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Toc 1 Biological Clock Gene in Plants of Nine-Planet Forest

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Abstract

The circadian clock is a timekeeping system that provides organisms with a mechanism to adapt to 24-h day-night cycles. *toc1* was the first plant gene that when mutated yielded a circadian phenotype. The *toc1* gene was initially discovered by Prof. Andrew Millar colleagues in 1995. According to Indian Traditional Knowledge, nine astronomical entities represent nine medicinal plants together known as Nine-Planet forest. In this research, DNA isolation, PCR amplification and Agarose gel electrophoresis of nine explants was done. *toc 1* gene was identified among certain plants, using sequence of *toc1* gene of *Arabidopsis thaliana* as reference. Further research on the behaviour of *toc 1* gene and TOC 1 protein in relation to sun, moon and other planets may reveal new frontiers in the field of Chronobiology, Divine Botany, Astroethnobotany, Astrobiology etc.,

Keywords: *Nine-Planet forest, Nine plants, toc 1gene, DNA isolation, PCR, Agarose Gel Electrophoresis, Amplicon*

1. Introduction

Adapting to changing environment is critical to all life. Some of these changes are predictable, such as day-night cycles and ever-changing seasons. Accordingly, organisms from all kingdoms of life have developed mechanisms to anticipate such predictable changes. Intrinsic clocks that generate circadian rhythms are present in most organisms, from cyanobacteria to land plants and animals **(Linde *et al.*, 2017).**Many physiological and biochemical processes in some prokaryotes and most eukaryotes exhibit a cyclic pattern of activity with a period that approximately matches that of the earth's rotation. These 24-h rhythms are regulated by an internal timing mechanism, the circadian clock that enables organism to anticipate rhythmic changes in the environment and to synchronize their physiological states accordingly **(Mas *et al., 2003*).** The circadian clock is a timekeeping system that provides organisms with a mechanism to adapt to 24-h day-night cycles. The clock activates biological processes at specific times during the daily cycle through synchronous expression of genes involved in related biological processes, such as preparing for colder temperatures in the evening or anticipating infection by pathogens at dawn **(Kamioka *et al.*, 2016).** Clock genes have been shown to be important in regulating many key agronomic traits. Therefore, identifying new players in this interconnected clock network will provide novel strategies toward developing new crop varieties (**Tripathi *et al.*, 2016).** The circadian clock is an endogenous timekeeper that allows organism to anticipate the day/night cycle, thus improving their fitness. In plants, the clock has been shown to regulate a wide variety of processes, including hypocotyls and root growth, flowering time, sugar metabolism, photosynthesis, nutrient homeostasis, hormonal signaling and immunity. Circadian oscillations originate at the cellular level form the interactions of at least a dozen clock genes **(Clauve *et al.*, 2016).** Genes subject to circadian clock regulation are central to many important physiological processes, including flowering time, phytochrome synthesis and signaling, growth control, metabolic activities, abiotic stress response and plant-pathogen interactions. At the molecular level, the core circadian clock is made up of genes that interact through a series of transcriptional and post-transcriptional feedback loops to create rhythmic gene expression. Although core circadian clock genes are expressed throughout the day, distinct morning, day and evening transcriptional phases exist, and each phase represents the activity of multiple core circadian clock proteins. The circadian clock regulates a number of central plant activities, including growth, development and reproduction, primarily through controlling a substantial proportion of transcriptional activity and protein function. Homologous genes from PRR (Pseudo-response regulator) group underlie quantitative trait loci that have beneficial influences on key agricultural traits, especially flowering time but also yield, biomass, and biennial growth habit.

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Abiotic and biotic stresses discussed to highlight promising avenues for further crop improvement. Drought tolerance, Latitudinal adaption, Hybrid vigour, Immunity/Defence, Photoperiodic flowering, GA (Gibberellin Acid) synthesis, Photosynthesis metabolism, JA (Jasmonic Acid) synthesis, ABA (Abscisic Acid) signalling, Growth, R(resistance)-mediated response, ROS (reactive oxygen species) Homeostasis are traits influenced by clock-regulated pathways**.** The PRR gene family is a major contributor to the clock system in *Arabidopsis*, with five genes (TOC 1, PRR 3, PRR 5, PRR 7 and PRR 9) serving as clock components. The *Arabidopsis* PRRs contribute to many clock-associated functions, including flowering time regulation, clock temperature compensation and entrainment, response to photosynthetic sugar, maintenance of mitochondrial homeostasis, heat shock response, cold stress response, oxidative stress response, and the regulation of stomatal conductance **(Bendix *et al.*, 2015).**

Timing of CAB (chlorophyll-a, b binding protein) expression 1 (TOC 1) is a protein that in *Arabidopsis thaliana* is encoded by the *toc1* gene. *toc1* was the first plant gene that when mutated yielded a circadian phenotype. It codes for the transcription factor TOC 1 which affects the period of plants circadian rhythm: built-in, malleable oscillations that repeat every 24 hours. The *toc1*gene was initially discovered by Prof. Andrew Millar colleagues in 1995**(Wikipedia TOC1 (gene)).**

Homolog's of TOC 1 have been found in *Lotus, grape vine, Potato, Tomato, Apple, Papaya, Cucumber, Strawberry, Soyabean, Peach, Western poplar (populus), Castor bean, Lyrate rockcress, Brassica* and *Chickpea***(Wikipedia TOC1(gene))**.

In the view of importance of *toc1* gene and TOC 1 protein as reviewed above, the present study focused on the detection **and sequencing** of *toc1* gene in nine unique and sacred medicinal plants of nine-planet forest described in Indian Traditional Knowledge.

Every nation has its own set of sacred plants **(Pandey and Pandey,2016).** Stars, Planets, and Zodiac are considered to be made manifest in specific plant and tree species. In Indian Traditional Knowledge/Vedic culture the plant species attributed to the planets were as stated in following Sanskrit verse **(Chandrakanth, 1999)**

Arkasam idam Adithyaya (*Calotropis gigantea* to represent Sun)
Palashagam Somaya (*Butea monosperma* to represent Moon)
Khadiram Angarakaya (*Acacia catechu* to represent Mars)
Apamargam Bhudhaya (*Achyranthus aspera* to represent Mercury)
Ashwatham Brihaspathaye (*Ficus religiosa* to represent Jupiter)
Audumbarag Shukraya (*Ficus racemosa* to represent Venus)
Shamigam Shanaischaraya (*Acacia catechu* to represent Saturn)
Rahuve Doorvaya (*Cynodon dactylon* to represent Rahu(Dragons head))
Kethuve Kushaya (*Desmostachya bipinnata* to represent Kethu(Dragons tail))

2. Materials and Methods

2.1 Collection of Plant material

Calotropis gigantea (Linn.), *Acacia catechu* (Rottler) Willd., *Achyranthes aspera* (Linn.), *Ficus religiosa* (Linn.), *Prosopis cineraria* (Linn.), *Butea monosperma* (Linn.) *and Ficus racemosa* (Linn.) and *Cynodon dactylon* (Linn.) Pers were identified in main campus of Osmania University, Hyderabad, Telangana State and *Desmosatchya bipinnata* (Linn.) Stapf was identified in coastal Andhra Pradesh. Plants were authenticated using standard keys and descriptions.

2.2 Explants used

Stems of *Calotropis gigantea* (Linn.), *Acacia catechu* (Rottler) Willd., *Achyranthes aspera* (Linn.), *Ficus religiosa* (Linn.) *and Prosopis cineraria* (Linn.); Bark of *Butea monosperma* (Linn.) *and Ficus racemosa* (Linn.); Leaves of *Cynodon dactylon* (Linn.) Pers. and *Desmosatchya bipinnata* (Linn.)Stapf.

2.3 DNA Isolation

Genomic DNA was isolated from the powdered explants by cell lyses and DNA Precipitation as stated below:

0.2 gm of powdered explants was grinded in 2 ml of Extraction buffer* and incubated at 65 ˚C for 40 min wherein after every 10 min, sample was gently vortexed. Sample was allowed to come to room temperature and centrifuged at 10000 rpm for 10 min. To the supernatant, 2 ml of Chloroform: Isoamyl alcohol in the ratio 24: 1 was added and centrifuged at 10000 rpm for 10 min. To the uppermost aqueous layer of the supernatant (the middle layer of organic matter of the leaf and the down most 3rd layer of Chloroform should not be taken) equal volume of Isopropanol /ethanol was added and incubated for overnight at 4 ^{o}C. Thereafter centrifuged at 12000 rpm for 10 min. Pellet was taken, dried and 50 µl of TE buffer was added.

Composition of Extraction Buffer*:

3% CTAB (Cetyl Trimethyl Ammonium Bromide)
100 mM Tris HCl
20 mM EDTA
1.4 M NaCl

0.2% Beta mercapta ethanol
0.2% PvP (Polyvinyl Pyrrolidone)

2.4 Primer Design and DNA amplification through Polymerase Chain Reaction (PCR)

Primer was designed using the *toc* 1 gene sequence of *Arabidopsis thaliana* as reference. Tools and database used were NCBI, Primer 3 and BLAST. Primer set was selected as showed in Fig.1. This primer was synthesized and used for amplification in all the 9 samples.

Ingredients in the PCR Tube:

- Genomic DNA/Template DNA=2 µl (sample)
- Two primers i.e. forward primer(F)= 2 µl and Reverse primer(R)=2 µl
- PCR buffer=4 µl
- DNTPs =4 µl
- Taq-DNA polymerase enzyme =0.2 µl
- $MgCl_2$ =2 µl
- Nuclease free water for making volume up to 25 µl

The primers used for the amplification:

- F GACGAAGTCCCTGTCGTTGT
- R GCGCTGCAAAACCCCTACTA

PCR Conditions:
1 cycle: 94°C for 5 min (Initial denaturing)
35 cycles: 94°C for 60 sec (denaturing)
53°C for 45 sec (annealing)
68°C for 90 sec (extension)
1 cycle: 68°C for 10 min (final extension)

2.5. Agarose Gel Electrophoresis of Amplified DNA

Amplified PCR product was subjected to electrophoresis using Agarose gel 1% in TAE buffer and visualized by staining with ethidium bromide.
Methodology was as follows:

Agarose gel electrophoresis (AGE) tray and chamber was wiped with spirit and AGE tray was sealed with cellophane tape and tested for leakage. It was leak proof. 1% agarose in TBE buffer was prepared by melting in oven. After cooling it to 45 ^{0}C, 5-10 µl of Ethidium bromide was added (gloves were worn while handling Ethidium bromide, as it is carcinogenic), mixed and poured into the agarose gel tray. The comb was inserted and left for setting. It was not disturbed until solidification. After setting, comb was carefully removed without puncturing the wells. Seal was removed and gel tray was placed in the electrophoresis chamber. Gel was covered with TBE buffer.

10 µl of each DNA sample was added to 3 µl of loading dye separately and carefully loaded into designated wells and order of loading was noted. The power supply was switched on and the gel was run at 50 V till the bands reach the 3/4th of the gel. Power was turned off and lid of the electrophoresis chamber, tray and gel were carefully removed using gloves. Gel was observed in UV transilluminator for visualization of DNA.

3. Results

3.1 Gel Picture of the amplicons showing PCR products of 9 plant samples (number in parenthesis indicates plant's PCR product on GEL) and 1 kb marker in the first lane (Fig 2).

toc1 gene amplicons were observed in six plants namely *Ficus religiosa* (3), *Cynodon dactylon* (4), *Achyranthes aspera* (5), *Desmostachya bipinnata* (6), *Prosopis cineraria* (8) *and Acacia catechu* (9). *toc 1* gene amplicons were not observed in explants of *Calotropis gigantea*, *Butea monosperma* and *Ficus racemosa*

4. Discussion

For more than 2000 years, certain forestry practices and rules regarding trees have been carried out in observance to Moon cycles. Without these strange reminders from past cultures we would, however perhaps never have conceived of initial and further leading scientific observations in forestry traditions **(Zurcher,2001).** J.Schultz, as an astronomer worked precisely in a limited area, on connection of leaf positions with the rhythm of the moon, the sun, and the planets. He convincingly pointed out the correlation of several leaf positions with the rhythmic movement of the planets. During the 1920's and 1930's A.Usteri attempted to establish a relationship between the families of flowering plants, and their individual genera and the spheres of the planets. As early as the 1940's, J.Schultz discovered a correlation between the seed and fruit formation of trees and Jupiter's twelve year cycle. For red beech, the years of high seed yields coincided with specific constellations of Jupiter against the zodiac. The seeding experiments by M.Thun pointed to diverse relationships between the course of the moon through the zodiac and the formation of certain plant organs (roots, shoot, flowers, fruit, and seed) **(Krainch, 1986).** Statistically significant lunar rhythmicities were revealed in germination rates from sowings shortly before full

moon, compared to those shortly before new moon **(Zurcher,2014).**

There is a great research gap in such one-to-one i.e., plants-to-planets studies. Identification of the biological clock genes such as *toc 1* among nine plants related to nine astronomical entities can create new insights in studying behavior of these unique nine plants in relation to sun, moon and other planets and also new insights in fields of 'Divine botany'**(Jain and Kapoor,2007)** and 'Astroethnobotany'**(Joshi and Gupta,2011).**

5. Conclusion

TOC 1 and *toc 1*'s behavior in these six plants need to be further studied in relation to sun, moon and other planets to know any correlations among them.

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Figures:

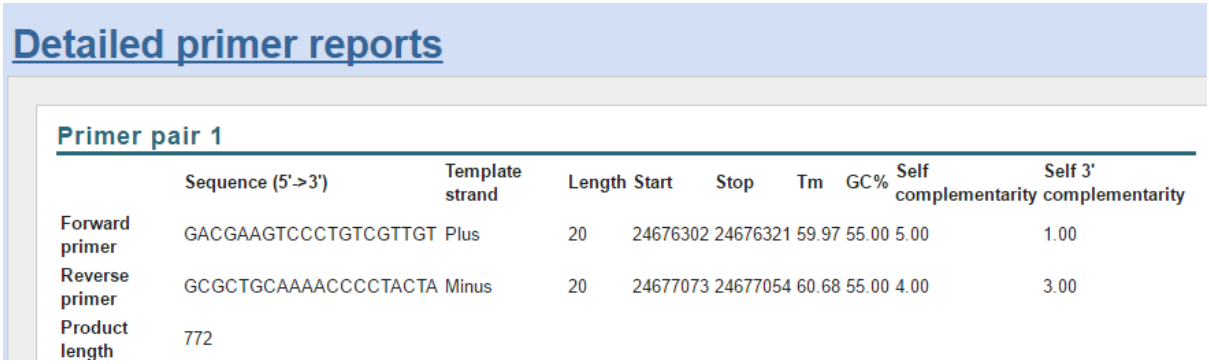

Fig. 1 Primer information

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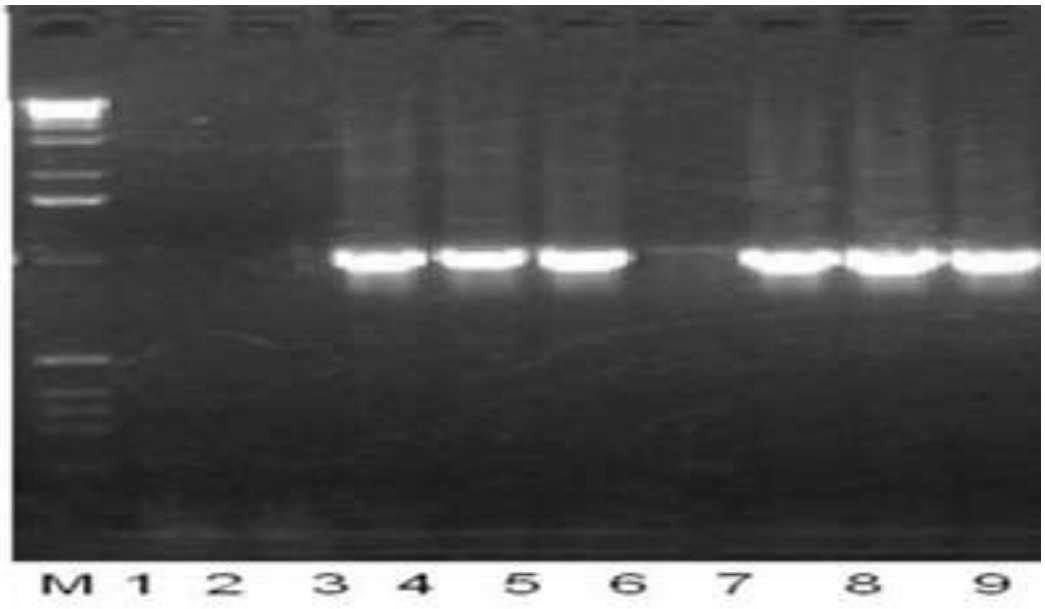

Fig. 2 **A**mplicons could not be obtained in samples ***Calotropis gigantea(1*****), *Butea monosperma(* 2) and *Ficus racemosa (*6).**