango the regulation of flowering involves *GI-FKF1-CDF1-CO* module. In this communication, we have studied these genes from Ratna cultivar and have characterized their expression patterns to understand their role in mango floral induction. Taking into consideration the diurnal changes in the expression patterns as also seasonal changes in their expression particularly during the flowering months it appears that *GI-FKF1-CDF1-CO* system may also be functioning in mango. Further, we suggest that the relation between flowering and low temperature at night observed in mango may be related to the presence of increased number of temperature sensing elements in the promoter region of MiGI2 of mango.

Materials and methods

Plant material

All samples were collected from the mango orchard of cv. Ratna located at Jain R&D Farm, Jain Hills, Jain Irrigation Systems Ltd., Shirsoli Road, Jalgaon, India. Cultivar Ratna is a regular and prolific bearer and has a tendency to flower and fruit every year. The tissues were collected and snap frozen in liquid nitrogen and stored at − 80 °C until further processing. Leaf and reproductive tissue (reproductive apex, inflorescence, and flower) samples were collected for isolation of the genes. Gene expression analyses during flowering were performed using leaf samples collected in the afternoon (2:00–3:00 pm) from the first week of October to first week of April. During that year flower initiation occurred around middle of December and was complete by 30th January with 50% flowering occurring around 15th January. Supplementary Fig. 1 gives maximum and minimum temperatures during that period. Flowering initiation occurred when the temperature at night fell below 15 °C while the day temperature was about 25 °C.

To monitor the circadian rhythm, leaf samples over a 52 h period were collected at three hour intervals starting from 6:00 am on 26th to 28th November. Samples for tissue specific gene expression analysis were collected between September to April based on tissue type, mature leaf (ML), vegetative apex (VA), reproductive apex (RA) and flower (FL). All samples were collected in three biological replicates and each replicate represents an average from three plants.

Nucleic acid isolation

Genomic DNA was isolated using a protocol described by Dellaporta et al. (1983). For cDNA library preparation and gene expression studies total RNA was extracted by using Spectrum ™ Plant Total RNA kit (Sigma-Aldrich, USA) following steps as described by the manufacturer. The concentration of RNA was determined by NanoDrop100 followed by monitoring its integrity by electrophoresis on 2% TBE agarose gel. First strand cDNA was prepared using 2 µg total RNA as template with Revert Aid H minus first strand kit (Thermo Fisher Scientific, USA).

Gene isolation and identification

Genome walking library was prepared using 10 µg of genomic DNA of mango digested with DRAI, EcoRV, HindIII, Stul (Fermentas Life Sciences, Lithuania) and genome walking was carried out using Genome walker Universal kit (Clonetech Laboratories, Inc. USA) following manufacturer's instructions. SMARTer RACE cDNA Amplification kit (Clonetech Laboratories, Inc. USA) was used for the preparation of 5′ SMARTer and 3′ SMARTer libraries. Preparation of cDNA liabrary was done by using RNA from a mixture of various tissues like vegetative tissues and reproductive tissues.

Isolation of MiGI genes

For the amplification of the *GIGANTEA* (*GI*) genes, a set of degenerate primers (Supplementary Table 1) were designed based on the sequence homology of dicotyledonous plants of different species by using iCODEHOP software (https://www.bitnos.com/info/icodehop). The putative gene portion was amplified using a set of degenerate primers, tenfold-diluted cDNA was used as a template in polymerase chain reaction (PCR) following PCR program 5 min at 95 °C, 30 s at 95 °C, 30 s at 55 °C, 1 min at 72 °C, 6 min at 72 °C, and final hold at 4 °C. The PCR products were cloned in pTZ57R/T vector using InsTAclone PCR cloning kit (Thermo Fisher Scientific, US). The fragment sequence that showed homology to the published GI sequences from other species was used to obtain full length *MiGI* sequence using 5'SMARTer and 3' SMARTer libraries. A full length complementary DNA sequence was obtained using appropriate primers and cDNA library. The obtained sequence was named as *MiGI1*. A BLAST query of full-length complementary

DNA sequence of *MiGI1* was performed with the recently published mango genome of Alphonso (Wang et al. 2020). This search resulted into two similar sequences one was identical to *MiGI1* and the other which was similar to *MiGI1* was named as *MiGI2*. Using the published sequences of MiGI2 of Alphonso, primers were designed to amplify coding region of *MiGI2* using cDNAs of Ratna. Obtained DNA bands were cloned in pTZ57R/T vector using InsTAclone PCR cloning kit (Thermo Fisher Scientific, US) and sequence was confirmed.

Isolation of MiCO genes

Amplification of the *CONSTANS* (*CO*) genes was done by using a set of degenerate primers (Supplementary Table 1) designed based on the sequence homology of dicotyledonous plants of different species by using iCODEHOP software (https://www.bitnos.com/info/icodehop). Degenerate primers (DegMiCO F1 and DegMiCO R1) were used on a tenfold diluted genomic DNA in a polymerase chain reaction (PCR) following PCR program 5 min at 95 °C, 30 s at 95 °C, 30 s at 55 °C, 40 s at 72 °C, 6 min at 72 °C and final hold at 4 °C. The obtained fragments were cloned in pTZ57R/T vector using InsTAclone PCR cloning kit (Thermo Fisher Scientific, US). Sequencing of these fragments identified one 393 bp fragment that showed homology with the *CO* like genes. This 393 bp sequence was extended to full length using Genome walker library**,** 5′ SMARTer and 3′ SMARTer libraries. We obtained two sequences that showed homology to the *CO* sequences from other plant species. These were designated as *MiCO1* and *MiCO2*.The intron–exon junctions of both *CO*s were identified by using web based (www.gsds.gao-lab.org) Gene Structure Display Server (GSDS) (Hu et al. 2015).

Identification of MiFKF1 and MiCDF1

MiFKF1 and *MiCDF1* genes were searched from recently published mango genome of Alphonso (Wang et al. 2020) using Blastn. Based on *MiFKF1* and *MiCDF1* sequence of Alphonso, primers were prepared to amplify coding region of *MiFKF1* and *MiCDF1* using cDNAs of Ratna. Obtained DNA bands were cloned in pTZ57R/T vector using InsTAclone PCR cloning kit (Thermo Fisher Scientific, US) and sequences were confirmed.

Bioinformatics analysis

The cDNA sequences of candidate genes were translated into amino acid sequences using NCBI protein blast. Multiple sequence alignments were performed using ClustalW 2.1 program (http://www.genome.jp/tools/clustalw/) for conducting phylogenetic analysis and a phylogenetic tree was constructed by the MEGA6 software using neighbor- joining (NJ) with 1000 bootstrap values with respective homolog for each of the candidate genes (http://www.megasoftware.net/). For the boxshaded background representation, BOXSHADE (version 3.21) was used (http:/ch.embnet.org/software/BOX_form.html). The molecular weight of proteins was calculated using the geneid WEB server (http://www.geneinfinity.org/sms/sms_proteinmw.html). The percent similarity and identity were calculated by online bioinformatics tool MatGAT (Matrix Global Alignment) software package (Campanella et al. 2003)**.** GI promoter analysis was performed using plantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Gene expression analysis

Real time quantitative PCR was performed using 5 µl Sso fast ^{TM} Eva Green Supermix (Bio-Rad Laboratories, USA), 250 nM of 0.3 µl of each forward and reverse primer, 2 µl 1:50 diluted cDNA and 2.4 µl Nuclease free water as PCR mixture. Three technical replicates were used for the gene expression analysis. Rotor—Gene Q (QIAGEN Inc. USA) was used to run real-time PCR for gene expression analysis.

Mango *Elongation factor* gene (*MiEF*) was used as a housekeeping gene for this study (Vyavahare et al. 2017). Relative gene expression calculation was done by using delta ct method (Livak and Schmittgen 2001). The gene specific primers used for the gene expression analysis (Supplementary Table 2) were designed using Primer3 (Version 0.4.0) software (https://bioinfo.ut.ee/primer3-0.4.0/).

Results

Identification of mango GI, CO, MiFKF1 and MiCDF1 genes

The cDNA libraries were prepared from a mixture of RNA isolated from leaves of vegetative to reproductive stages to obtain a full length cDNA sequence using 5′ and 3′ RACE strategy as mentioned in Materials and Methods. The full-length sequences of *MiGI* have been submitted to NCBI genBank, named as *MiGI* (GenBank accession no. MK521809). After the publication of mango genome (Wang et al. 2020), we scanned the genome for the presence of *MiGIs*. We identified two similar genes. One of them was identical to *MiGI,* designated as *MiGI1* (already submitted by us) and the other was named as *MiGI2*. Based on the sequence of published Alphonso *MiGI2*, primers were designed to amplify coding region of *MiGI2* using cDNAs of Ratna (Supplementary Table 2). Obtained DNA

bands were cloned, sequenced and submitted to NCBI as *MiGI2* (accession no. MZ357241). A comparison of the two showed that both the *GIs* have 14 exons and 13 introns. The organization of *AtGI* consists of 14 exons and encodes a protein of 1173 amino acids (Fowler et al. 1999). *MiGI1* and *MiGI2* are 3516 bp and 3522 bp respectively encoding proteins of 1171 and 1173 amino acids. The predicted molecular weights of MiGI1 and MiGI2 proteins are 128.42 kD and 128.82 kD. Presence of two MiGIs is not surprising since the presence of two GIs has been documented in other species such as poplars (Ke et al. 2017) and onion (Taylor et al. 2010) besides some others. The genomic organizations of *MiGI1* and *MiGI2* genes are shown in Fig. 1. The genomic organization of *MiGI1* and *MiGI2* has been observed as similar except the 14th exon of *MiGI2* is six nucleotides longer than *MiGI1* (Fig. 2A). The intron lengths were different in the two GIs. The first intron of *MiGI1* was observed as much longer than in *MiGI2* while the 10th intron in *MiGI1* was shorter than the corresponding intron in *MiGI2*. The number of exons and introns varies in different plants: 14 exons and 13 introns in Arabidopsis (Fowler et al. 1999; Park et al. 1999), 16 exons and 15 introns in Brachypodium (Hong et al. 2010), 14 exons and 14 introns (one in the 5′UTR) in almond (Barros et al. 2017).

The amplification of *MiCO* was achieved through genome walking using degenerate primers (Supplementary Table 1) as mentioned in Materials and Methods. The full-length sequences of *MiCO1* and *MiCO2* have been submitted to NCBI genBank, named as *MiCO1* (GenBank accession no. MW969646) and *MiCO2* (GenBank accession number MW969647). *MiCO1* and *MiCO2* are 969 bp and 1116 bp long respectively encoding proteins of 322 and 371 amino acids respectively. The predicted molecular weights of MiCO1 and MiCO2 protein are 35.13 kD and 41.08 kD. The genomic organizations of *MiCO1* and *MiCO2* are shown in Fig. 1. The *CO* was predicted to possess two exons and one intron in both *MiCO1* and *MiCO2* (Fig. 1). The lengths of the introns and exons as well as 3' UTR are different in the two *CONSTANS* genes.

MiFKF1 and *MiCDF1* genes were searched and identified from recently published mango genome (Wang et al. 2020). Based on *MiFKF1* and *MiCDF1* sequence of Alphonso, primers were prepared to amplify coding regions of *MiFKF1* and *MiCDF1* using cDNAs of Ratna (Supplementary Table 2). Obtained DNA bands were cloned in pTZ57R/T vector using InsTAclone PCR cloning kit (Thermo Fisher Scientific, US) and sequenced. The full-length coding sequences of *FKF1* and *CDF1* genes were submitted to NCBI as *MiFKF1* (accession no. MZ357240); *MiCDF1* (accession no. MZ357239). *MiFKF1* and *MiCDF1* genes are 1881and 1407 bp long and encoding proteins of 626 and 468 amino acids respectively. The genomic organizations of *MiFKF1* and *MiCDF1* are shown in Fig. 1. The *FKF1* and *CDF1* possess two exons and one intron each (Fig. 1). The exons and introns were identified by using gene structure display server tools. The predicted molecular weights of MiFKF1 and MiCDF1 proteins are 69.46 kD and 51.00 kD respectively.

Sequence analysis of MiGI, MiCO, MiFKF1 and MiCDF1

The complete deduced amino acid sequences of MiGI1 and MiGI2 are shown in supplementary Fig. 2 while the relevant portion of the same are shown in Fig. 2A using BOXSHADE (version 3.21). Both the MiGIs were observed as 95% similar and 91% identical to each other on the basis of protein sequence. However there were some noticeable differences. One major difference is the absence of glycine and histidine in MiGI1 at position 1119 and 1120. MiGIs possess nuclear localization region located between the residues 543 to 783 ($\sim$ 241 amino acid region). This region consists of four clusters of basic amino acids as in Arabidopsis AtGI and the residues 749(S) and 750(E) bisect this region as in AtGI whose disruption disturbs the nuclear localization determinant as proposed by Huq et al. (2000) (Fig. 2A). We have not looked at the 5′ UTRs in these two genes. Apparently they must be different since there were differences in the expression levels of the two transcripts as shown later. The mango *GI* genes showed highest similarity (92%) to citrus *GI*.

The deduced amino acid sequences of MiCO1 and MiCO2 are shown in Fig. 2B using BOXSHADE (version 3.21). These two *CONSTANS* like genes belong to group 1 possessing characteristic two B-boxes that appear at positions 5–48, 48–91 in MiCO1 and 17–60, 60–103 in MiCO2. They also possessed group 1 characteristic CCT domain near the carboxy terminus which appeared between amino acids 255–297 in MiCO1 and 302–344 in MiCO2. The percent similarity and identity matrix showed that the MiCO1 protein was observed as 61.7% and 83.6% similar and 45.7% and 47.9% identical to that of Arabidopsis and citrus CO protein and similarly MiCO2 protein was 69.7% & 62.8% similar and 56.7% and 46.5% identical to Arabidopsis and citrus CO protein. The data showed that the MiCO1 protein is 83% similar and 75% identical to citrus CO whereas MiCO2 gene showed 70% similarity and 56% identity to Arabidopsis CONSTANS (AtCOL1). In addition, MiCO1 and MiCO2 proteins were 99.1% and 60.6% similar and 98.8 and 45.3% identical to MiCO proteins of mango cv. SiJiMi and also 98.1% and 60.6% similar and 97.5% & 45% identical to mango cv. TaiNong No. 1 (Liu et al. 2020). The MiCO1 protein similarity to MiCO (Liu et al. 2020) indicated that this gene is conserved in most of the mango cultivars/varieties. However, minor changes in

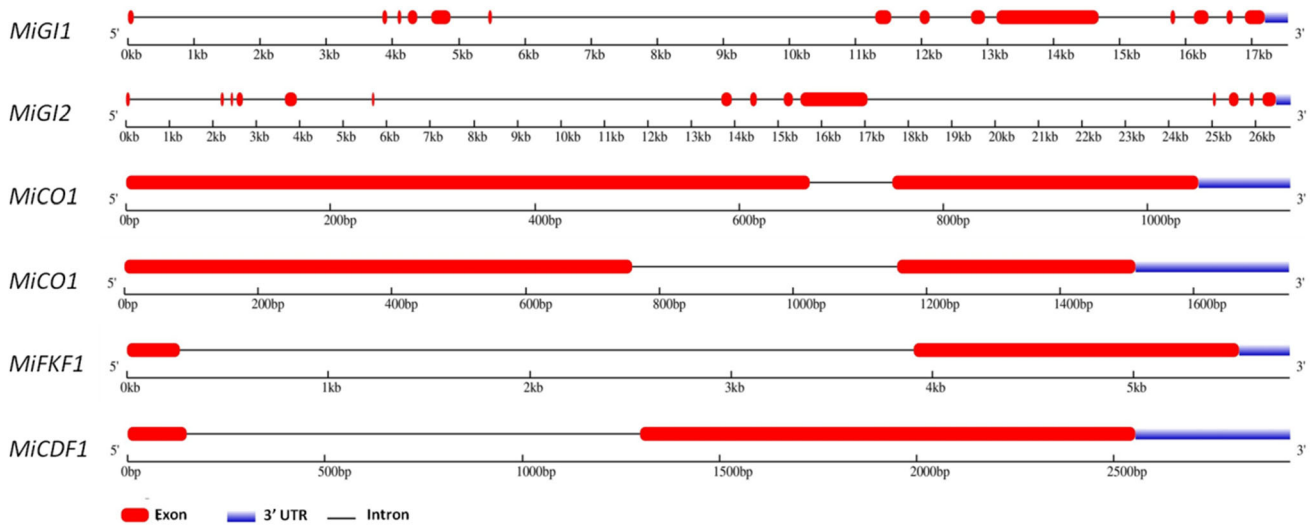

Fig. 1 Genomic organization of *MiGI (MiGI1 and MiGI2), MiCO (MiCO1 and MiCO2), MiFKF1 and MiCDF1*. Red/thicker box indicates exons, black line indicates introns and blue/thinner box indicates the 3' UTR regions

the protein sequences may have been incorporated during evolution of varieties.

The deduced amino acid sequences of MiFKF1and MiCDF1 obtained by using BOXSHADE (version 3.21) are shown in Fig. 3A and Fig. 3B. The FKF1 possesses the LOV domain which has been observed near the N terminal, the central region contains the F-box and the C terminal possesses six Kelch repeat domains (Zoltowski and Imaizumi 2014). The percent similarity and identity matrix showed that the MiFKF1 protein was 88.7% and 91.4% similar and 79.0% and 85.2% identical to Arabidopsis and citrus FKF1 protein respectively. The deduced amino acid sequence of *CYCLING DOF FACTOR 1-like* CDF1 proteins (CDF1) has a conserved Zinc finger DNA binding domain at position 110–164, 147–203, 131–187 and 119–175 in Arabidopsis, citrus, pistachio and mango respectively. The MiCDF1 protein is 45.7% and 65% similar and 31.7% and 50.2% identical to Arabidopsis and citrus CDF1 proteins respectively.

Since we have the amino acid sequences of Alphonso, one of the parents of Ratna, it was interesting to compare to what extent the sequences of genes under study were different. A comparison of sequences of Ratna and Alphonso MiGIs showed that MiGI1 Ratna was 99.1% similar and 98.5% identical to MiGI1 Alphonso while MiGI2 Ratna was 97.8% similar and 96.2% identical to MiGI2 Alphonso on amino acid sequence. It appeared that Ratna has inherited these two genes from Alphonso. A comparison of other 3 genes also suggested that Ratna and Alphonso has showed over 99% similarity and identity between the two varieties. These results suggests that Ratna has probably inherited these genes from Alphonso.

Phylogenetic analysis of circadian clock controlled genes

The phylogenetic tree of mango GI (MiGI1 and MiGI2) protein (shown in Fig. 4A) was constructed including several eudicots and monocots as mentioned in the legend of the figure. All the dicots cluster together while the monocots form a separate clade. MiGIs are closest to the citrus GI. A phylogenetic analysis of mango zinc finger CONSTANS like (MiCO1 and MiCO2) protein is shown in Fig. 4B. It includes several eudicots and monocots as mentioned in the legend to the figure. The dicots and monocots separate into different clusters. The previously published MiCOs of mango varieties SiJiMi and TaiNong No. 1 (Liu et al. 2020) have been reported as similar to MiCO1 of this study. Liu et al. (2020) however have not reported about the other MiCO.

Phylogenetic analysis of mango FLAVIN BINDING KELCH REPEAT F BOX 1 protein (MiFKF1) was carried out (Fig. 4C) being compared with several dicots and monocots as detailed in the legend to the Figure. Similarly phylogenetic analysis of mango CYCLIC DOF FACTOR 1-like CDF1 proteins (MiCDF1) was carried (Fig. 4D) including CDFs from other eudicots and monocots. Mango CDF1 also showed close similarity with PvCDF1, PvCDF2 and CsCDF3.1 whereas PvCDF3 and CsCDF3.2 have been clustered in a separate node.

Expression of circadian clock controlled genes follow circadian clock

The primers which were used in all the expression analyses are given in Supplementary table 2. In Arabidopsis,

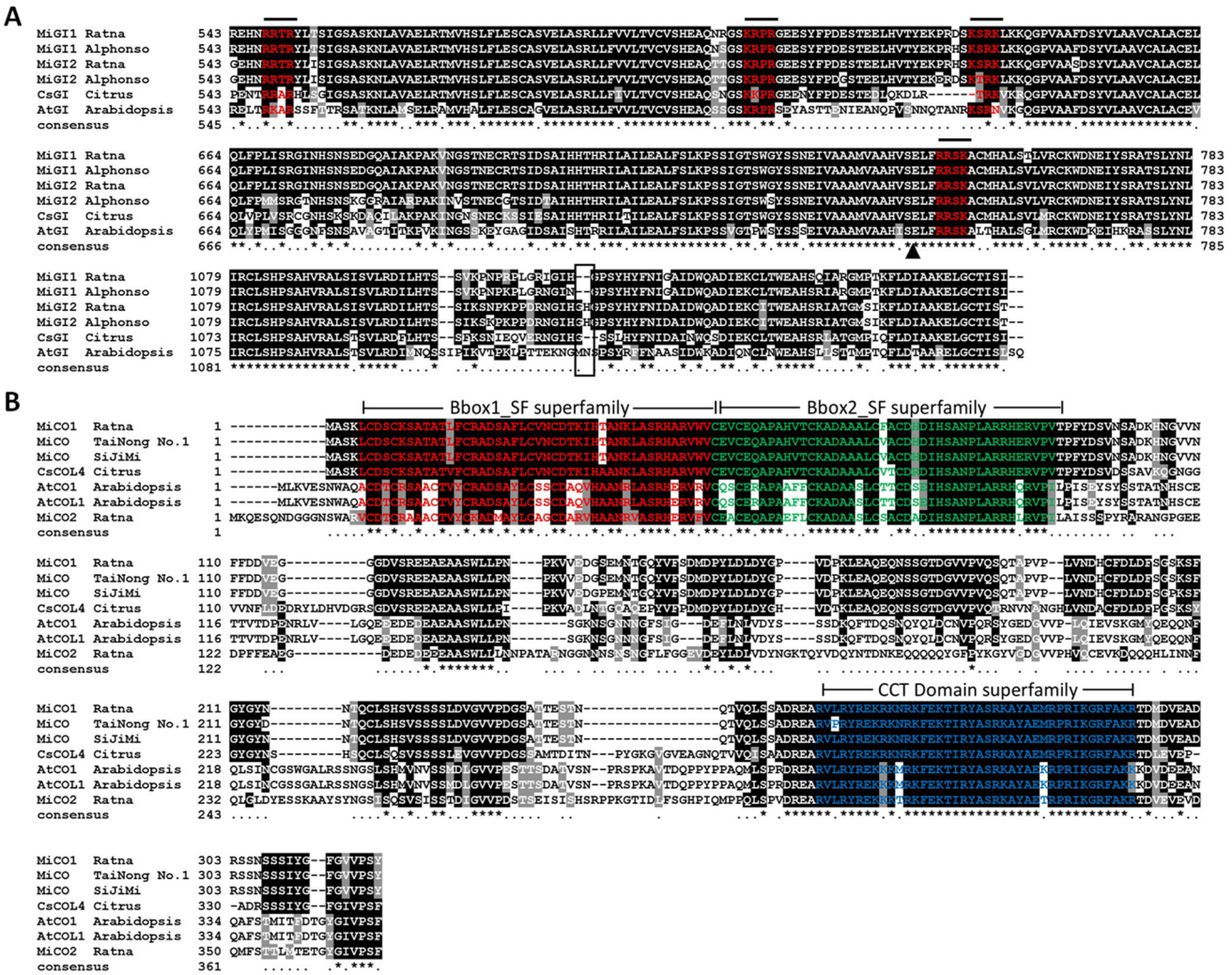

Fig. 2 Boxshade alignment of relevant portions of deduced amino acid sequences of GIGANTEA (GI) protein and full sequences of *CONSTANS* like protein. **A** Boxshade alignment of part of the deduced amino acid sequences of GIGANTEA (GI) gene family including mango GI Ratna (MiGI1 and MiGI2) and Alphonso (MiGI1 and MiGI2 taken from mango genome published by Wang et al 2020), Arabidopsis (AtGI) and citrus (CsGI) showing the nuclear localization region containing four clusters of basic amino groups shown in red colored letters (and black line at top) and red/black color triangle indicating junction between amino acid 749 to 750 whose disruption disturbs the function of GI. Red line box indicates the presence of two additional amino acids in MiGI2 accounting for the difference of two amino acids between the two genes. The full sequence of GIs is given in Supplementary Fig. 2. **B** Boxshade alignment of deduced amino acid sequences of CONSTANS LIKE (CO) protein family including mango (MiCO1 and MiCO2), mango SiJiMi (MiCO S), TaiNong No. 1 (MiCO T), citrus (CsCOL4) and Arabidopsis (AtCO1, AtCOL1). B-box1, B-box2 and CCT domain are indicated in the figure.

expression of flowering time genes like *LHY*, *CCA* and *AtGI* are regulated by the circadian clock and show rhythmical patterns in light/dark cycles (Fowler et al. 1999). To examine whether *GI* transcript levels fluctuate in a cycle, leaf tissue samples were collected at three hour intervals over a 52 h period. For the expression analysis of *GI* circadian pattern, biological triplicates were averaged and statistically treated using standard error bars. Times of sampling are expressed in hours as zeitgeber time (Zerr et al. 1990). It was observed that the expression levels of *MiGI1* and *MiGI2* followed the circadian clock. Expression levels of *MiGI1* and *MiGI2* started rising from 3 pm reaching a peak at 6 pm in case of *MiGI1* and 9 pm for *MiGI2* after which both declined reaching a minimum by around 6 am and then remained low until 3 pm. The pattern was repeated in the next 24 h (Fig. 5A). Thus both *MiGI* transcripts followed the circadian rhythm. The expression level of *MiGI2* was slightly higher than *MiGI1*. We also analyzed the expression patterns of other key circadian clock controlled gene namely *CONSTANS like*. We have observed that the expression level of *MiCO1* and *MiCO2* followed the circadian clock. The expression levels of *MiCO1* and *MiCO2* began rising beginning early morning and reached a peak around 6 am and then started

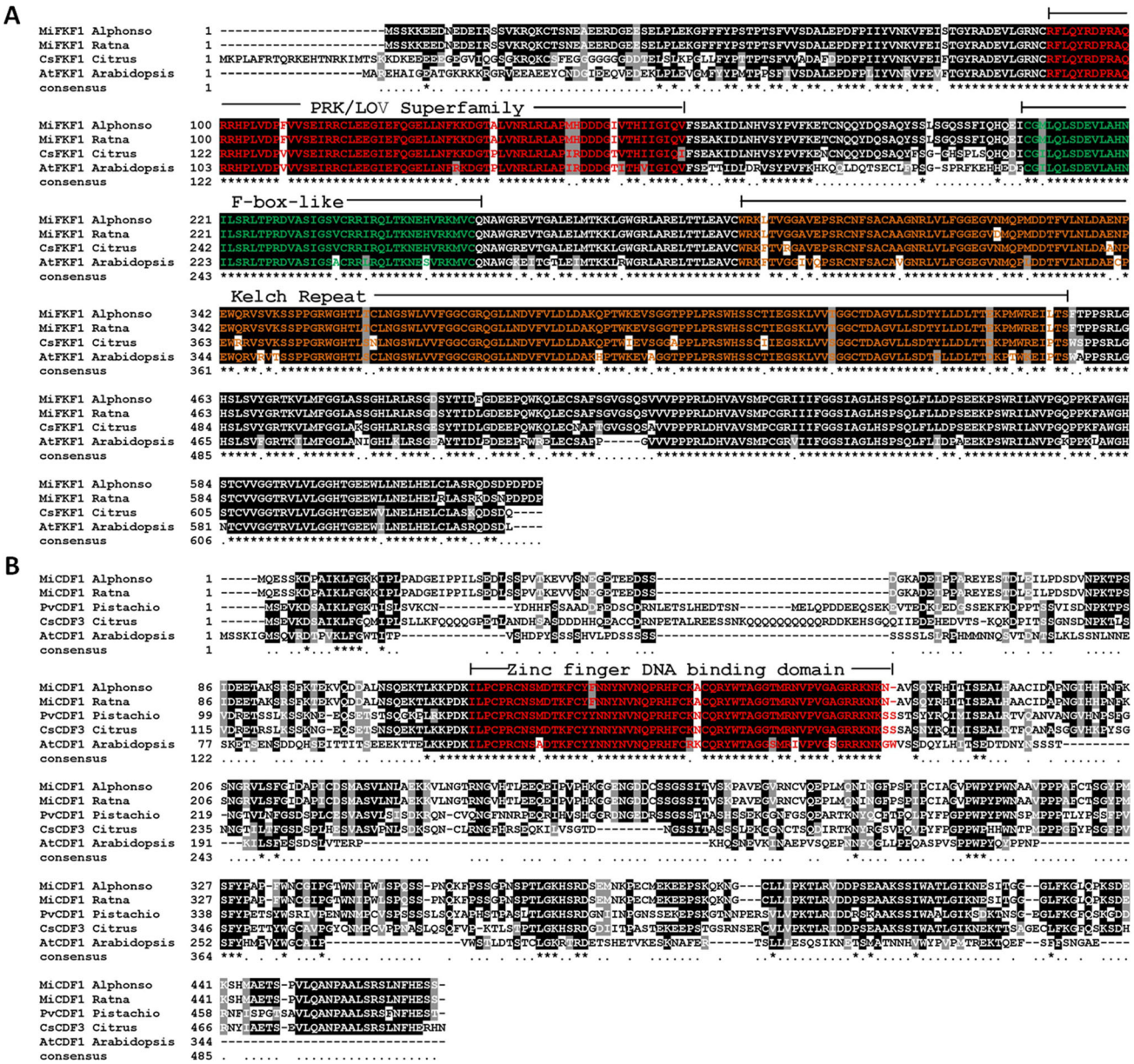

Fig. 3 Boxshade alignment of deduced amino acid sequences of FLAVIN BINDING KELCH REPEAT F BOX 1 (FKF1) proteins and CYCLING DOF FACTOR1 (CDF1) proteins. **A** Boxshade alignment of deduced amino acid sequences of mango MiFKF1 protein in comparison with FKF1 proteins of Arabidopsis (AtFKF1) citrus (CsFKF1) and mango cv. Alphonso (MiFKF1). PAS/ LOV domain, F-Box domain and Kelch repeats are indicated in the figure. **B** Boxshade alignment of deduced amino acid sequences of mango MiCDF1 in comparison with CDF1 proteins of Arabidopsis (AtCDF1), pistachio (PvCDF1) mango cv. Alphonso (MiCDF1) and citrus (CsCDF3). Zinc finger DNA binding domain is indicated in the figure. Accession numbers of all the genes is given in Supplementary Table 3

decreasing gradually reaching its low value at 12 noon in case of *MiCO2* while the *MiCO1* maintained a somewhat high expression reaching a minimum at 6 pm. In mango, it has been shown that the level of expression of *MiCO* was higher in the morning around 9 am (Liu et al. 2020). This variation in the expression peak of *MiCO* genes may be due to the difference in the experimental location or due to a change in the varieties used for study. The cycle was repeated in the next 12 h showing low expression at night and then starting to rise beginning early morning. The *MiCO2* was somewhat earlier than *MiCO1* (Fig. 5B). The real time expression data showed that both *MiCO*s (*MiCO1*and *MiCO2*) followed the circadian clock such that when the expressions of *MiGIs* came down *MiCOs* expression began to rise.

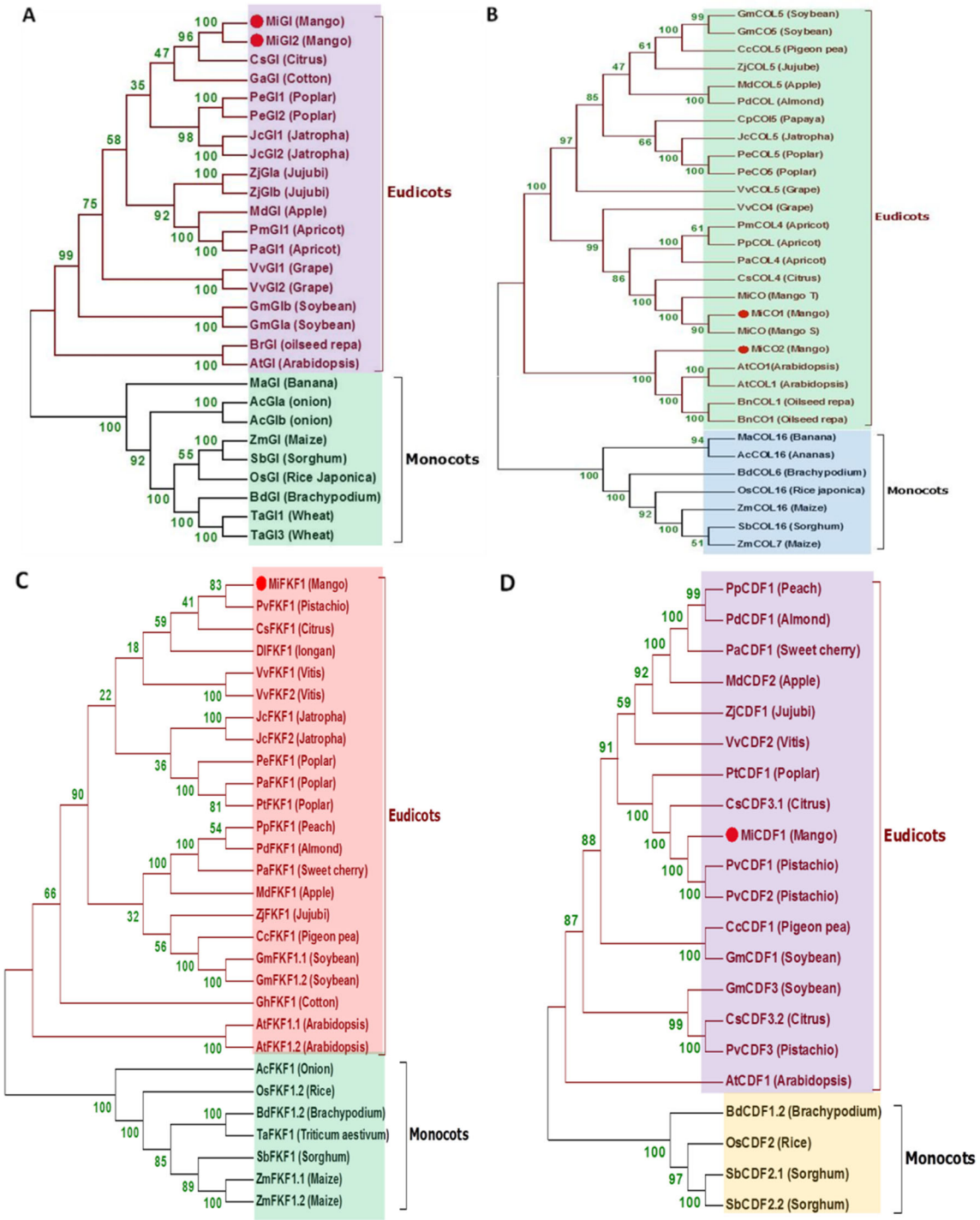

Fig. 4 Phylogenetic analysis of mango GIGANTEA proteins (MiGI1 and MiGI2), mango CONSTANS proteins (MiCO1 and MiCO2), mango FKF1 protein (MiFKF1) and mango CDF1 protein (MiCDF1). The tree was constructed using the MEGA6 program using the neighbor- joining (NJ) method with 1000 bootstrap value. Number at nodes indicates bootstrap values (in percentage). **A** Phylogenetic tree of mango GIGANTEA proteins (MiGI1&MiGI2). **B** Phylogenetic tree of mango CONSTANS like (MiCO1and MiCO2) proteins, **C** Phylogenetic tree of mango FLAVIN BINDING KELCH REPEAT F BOX 1 protein (MiFKF1) and **D** phylogenetic analysis of mango CYCLIC DOF FACTOR 1-like CDF1 proteins (MiCDF1). The gene sequences from the following dicots and monocots have been compared with the mango proteins. Dicots compared: almond (Pd), apple (Md), apricot (Pm), Arabidopsis (At), citrus (Cs) cotton (Ga),grape (Vv) jatropha (Jc) jujube (Zj) longan (Dl), mango SiJiMi (Mi S); TaiNong No. 1 (Mi T) oilseed repa (Br), papaya (*Cp*), pigeon pea (*Cc*), poplar(Pe2), soybean (Gm), sweet cherry (PaFKF1). Monocots compared: ananas (Ac) banana (Ma), Brachypodium (Bd) maize (Zm) onion (Ac) rice japonica (Os) sorghum (Sb) and wheat (Ta). Accession numbers of all the genes used for comparision have been given in supplementary Table 3

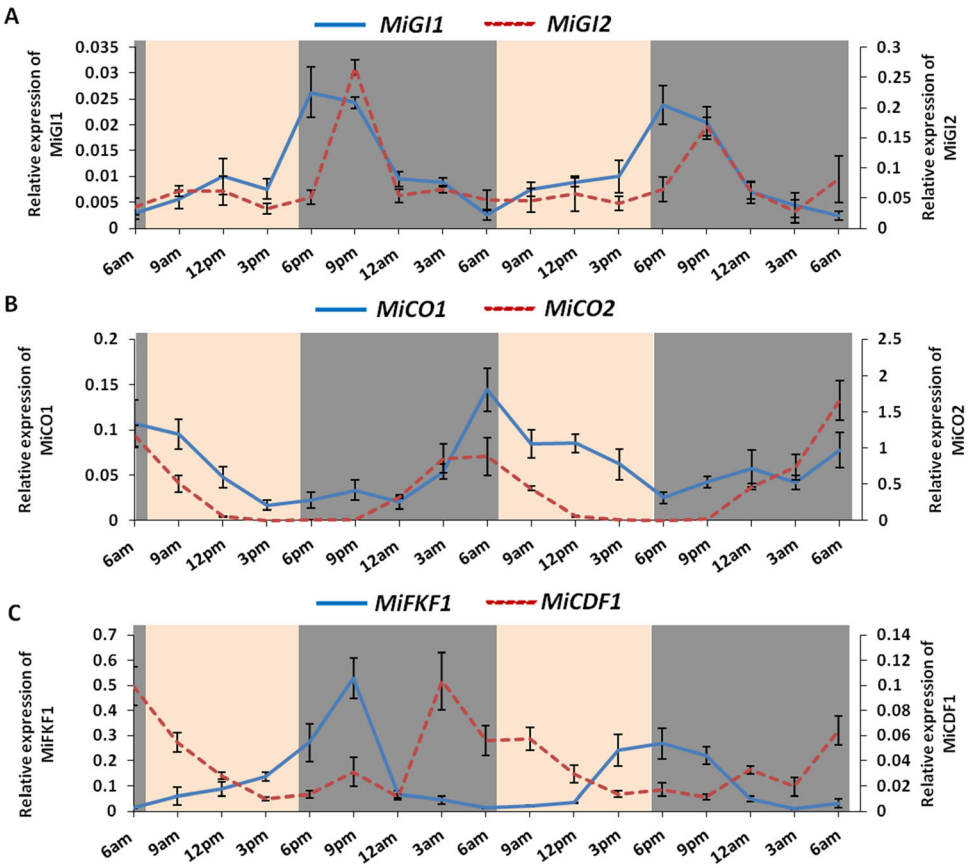

Fig. 5 Relative expressions of *MiGIs* (**A**), *MiCOs* (**B**), *MiFKF1* and *MiCDF1* (**C**) genes in leaf tissues of Ratna cultivar. The X- axis represents time interval and Y-axis (primary axis indicates relative expression of *MiGI1*, *MiCO1* and *MiFKF1* in **A**, **B** and **C** respectively and secondary axis indicates relative expressions of *MiGI2*, *MiCO2* and *MiCDF1* in **A**, **B** and **C** respectively). Gene expression is the mean of three biological replicates and bars indicate standard error of the mean

Further, we checked the expression patterns of *MiFKF1* and *MiCDF1* genes which play an important role in regulation of circadian rhythms. Interestingly, the expression pattern of *MiFKF1* followed the expression pattern of *MiGI1* while the expression pattern of *MiCDF1* followed the patterns of *MiCOs* (Fig. 5C) but peaking about 3 h earlier. The expression patterns showed that when *MiGI* expression was high the *FKF1* expression was also high. This is accompanied by low expression of *CDF1*. When the expression of *FKF1* was at its lowest around 12 am, the expression of *CDF1* begins to rise yielding a peak around 3 am. When its expression started decreasing beginning around 3 am *MiCO1* expression starts rising giving a peak around 6 am and the expression remains elevated during the day and started decreasing later in the evening.

Tissue specific expression analysis of circadian clock controlled genes

The expressions of circadian clock controlled genes (*MiGI1*, *MiGI2*, *MiCO1*, *MiCO2*, *MiFKF1* and *MiCDF1*) were monitored in different tissue samples namely from vegetative tissues like mature leaf (ML), vegetative apex (VA),) and reproductive tissues namely reproductive apex (RA) and flower (FL). The samples for this study were taken when the tree was flowering.

MiGI1 and *MiGI2* mainly expressed higher in the vegetative tissues such as leaves. The expression of *MiGI* was low in vegetative apex (that culminated in producing vegetative shoot and not reproductive tissue) but its expression was relatively high in reproductive apex (which culminated in producing the floral structures). The expression was comparatively lower in reproductive tissues namely flower as compared to leaf tissues (Fig. 6A, B). The expression level of *MiGI2* was four fold higher compared to the expression of *MiGI1*. It suggests that *MiGI2* may have a key role in flowering of mango.

The expression patterns of *MiCO1* and *MiCO2* were also monitored in different tissue samples. *MiCO1* and *MiCO2* were highly expressed as were *MiGIs* in vegetative tissues such as mature leaf while *MiCO1* expressed well in flower (Fig. 6C, D). The expression of *MiCO1* was higher; almost five times higher compared to the expression of *MiCO2* indicating *MiCO1* may be the active CO during flower induction.

The expression of other two circadian clock controlled genes namely *MiFKF1* and *MiCDF1*were examined in all the tissues. The data showed that the *MiFKF1* gene was expressed in leaf tissue and gradually decreased in apex tissue. Interestingly, it was seen that the expression of the

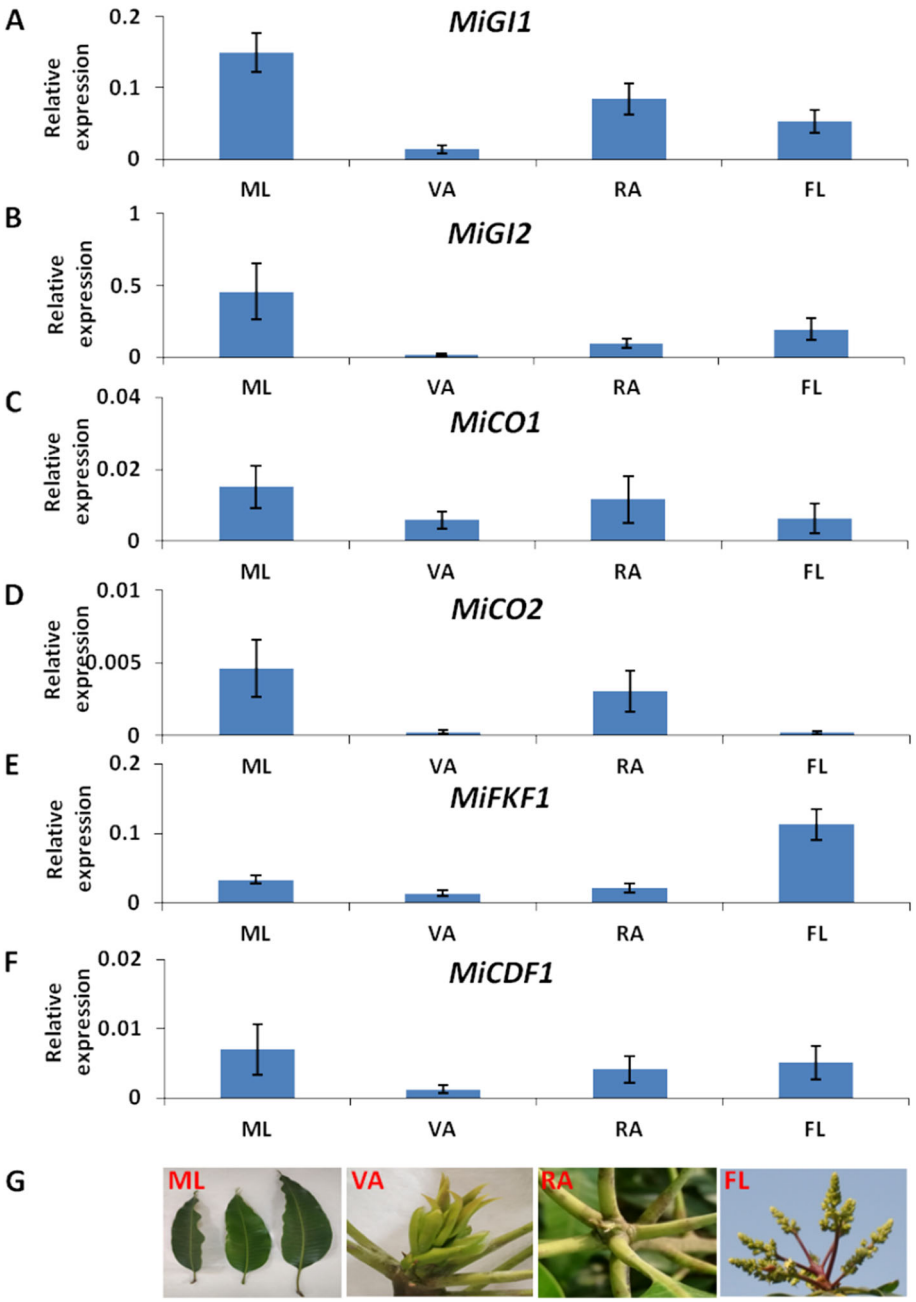

Fig. 6 Expression levels of *MiGI1* (**A**), *MiGI2* (**B**), *MiCO1* (**C**), *MiCO2* (**D**), *MiFKF1* (**E**) and *MiCDF1* (**F**) genes in different plant tissues as indicated in (**G**). Gene expression is the mean of three biological replicates and bars indicate standard error of the mean. **G** Photographs of different developmental stages of plant, ML (mature leaf), VA (vegetative apex), RA (reproductive apex) and FL (flower)

MiFKF1 gene was high in flowers. This indicates that *MiFKF1* gene may have some role in flower development (Fig. 6E).The expression of *MiCDF1* gene was higher in vegetative tissues as compared to the reproductive tissue (Fig. 6F).

Expression analysis of Circadian clock controlled genes in leaf samples during developmental stages in mango plants

The expression profiling of circadian clock controlled genes in leaf tissues of mango variety Ratna plants during all developmental stages was conducted by using the leaf samples collected in the afternoon around 2 pm beginning about two months prior to flowering and continued until the fruiting phase. The samples were collected in a year when the cooler temperatures occurred somewhat later than usual. The temperatures during the flowering period are given in supplementary Fig. 1.

The expressions of both *MiGI1* and *MiGI2* were upregulated beginning prior to initiation of flowering (late November to early December) and reached a peak coinciding with the time when flowering started (around 20th December). The expression then started to come down

reaching a low level by the end of January. The upregulated expressions stayed for about 45 days (Fig. 7A). Of the two *MiGI*s, *MiGI2* expression began rising again after the fruits were formed and during their development (Fig. 7B). It appears that *MiGI2* may have a role during the fruit development.

The expression patterns of *MiCO1* and *MiCO2* did not follow any specific pattern (Fig. 7C, D). Rather they increased and decreased during the floral induction as also during fruit development. However the expression peaks of *MiCO1* correlated with the peak patterns of *MiFKF1* indicating that *FKF1* expression influences the patterns of *MiCO1*. It may be emphasized that, Ratna, the variety under study, continues to flower for almost three months such that it is possible to see the inflorescence when fruits have matured on the same tree.

Further, we examined the expression patterns of other two genes *MiFKF1* and *MiCDF1* genes which play important roles in circadian clock regulation. The expression profiles showed that the expression of *MiFKF1* gene was upregulated just prior to flowering in consonance with the expression of *MiGI* (Fig. 7E). The *MiFKF1* showed peaks very similar to *MiCO1*. The relative expression of the *MiCDF1* gene had a peak about a month before flowering. Its expression decreased after that and remained low for one and half month during the flowering (Fig. 7F). After March, during peak fruit growth, the expressions of both these genes continued to rise as was the case with *MiGI2*.

Discussion

Keeping in view the important role of *GI*, a circadian clock controlled gene in induction of flowering by activating the expression of *FT* either directly (Brandoli et al. 2020) or through the intervention of *CO* through *GI's* interaction with *FKF1* and *CDF1*, the possible roles of these 4 genes were investigated in a day neutral fruit crop mango through their expression patterns and sequence analysis.

Our results showed that mango has at least two *GI* genes that are distinct from each other in terms of the location of their introns although both have many similarities. They have been observed as 95% similar and 91% identical in their protein sequence. The MiGI2 has additional two amino acids namely glycine and histidine at positions 1120 and 1121. There were other differences as shown in Fig. 2A between the two *GIs*. As discussed later, of these two, the functional *GI* on the basis of their temporal characteristics of expression and expression coinciding

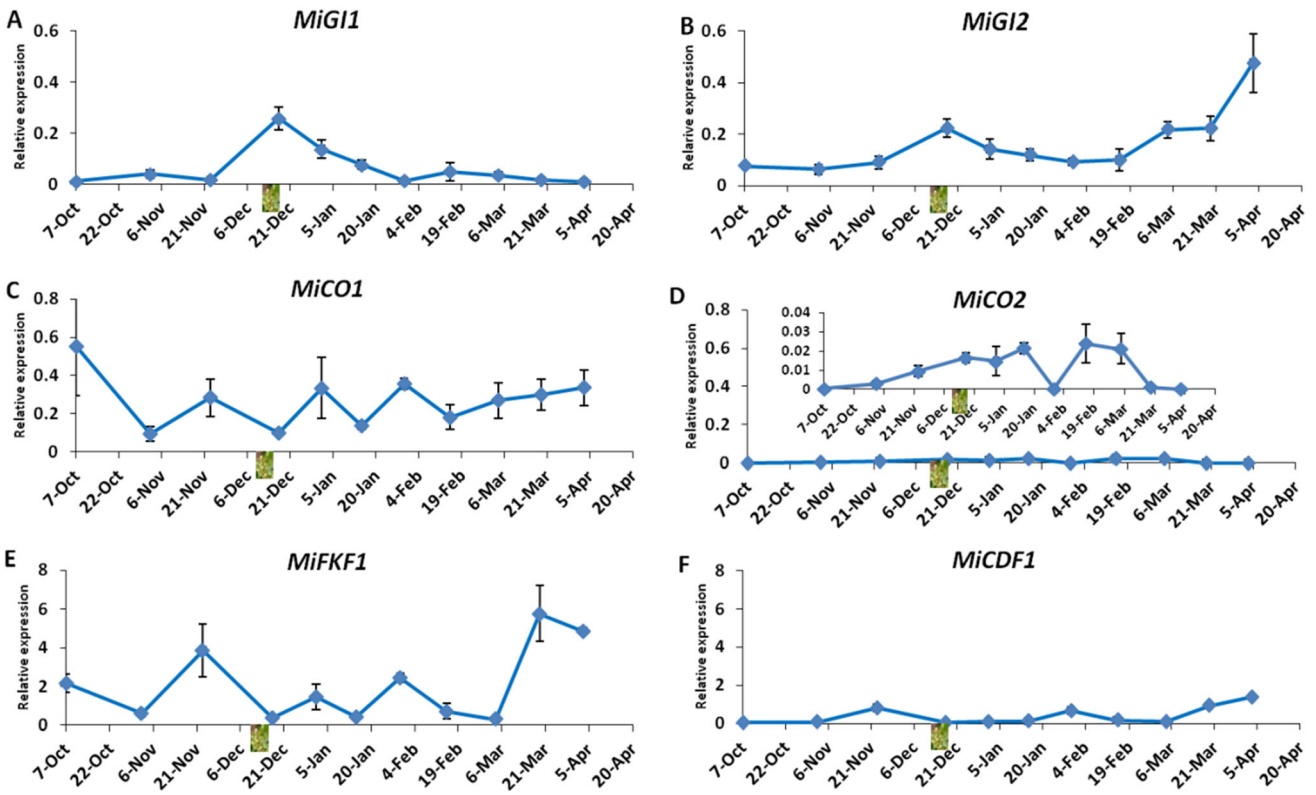

Fig. 7 Relative expressions of *MiGI1* (**A**), *MiGI2* (**B**), *MiCO1* (**C**), *MiCO2* (**D**), *MiFKF1* (**E**) and *MiCDF1* (**F**) during phenological stages in leaf tissues of mango. Gene expression is the mean of three biological replicates and bars indicate standard error of the mean. Flowering started around 17 Dec giving a peak around 7 January and was over around 30 January

with the induction of flowering, *MiGI2* appears to be important. It is not clear whether this is due to the changes in the coding region or due to the promoter characteristics.

The number and length of exons and introns in different species are different (Fowler et al. 1999; Park et al. 1999; Hong et al. 2010; Taylor et al. 2010; Barros et al. 2017; Ke et al. 2017). Both the GIs have 4 clusters of basic amino acids (arginine and lysine) (Fig. 4) like in Arabidopsis. In Longan the lone GI has only 1171 amino acids (Huang et al. 2017) while the homologue in the closest species *Citrus sinensis* has 1165 amino acids. *GIGANTEA* from several species have been sequenced and they show considerable variation in the length of the protein, most having an amino acid chain ranging between 1150 and 1200 (Fowler et al. 1999; Park et al. 1999; Taylor et al. 2010; Wang et al. 2020; Barros et al. 2017; Ke et al. 2017).

An analysis of the GI sequences from several species as mentioned in Fig. 4A showed that except onion GI (AcGIa) all the known GIs seem to bisect the 4th basic amino acid region from the other three by serine and glutamate peptide bond located at position 749 and 750 in AtGI. This suggests that this is a highly conserved peptide whose disruption has been shown to disrupt the function of GI in Arabidopsis (Huq et al. 2000). In mango also the same amino acids are present at the same position and probably function the same way. Even in onion, in this peptide, glutamate is replaced by aspartate which is not structurally very different. This conservation is irrespective of whether the plant is a monocot or dicot although the sequences of GIs of these two separated in different clades. Serine is known to be an amino acid that gets phosphorylated in many proteins and its phosphorylation/dephosphorylation could activate or inactivate the function of the protein. Does the serine at position 749 have such a function in GIs? This needs to be looked into and is under study in our laboratory.

FKF1 protein forms a complex with GI and regulates the degradation of a repressor CDF which binds to the promoter of *CONSTANS* (Sawa et al. 2007). FKF1 protein sequence in mango (Fig. 3A) shows that the FKF1 protein is similar to FKF1 from Arabidopsis and citrus possessing the Bacterio-opsin activator and kelch repeats. The other protein namely CDF1 (Fig. 3B) is also similar to the proteins in Arabidopsis and citrus with characteristic zinc binding domains. CONSTANS, a protein whose expression is regulated by photoperiod in long day and short day plants, has been sequenced in mango and there are at least two CONSTANS proteins (Fig. 2B) with sequence characteristics similar to *CO* of Arabidopsis and many other plant species. Of the two, we have studied the functional characteristics of *MiCO 1* that restores the function of *CO* in Arabidopsis mutant lacking functional *CO* (co-2). The *MiCO2* has not been functionally studied. The two *COs* apparently function redundantly. Thus, the entire machinery functioning in modulating the expression of *FT*, the inducer of flowering in plants, is present in mango. It was of interest to find out whether this *GI-FKF-CDF-CO* system works in a day neutral mango for induction of flowering. Flowering in mango as mentioned earlier is temperature dependent and not photoperiod regulated. Our own observations in mango flowering in several varieties have suggested that induction of flowering in mango is triggered by a drop in temperature. In some years, there can be two flushes of flowering if the temperature, after the first flush, increases and then again decreases. Thus, temperature fluctuations do seem to induce flowering irrespective of the photoperiod.

To understand the relationship between the expressions of these 4 genes with the induction of flowering in mango we studied the expression patterns of these genes during the flowering time of mango. The expression of both *MiGI1* and *MiGI2* started increasing from around 21 November giving a peak around 21 December. Flowering also began around December 21 yielding a peak in the middle of January coinciding with the expression patterns of *MiGI1* and *MiGI2* (Fig. 7). *MiFKF1* gave a peak expression around 21 November which also coincided with the expression of *CDF1* probably resulting in neutralizing the repressor effect of *CDF1*. The samples for studying the expressions were the same and the reference gene used was also the same. The *MiCO's* expressions particularly the *MiCO2* seemed to be higher when the *CDF1* expression was low. These data suggest that when *MiGI* and *MiFKF1* expressions were higher and *MiCDF1* expressions were lower, the expressions of the two *MiCOs* were higher as would be expected. The protein complex of GI and FKF1 as mentioned above degrades the CDF protein that is present on the promoter of *CO* (Sawa et al. 2007). Higher *CO* expression induces the expression of *FT*, the inducer of flowering. Taken together, these data suggest that the *GI-FKF1-CDF1-CO* module may be functioning in the day neutral plant mango as it does in long day/short day plants. A confirmation of this suggestion can come through the functional analysis of these genes in Arabidopsis since transformation of mango is not possible with present available techniques. The functional analysis of these genes is under study in our laboratory.

Further, support for this comes from the diurnal changes in the expression patterns of two *MiGIs*, *MiFKF1* and *MiCDF1* expressions. The diurnal expression patterns of *MiGI, MiFKF1* and *MiCDF1* showed that *GI* and *FKF1* expressed simultaneously at night while the *CDF1*, the repressor of *CO* showed a peak just before dawn. When the expression of this repressor started decreasing, coinciding with the beginning of the daylight period, the *CO* expression of both the homologues begins to rise in the morning

yielding a peak around 6 am. Thereafter, the expression decreased gradually reaching a minimum for *MiCO1* around 10:30 am while the *MiCO2* remain expressed until late in the evening (Fig. 6). The circadian expression pattern of *CO* gene showed different patterns in different species. In Arabidopsis, under LD condition the level of expression of *CO* gene was high at the end of day but under SD conditions its expression was higher during only night (Suárez-López et al. 2001). In Loquat, *EdCO* mRNA peaks at noon (Zhang et al. 2019), in bamboo *PvCO1* was highly expressed throughout the night whereas the *PvCO2* showed higher expression in the morning (Xiao et al. 2018). Mango *CO* (*MiCO*) was highly expressed at morning 9 am (Liu et al. 2020). Our results also showed that the level of expression of both *CO*s (*MiCO1* and *MiCO2*) was higher in the morning around 6 am. In photoperiod dependent species, the *CO* expression peaks in the afternoon (Andrés and Coupland 2012) have reportedly induced expression of *FT*. Unlike in long day plants, where the *CO* expression is higher in the afternoon, in the day neutral plant mango the *CO* expression peaks in the morning. It is not surprising that *CO* expression is higher in the morning since day length in mango does not influence the flowering unlike in long day plants. The presence of DNF, the protein that expressed in the morning and repressed the expression of *CO* in the morning hours in the photoperiod dependent long day plant Arabidopsis (Morris et al. 2010), is not present in the mango sequence genome database. We therefore believe that *CO* expression in the morning hours have not repressed in mango and this probably prevents photoperiodic control of flowering in mango.

Additional support for the possible involvement of *GI-FKF-CDF-CO* system comes from their expressions in different tissues which are important in flowering. Figure 7 shows that in mature leaf the *MiGI*s, *FKF1* and *CDF1* were well expressed and their expression was very low in the vegetative apex. Both the *CO*s expressed high in the mature leaf as also in the reproductive apex but not in vegetative apex. These patterns of expressions also suggest that *GI-FKF-CDF-CO* system have a role to play in the induction of flowering in mango.

Mango flowering as mentioned earlier is temperature dependent. We therefore examined the relationship between the temperature changes and the flowering. Earlier studies by Fernández et al. (2016) have shown that under short day conditions which are non-inductive in Arabidopsis, an increase in temperature makes the photoperiodic pathway more sensitive. Thus temperature changes are also sensed even in photoperiodic pathway. Further, studies by Kinmonth-Schultz et al. (2016) using different mutants/transgenics of Arabidopsis have shown that exposure of plants to lower temperatures increased *CONSTANS (CO)* transcript levels at night. These workers elegantly showed that in plants maintained at 22 °C during the day and 12 °C during night *CO* was highly expressed. However plants that were maintained under short day at 22 °C both during the day and night and subsequently transferred to long day conditions at 12 °C during the day and also at night, did not show strong night time induction of *CO*. This suggested that upregulation of *CO* at night was due to a drop in temperature at night. It appears that in mango exposed to high temperature during the day, as it happens in tropics even in winter, a drop in temperature at night from 25 to 30 °C during the day to 11 to 19 °C at night triggers *CO* expression at night under the conditions of the present experiment. This expression is associated with expected changes in the expression patterns of *GI-FKF1-CDF1* genes that control the induction of *CO*.

We wondered whether the promoters of the *MiGIs* of mango have an element in their promoter which senses temperature and distinguishes itself from those *GIs* that are photosensitive. Dunn et al. (1998) while working with barley blt4.9 showed that the hexanucleotide CCGAAA, at − 195 from the first ATG, is involved in the low-temperature response (LTR) of blt4.9 in barley. A comparison of the promoter region of Arabidopsis, a long day plant and two *GIs* out of several of banana, a day neutral plant, showed that the Arabidopsis lone *GI* also has as many as 13 light sensing elements but only one low temperature sensing (LTR) element at position − 1333 from the first ATG. In banana also two *GIs* have only one temperature sensing element at position − 1724 and − 1934 respectively from the start ATG but about 15 light sensing elements in the 5000 nucleotide stretch upstream of transcription start site. Flowering in banana is not induced by temperature.

We analyzed a stretch of 5000 nucleotides upstream of the transcription start site of both the *GIs* of mango to find out if LTR elements, CCGAAA, sensing low temperature is present in the promoters of *GIs* of mango. The presence of light and temperature sensitive elements in *MiGI1* and *MiGI2* are shown in Supplementary Table 4. Most interesting was the presence of 3 LTR elements at − 628, − 3372 and − 4944 from the start ATG in *MiGI2* which are absent in *MiGI1*. LTR elements confer low temperature sensitivity. Further, the *MiGI1* has 24 light sensitive elements while the *MiGI2* has only 12 light sensitive elements. Thus it appears that *MiGI2* could be the important and functional *GI* responding to low temperature for inducing *MiCOs*.

In the present experiments, the temperature started dropping at night from around 23 °C until first week of October to about 17 °C by the third week of November and then decreased further at night to around 11–13 °C until the middle of January (supplementary Fig. 1). This is also the period during which flowering occurred with a

peak around middle of January. The day temperatures during this time were still well above 25 °C (supplementary Fig. 1). As mentioned above during this flowering period the expressions of both the *MiGIs* and *MiCDF1* were substantially upregulated and *MiCDF1* expression, although present, was probably controlled by the presence of MiGI-FKF1 protein complex as these two were expressed during the same time as the *MiCDF1* expression. Further, if one compares the diurnal expression patterns at the peak flowering day it is evident that the expression patterns of *GI-FKF1-CDF1* were conducive to the expression of *MiCOs* in the morning. Since, mango genome does not seem to possess the *DNF* gene the *MiCO* expressions are not repressed in the morning and hence photoperiodic control is not exercised in mango. Considering the results of Kinmonth-Schultz et al. (2016) we suggest that the *GI-FKF1-CDF1-CO* system may be functioning as it does in photoperiod dependent plants except the expression of *CO* occurs in the morning as a result of the expressions of *GI-FKF1-CDF1* during night. Taken together the results of the present study suggest that in the day neutral mango the temperature drop at night brings about changes in the expressions of the *GI-FKF1-CDF1-CO* genes resulting in high expression of *MiCOs* which may be inducing the expression of *FT*, the signal for flowering.

Conclusion

The presence, sequencing homology and expression patterns in relation to induction of flowering and the tissue specific expressions of the circadian clock regulated genes of the GI-FKF1-CDF1-CO module indicate that this module may also be functional in mango, a day neutral fruit tree. This module has been shown to be functional in plants whose flowering is photoperiod controlled. Further, the temperature dependent floral induction may be the key regulator in mango due to the presence of temperature sensing elements present in the promoter region of MiGI2.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12298-021-01053-8.

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Author contribution statement PVS and BK conceived and designed the research. SIP and SNV conducted the experiments and performed bioinformatics analysis. SIP, SNV, BK and PVS wrote the manuscript. All authors read and approved the manuscript.

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Declarations

Conflicts of interest/Competing interest Authors have no conflict of interest.

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