RESEARCH ARTICLE

Effect of the Static Magnetic Fields on the Circadian Rhythm in *Arabidopsis thaliana*

Sunil K. Dhiman*, Ashish Agnihotri

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Abstract

The influence of geomagnetic field on various biological processes is certainly an interesting research field, and more work is being performed across the globe. However, the impact of varying magnetic flux density on plant circadian rhythms has not yet been completely investigated. Different researchers have mostly focused their attention on the molecular workings of circadian rhythms in various plant systems and the circadian architecture of transcriptomes under varying conditions. The present investigation evaluated the influence of variable geomagnetic field on the expression of genes that are under the control of circadian rhythm, and checked whether the expression pattern of these genes with respect to time (circadian nature) under different magnetic flux density changes or not in *Arabidopsis* seedlings. This study examined the impact of varying magnetic flux densities on the mRNA expression levels of six genes in *Arabidopsis thaliana* during the final 33 hours of their total 120 hour growth period. *A. thaliana* seedlings were subjected to four distinct magnetic flux densities (0, 25, 50, and 90 µT), and the abundance of transcripts for chlorophyll a/b binding protein 4, the large subunit of RuBisCO, rubisco activase, chalcone synthase, porphobilinogen synthase, and phytoene dehydrogenase genes was examined. While the present study's findings lend credence to the idea that the aforementioned genes are differentially expressed in response to changes in magnetic flux density, it also proved that the circadian nature of these genes was largely unaffected, with their expression pattern remaining largely unaltered regardless of the strength of the magnetic field.

Keywords: Circadian rhythm, Magnetic flux density, mRNA expression, Static magnetic fields.

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Introduction

The duration of one cycle of Earth's rotation on its own axis is 24 hours (h). This cycle influences various environmental factors such as the quantum and light direction, relative humidity, temperature, etc. Moreover, there is a tilt of 23.5° in the Earth's axis, which results in seasonal variations in the annual photoperiod and other environmental factors (Fedorov and Frolov, 2022). These daily cyclic changes are believed to have resulted in the evolution of circadian rhythm in plants and animals. Circadian rhythms, as a subdivision of biological rhythms, are distinguished by their period, which refers to the time needed to finish a cycle of about 24 hours (Dunlap *et al.,* 2004). Circadian rhythms possess a notable attribute of being endogenously generated and persisting even in the absence of a 24 hours environmental cycle. These endogenous cycles continue to operate consistently under constant light and temperature conditions. Another fundamental characteristic of circadian rhythms in living systems is their control over the daily phase of many crucial biological processes. This control enables the regulation of the timing of gene transcription rates, subsequently influencing metabolic processes at specific times of the day. By coordinating these activities, circadian rhythms facilitate the synchronization of various biological processes, ensuring that they take place at suitable times and in a coordinated way (Harmer *et al.,* 2000). Higher plants, especially agricultural plants, perform better and are consequently more fit when gene(s) expression is regulated in line with the circadian cycle (Green *et al.,* 2002; Dodd *et al.,* 2005; Turner *et al.,* 2005; Graf *et al.,* 2010; Izawa *et al.,* 2011; Müller *et al.,* 2015).

The geomagnetic field refers to the magnetic field that surrounds the Earth, resembling a bar magnet with its poles positioned away from geographic poles and at an 11° angle in relation to Earth's rotational axis (Buis, 2020). Earth's magnetic field is generated by a self-sustaining geo-dynamo in the core, powered by convective forces resulting from the movement of molten iron. This geodynamo has been active for about 3.5 billion years (Tarduno *et al.,* 2010). Across the Earth's surface, the magnetic field's strength varies, ranging from 25 µT at the equator to gradually increasing values of up to 75 µT at the poles (König *et al.,* 1981; Merrill *et al.,* 1998, Jin *et al.,* 2019). In a specific location, the geomagnetic field remains relatively stable and consistent, influenced by the electromagnetic radiation released by the Sun. While magnetic storms and solar winds has the potential to impact the strength of the Earth's magnetic field, any resulting variations are typically insignificant (Jin *et al.,* 2019). Studies of the Earth's magnetic field history over the previous 160 million years indicate that the field's strength was approximately half of its present strength during that time period (Juárez *et al.,* 1998). The magnetic field's strength measurement is expressed as magnetic flux density (MFD), symbolized as 'B,' with the unit Tesla (T). The magnetic flux itself is denoted as 'Φ'

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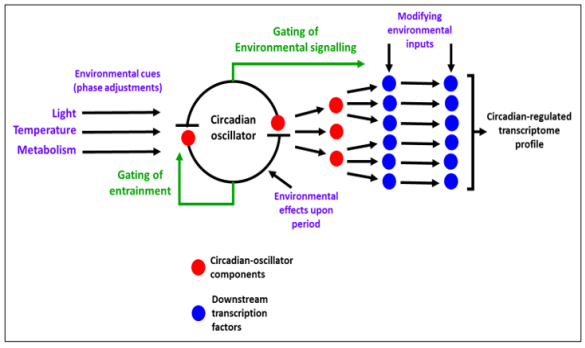
or 'Φ_B' and is measured in SI unit Weber (Wb), thus defining one Tesla equivalent to one Weber per square meter.

The studies on circadian rhythms over the years have brought about certain characteristic features of the circadian system (Fig. 1), which include many conceptual fundamental units (Nagel and Kay, 2012; Hsu and Harmer, 2014; Huang and Nusinow, 2016; Millar, 2016; Panter *et al.,* 2019). Input mechanisms (environmental cues), being the first one, also termed as entrainment pathways, alter the circadian oscillator's phase in harmony with the phase of the surrounding environment in response to the environmental cues, called as 'zeitgebers.' The molecular network is the second one, known as circadian oscillator, which accomplishes the assessment of the time of the 24-hour cycle time. The circadian oscillator in plants is a system of feedback loops consisting of an interconnected network of various genes and proteins (Wang *et al.,* 2022). In *Arabidopsis*, for example, oscillator components are CCA1, LHY, the PRR, GI, and the evening complex (Panter *et al.,* 2019; Nakamichi *et al.,* 2012; Liu *et al.,* 2013; Nagel *et al.,* 2015; Liu *et al.,* 2016; Ezer *et al.,* 2017; Adams *et al.,* 2018; Nohales *et al.,*2019). The Evening complex, being the integral module of the circadian clock of *Arabidopsis*, is responsible for suppressing the target genes during the evening. Besides it coordinates environmental and endogenous signals by integrating temperature information (Tong *et al.,* 2020). The estimated time of the day gauged by the circadian oscillator is then communicated inside the cell to clock-controlled processes by the circadian system's third unit, known as the output mechanism. Eventually, the signaling pathway of the cells becomes circadian regulated, which leads to a calculated response of a certain magnitude that is optimum for the current time of the day and night. This phenomenon of circadian regulation has been described as circadian "gating" of signal transduction (Hotta *et al.,* 2007).

Researchers worldwide have directed significant attention to the impact of the geomagnetic field on a range of biological processes, including biological rhythms, orientation and development (Galland and Pazur, 2005; Wang *et al.,* 2022). Among these, animal orientation has particularly garnered widespread interest from the scientific community (Okano and Ueno, 2022). On the other hand, studies on the influence of geomagnetic fields on various aspects of plants have picked up recently and generated some literature that needs to be deeply explored further (Teixeira da Silva and Dobránszki, 2016; Agliassa *et al.,* 2018; Nyakane *et al.,* 2019; Dhiman *et al.,* 2022). Different researchers have primarily explored the molecular mechanism(s) underlying circadian rhythms in plants and transcriptome coordination and organization in laboratory settings with varying conditions. However, the influence of the geomagnetic field or the variable magnetic flux density has still not been deeply explored on the circadian rhythm. The mustard family (Brassicaceae), which includes *Arabidopsis thaliana*, has a wide geographic range across Europe, Asia, and North America, and it has become the preferred organism for a variety of studies as it completes its entire life cycle in six weeks from seed germination to maturity. Also, all the aspects of *Arabidopsis* are diminutive in scale (Schneijderberg *et al.,* 2020). Therefore, the present study attempts to investigate the potential role of Earth's geomagnetic field on expression of the genes under the control of circadian rhythm in *A. thaliana*.



Note: Purple coloured text represents possible targets of environmental influence for circadian regulation. Red circles denote circadian oscillator components which are transcriptional regulators, and are also responsible for initial output signals from the circadian oscillator that finally control the downstream transcription factors (blue circles) (Adapted from Panter et al. 2019).

Fig 1: The primary component of circadian system is the circadian oscillator (system of feedback loops consisting of interconnected network of genes and proteins), which receives information from environmental cues (zeitgebers) further providing inputs to circadian oscillator for the entrainment. Circadian oscillator generates output timing signals modulating the transcriptome through a transcription factor cascade.

Materials and Methods

Plant Material and Growth Conditions

In the present study, we used wild-type strains of *A. thaliana* (L.) Heynh., specifically the Landsberg erecta (Ler) ecotype procured from LEHLE SEEDS (Tuscon, USA). The seeds were subjected to surface sterilization by treating them with 70% ethyl alcohol followed by 5% sodium hypochloride. Subsequently, the sterilized seeds were positioned at the centre (2-2.5 cm diameter) of petri dishes (9.5 cm diameter) containing a semi-solid culture medium. The culture medium was prepared by combining Phytagel (Sigma) as the gelling agent at a concentration of 3 gL^{-1}, Murashige-Skoog Medium (Sigma) at 2.165 gL^{-1}, MES buffer (Roth) at 0.5 gL^{-1}, and sucrose (Roth) at 1.5%. Following the inoculation, the petri dishes were placed in a dark and cool room maintained at a temperature of 5-6°C for 48 h to facilitate dormancy breakage. Prior to being subjected to the Helmholtz coils, the plants were exposed to white fluorescent light (1.2 Wm^{-2}) for approximately 6 h. Once transferred to the Helmholtz coils, *Arabidopsis* seeds were exposed to a consistent and uninterrupted combination of blue light and magnetic flux density. Fig. 2 summarizes the scheme of the protocols followed for the experiments. The experiment was conducted under controlled conditions, maintaining a constant temperature of 22°C throughout. Four distinct experiments were carried out, each focusing on different points of the magnetic flux density scale: 0 µT, 25 µT, 50 µT, and 90 µT. The geomagnetic field is about 25 µT that increases gradually to about 75 µT as we move from equator to either of the poles. Taking 0 µT was considered significant as it can be used as a good control. 90 µT was selected to consider a point that extends beyond the limits of the Earth to understand plant's reaction to a field that is not experienced by them normally. For each experiment, a set of 12 separate

Helmholtz coils was employed. The motive was to investigate the effects of varying MFD on the chosen parameters. This was performed in order to avoid any disturbance to the seedlings of the neighbouring Helmholtz coils while harvesting them at different time points. The seedlings were allowed to grow for a total duration of 120 h. However, for the purpose of this study, the final 33 hrs, that is, from 87 h to 120 h were selected. The seedlings were harvested at regular intervals of 3 h during this period. Fig. 2 provides a comprehensive visual representation of the seedlings' cultivation protocol. To provide overhead blue light (475 nm, 40 mol $m^{-2}s^{-1}$), two arrays consisting of ten light emitting diodes each (Conrad, Germany) were affixed to plastic holders made of acrylic measuring 6x3 cm (Fig. 3b). The fluence rate was measured using a UV-enhanced photodiode (Meßkopf BN-9102-4) obtained from Gigahertz-Optik GmbH (Germany), and a calibrated readout apparatus (Optometer P-9201 from Gigahertz-Optik GmbH).

Magnetic Fields Generation

All four experiments were performed in a shielded room known as Faraday-cage (5.04m x 2.04m x 2.1m). A Faraday cage was constructed using solid iron walls that were 1.5 mm thick. The cage included two metal doors measuring 2 meters in height and 0.8 meters in width. Additionally, electrical plugs were installed to effectively eliminate alternating magnetic fields within the cage. The Faraday cage effectively reduced the magnetic fields to 2 – 3 µT from about 40 µT, the geomagnetic field present in the Marburg. Helmholtz coils contained in a Faraday cage were utilized to produce the necessary magnetic field strength. Thyssen Krupp Plastics GmbH, Köln supplied the acryl glass cylinder used to make the coils (which were 18 cm in diameter and 9 cm in height). Insulated copper wire (with a diameter of 1.5 mm) from Hopf, Schwalbach, Germany, was used for winding the coils. Inside each cylinder were two windings made of continuous wire positioned on opposite edges. The number of coils within each pair remained constant for all the Helmholtz coils used in the experiment. Furthermore, a support system was incorporated into the Helmholtz coil setup to securely hold two LED arrays, which were used to provide illumination for the growing seedlings during the experiments. For housing each Helmholtz coil system and its irradiation apparatus (Fig. 3a), a Mu(µ)-metal cylinder (25 cm diameter, 40 cm height) constructed using 0.5 mm thick Mu-metal (sourced from Henry Electronic, Germany) with a securely closed lid was utilized. These Mu-metal cylinders together with Faraday-cage, effectively shielded external contaminating magnetic fields. Four Helmholtz coils set-ups having a similar number of windings were arranged in a series and were connected to a single power supply (Hewlett Packard, 0-40 Volt, 0-10 Ampere), to generate the magnetic fields (Fig. 4). The magnetic fields generated corresponded to northern geographical hemisphere (magnetic south pole). This whole system resulted in a homogenous magnetic field in the central region within the Helmholtz coils, where *Arabidopsis* seedlings have been grown, a necessary requirement for the reliability of experiments. To measure the MFDs, a Fluxgate magnetometer was used which was obtained from Stefan Mayer Instruments, Germany. The power sources were placed inside a different compartment within the Faraday cage in order to maintain isolation and reduce interference. The Mu-metal cylinders containing the Helmholtz coil pairs were completely separate from this power compartment, ensuring ideal separation and reducing any disturbances.

Seedling Irradiation

The experimental seedlings were exposed to the desired light using LED arrays specifically designed for this purpose. The blue LED arrays were custom-made by our biology workshop to ensure uniform illumination for the seedlings within the Helmholtz coils. Each array was made up of 10 LEDs connected in series over a 6x3 cm plastic plate. These blue LEDs' 475 nm spectral radiance peak provided ideal lighting to the set up.

mRNA Isolation, cDNA Synthesis and Quantitative PCR

Arabidopsis seedlings were removed from the experimental setup and instantly flash-frozen in liquid N_2 to maintain

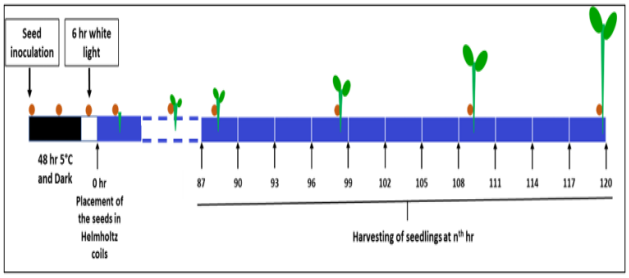


Fig. 2: Schematic representation of the experimental protocol for studying the kinetics of the gene expressions. (Blue colour bar represents the timeline of the seedling growth under blue light besides specific magnetic field. The numbers 87 to 120 along with the arrows denote the time-points in hours when the seedlings were harvested).

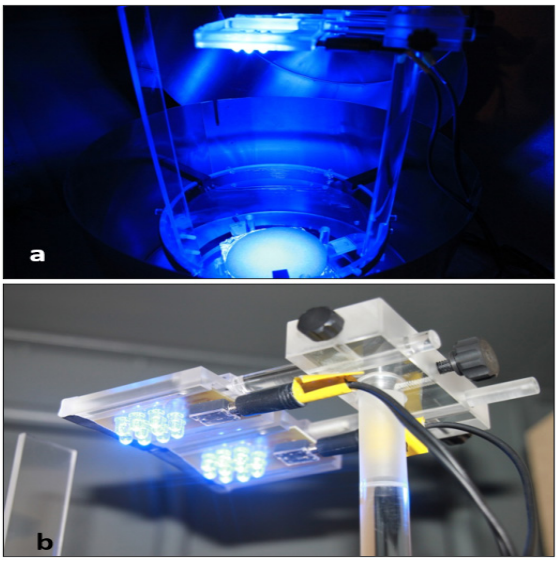


Fig. 3: (a) Helmholtz pair (diameter 18 cm) and two LED-arrays placed inside a Mu metal box cylinder. Helmholtz pair has a pair of windings, one on each edge of the cylinder that generates a uniform magnetic field at the center of the cylinder. Mu metal is an Iron-Nickel alloy having ferromagnetic properties and helps in shielding against static magnetic fields (b): Plastic holder made of acrylic having two LED-arrays (10 LEDs per array). Illumination from 20 LEDs provide a uniform focused blue light for the growing seedlings at the center of the Helmholtz pair.

the metabolic status. The harvesting was performed for 12 time-points. Starting at the 87th h, with an interval of 3 h, the harvesting of seedlings continued overnight until 120th h. The harvested seedlings were homogenized using steel beads to achieve a uniform plant material mixture (Plant Miller M200, Germany). Following homogenization, total RNA was isolated from the resulting plant material using the RNeasy kit (QIAGEN RNeasy Mini Kit 250) according to manufacturer's instructions. The isolated RNA was subsequently quantified using a Nano Drop spectrophotometer. Accordingly, 4µg RNA was used for cDNA synthesis. Before proceeding with cDNA synthesis, the RNA sample underwent DNase I (Fermentas) treatment to remove any genomic DNA contamination. For initiating cDNA synthesis, oligo (dT)18 primer (Fermentas) was introduced to the RNA samples containing total RNA. The oligo dT primer specifically annealed to the polyA tail of mRNA under suitable annealing conditions (70°C for 5 minutes). Following the RNA extraction and quantification, cDNA was synthesized following the manufacturer's protocol of M-MLV reverse transcriptase (Promega). cDNAs were used as templates for the quantification of mRNA transcripts of chlorophyll a/b binding protein 4, large subunit of RuBisCO, rubisco activase, chalcone synthase, porphobilinogen synthase, and phytoene dehydrogenase by qRT-PCR assays. The real-time PCR was done utilizing gene-specific primers (Table 1) and qPCR SYBR Green mix (Thermo Scientific) according to the instructions provided by the manufacturer. The PCR was run on Eppendorf Mastercycler.

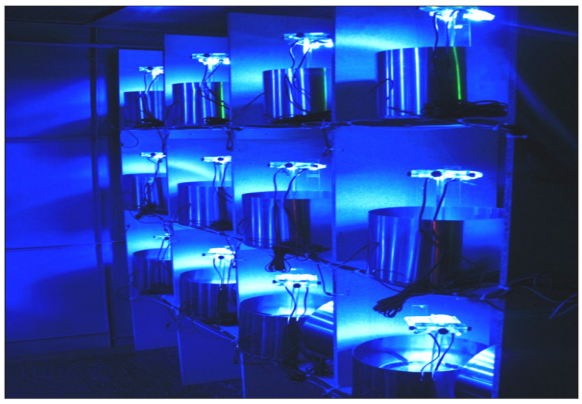


Fig. 4: Picture showing the series of 12 separate experimental Helmholtz coil boxes. Each experimental box was used for harvesting the *Arabidopsis* seedlings at a single time point from 87h to 120h at an interval of 3 hours in these kinetics experiment.

Results

To examine the impact of MFD on the plant circadian rhythm, final 33 h window of the 120 h seedling growth was taken in to consideration (Fig. 2). 120 h (5 days) of seedling growth was considered necessary as it allowed *Arabidopsis* seedlings to attain enough biomass for the RNA extraction. Four points i.e., 0 (near null), 25, 50 and 90 µT were selected on the MFD scale for performing the experiments. The orientation of the magnetic field lines of these four points in present experiments corresponded to the northern hemisphere of the Earth.

The gene responsible for encoding chlorophyll a/b binding protein 4 (*cab4*), a component of chloroplast's light-harvesting complex, exhibits a distinct 24-hour circadian rhythm cycle. This pattern was observed consistently across all four kinetic experiments conducted at MFDs of 0 (near null), 25, 50, and 90 µT (Fig. 5). At varying MFDs, different amounts of mRNA transcripts were produced; the maximum expression was found at 0 (near null) and the minimum was at 50 µT.

The pattern of transcript abundance for the chalcone synthase gene (*chs*) (Fig. 6), which encodes the enzyme chalcone synthase involved in the production of anthocyanins, is more or less similar at all the MFDs investigated, being higher from 90th h to 105th h and then progressively declined after that. Similar to the observations made in *cab4* gene expression, the transcript abundance was found to be lowest at 50 µT, while the quantum of mRNA transcripts was apparently similar at other magnetic flux densities.

Considerably, similar expression pattern was exhibited by the genes porphobilinogen synthase (*hemb2*) (Fig. 7), encoding an essential enzyme involved in the biosynthesis of tetrapyrroles), and phytoene dehydrogenase (*pds*) (Fig. 8), a significant enzyme in carotenoid biosynthesis. A significant increase in the abundance of transcripts is observed between the 95th and 105th h, followed by a subsequent decrease. This pattern is consistent with the influence of variable MFDs over these genes, also observed in other genes studied. The transcript abundance at 0 µT (near null) was highest, and found to be lowest at 50 µT, indicating the effect of Earth's MFD on the expression of genes.

Table 1: Genes and Primers used for qRT-PCR

Gene/ Locus	*Primer*	*Sequence*
Chalcone Synthase/ AT5G13930	Forward- Chsy_72_f Reverse-Chsy_226_r	5`-ATAATGGTGATGGCTGGTGCT-3` 5`-CTGTTGGTGATGCGGAAGTAG-3`
Porphobilinogen synthase/ AT1G44318	Forward- HEMB2_734_f Reverse HEMB2_984_r	5`-GTGAGATGTTGGATGGTCGC -3` 5`-GAGAGATGGGAGTGCTGGCTT-3`
RBCL, RuBisCO/ ATCG00490	Forward- Lsu_1026_f Reverse- Lsu_1266_r	5`-TTTGGGCTTTGTTGATTTACTG-3` 5`-TACTCGGTTGGCTACGGCAC-3`
CAB-4/ AT3G47470	Forward- CAB4_576_f Reverse- CAB4_752_r	5`-GGTGTCGCTGGGATGCTTT-3` 5`-TGGGTTCTTGATGTCTTGCC-3`
Phytoene dhydrogenase/ AT4G14210	Forward- Phytdehy_224_f Reverse Phytdehy_448_r	5`- GCTGCGTCTCCTGTTTCTCTACTT-3` 5`- ACTCCTCCTCCTTGTTCTTGTCTTA-3`
Rubisco activase/ AT2G39730	Forward-Ruac_595_f Reverse- Ruac_814_r	5`-TTTACATTGCTCCTGCTTTCAT-3` 5`-TTTGCGGGTTCTCCTGCGT-3`

The large subunit of RuBisCO (*rbcl*) gene (Fig. 9) exhibits an unexpected pattern. With the exception of the MFD of 25 µT, the mRNA transcript abundance remains consistently similar without displaying any discernible circadian rhythm. However, significant differences are observed in the production of mRNA transcript at the different MFDs examined, with the lowest transcript abundance observed at 50 µT. In contrast, the Rubisco activase gene (*rca*) (Fig. 10), which encodes a chloroplast protein involved in the light activation of RuBisCO, demonstrates a circadian rhythm similar to the *cab4* gene. However, the rhythm is more pronounced at MFDs of 0 (near null) and 50 µT, while it becomes challenging to identify the rhythm at 25 and 90 µT. Likewise other genes analyzed, the mRNA transcript abundance

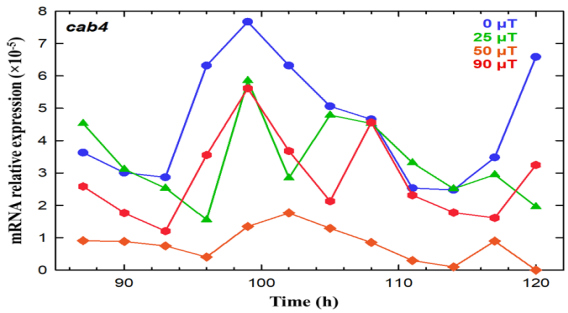


Fig. 5: Kinetics of the transcription of chlorophyll a/b binding-4 gene (*cab4*) from 87 h to 120 h at four different magnetic flux densities (0, 25, 50 and 90 µT). All the original values of mRNA expression were multiplied by 10^5 to present in the graph. Each data point represents the mRNA from approximately 50 seedlings.

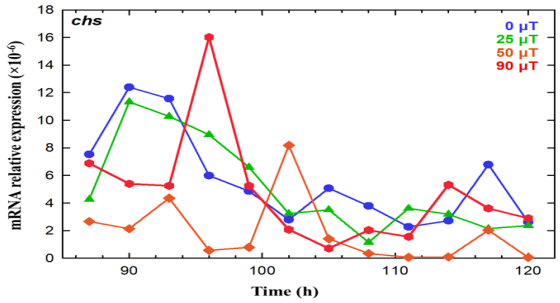


Fig. 6: Kinetics of the transcription of chalcone synthase gene (*chs*) from 87 h to 120 h at four different magnetic flux densities (0, 25, 50 and 90 µT). All the original values of mRNA expression were multiplied by 10^6 to present in the graph. Each point represents the mRNA from approximately 50 seedlings.

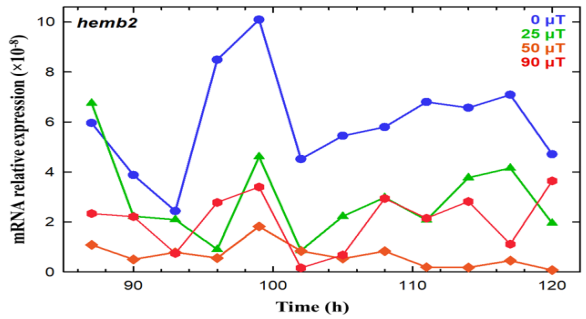


Fig. 7: Kinetics of the transcription of porphobilinogen synthase gene (*hemb2*) from 87 h to 120 h at four different magnetic flux densities (0, 25, 50 and 90 µT). All the original values of mRNA expression were multiplied by 10^8 to present in the graph. Each point represents the mRNA from approximately 50 seedlings.

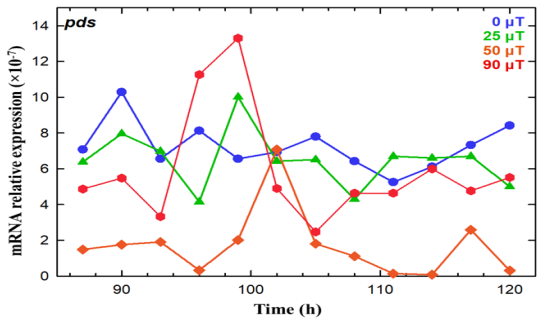


Fig. 8: Kinetics of the transcription of phytoene dehydrogenase gene (*pds*) from 87 h to 120 h at four different magnetic flux densities (0, 25, 50 and 90 µT). All the original values of mRNA expression were multiplied by 10^7 to present in the graph. Each point represents the mRNA from approximately 50 seedlings.

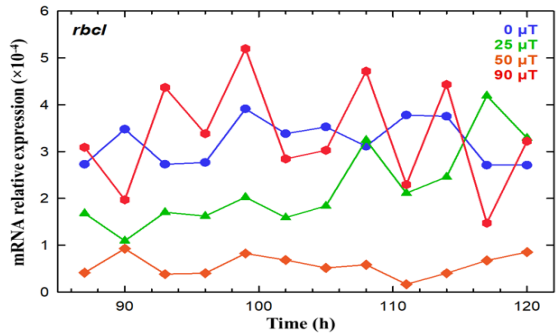


Fig. 9: Kinetics of the transcription of large subunit of RuBisCO (*rbcl*) from 87 h to 120 h at four different magnetic flux densities (0, 25, 50 and 90 µT). All the original values of mRNA expression were multiplied by 10^4 to present in the graph. Each point represents the mRNA from approximately 50 seedlings.

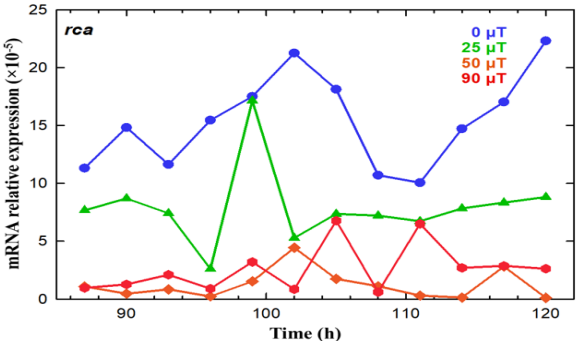


Fig. 10: Kinetics of the transcription of rubisco activase gene (*rca*) from 87 h to 120 h at four different magnetic flux densities (0, 25, 50 and 90 µT). All the original values of mRNA expression were multiplied by 10^5 to present in the graph. Each point represents the mRNA from approximately 50 seedlings.

of *rca* varies across different MFDs. Interestingly, at 90 µT, the *rca* mRNA levels found to be lower and similar to the levels observed at 50 µT, whereas the expression level of other genes at the same MFD (90 µT) are notably higher.

Discussion

The present study's findings lay out evidence for the differential expression of genes regulated by circadian rhythms in plants under different magnetic flux densities. While the *cab4* gene

maintains its typical circadian rhythm, the mRNA transcripts exhibit different levels of expression at various MFDs. Notably, the lowest transcription occurs at 50 µT, which clearly aligns with the stimulus-response curves. The second peak is observed at the range (35-50 µT), followed by a sudden decline in both the gene expression and response curves for blue light suppression of anthocyanin accumulation and hypocotyl length (Dhiman and Galland, 2018). However, the analysis of kinetics conducted at a near null MFD magnetic flux density, the mRNA transcript levels are found to be highest, which contrasts with the patterns observed in the stimulus-response curves. However, the majority of the genes still demonstrate relatively higher transcription even in these curves. The elevated transcript levels found at almost zero MFD are consistent with what the ion-interference mechanism predicts (Binhi *et al.,* 2001). The transcriptional kinetics of the large subunit of the rbcl gene also demonstrates the differential effect of variable MFDs. While the observed mRNA transcript abundance remains relatively stable during the observation period (except at 25 µT), the abundance differs across different MFDs, thus reinforcing the notion of the differential influence of Earth's magnetic fields with varying strengths on the transcription of genes. A more or less uniform magnitude of mRNA transcripts of the larger subunit of RuBisCO, a gene that encodes for world's most abundant enzyme, indicates that its expression is not under the control of circadian control. The continuous increase in mRNA transcripts at 25 µT throughout the observation period poses a challenge in terms of our current understanding of the impact of MFD on the expression of genes. The kinetics data of other genes studied, such as the *rca* gene (Fig. 10), *chs* gene (Fig. 6), *hemb2* gene (Fig. 7), and *pds* gene (Fig. 8), also support this observation.

Entrainment of the circadian rhythm, forms the basis of any experimental set up, that studies the phenomenon of circadian rhythm. This entrainment is usually provided by exposing the experimental plants to multiple light/dark cycles. However, the temperature is also very significant stimuli that can synchronize the plants endogenous rhythm with the external environmental conditions (Millar, 2004; Salome and McClung, 2005b). These periodic cycles entrain the endogenous timing system to a 24 h, as it is in the natural environment. In the current studies, experimental seedlings were not entrained. The seeds were merely exposed to 6 h white light after breaking their dormancy, before placing the seeds in Helmholtz coils. In the Helmholtz coils, the seeds were provided with continuous blue light, constant temperature, and constant magnetic fields. It appears that the seeds assume the commencement of the 6 h white light, provided to them, for initiating the germination process (Hennig et al., 2002), as a signal for initiating the endogenous circadian rhythm. This can be stated on account of the observation in the circadian cycle of mRNA transcripts of CAB4 (Fig. 5), where the 24 h cycle completes 6 h before 120 h duration of the study, or the completion of the 5th day in the Helmholtz coils. So, exposure to continuous light and constant temperature with no entrainment of the *Arabidopsis* seedlings still retained the characteristic features of the circadian rhythm. These observations bring out the fact very clearly that the circadian rhythm is endogenously generated apart from being self-sustaining (McClung, 2006).

The quest for periodic rhythmicity in plants had begun quite early i.e., in 4th century BC, when leaves sleep movements were reported by Androsthenes, in the time of the quest of Alexander the Great (Tuebner, 1903). But major work on understanding the different aspects of periodic cycles in plants started in 1731, when it was recognized that the rhythmic leaf movements in *Mimosa* continued to be exhibited even in constant conditions and, therefore are endogenous (Mairan, 1731). Later investigations on the rhythmic leaf movements in plants established some of the defining characteristics of the circadian rhythm-entrainment to the environment, endogenous origin of the rhythm, and time span of 24 hours of a single cycle (Cumming, 1968). With the advancement of molecular techniques, investigators began to look into plant circadian rhythm from the point of view of genetic expression. It began with the study of gene expression of the three genes involved in the photosynthesis process in pea. Light-harvesting chlorophyll *a*/*b* binding protein (*LHCB* or *CAB*) encoding gene was among the three genes studied, which showed periodic rhythmicity in its expression (Kloppstech, 1985). Afterward, circadian rhythmicity was also observed in the *CAB* gene of wheat (Nagy *et al.,* 1988). Subsequent investigations have revealed that about 1/3 of the genes of the *Arabidopsis* are under the control of circadian rhythm (Harmer *et al.,* 2000; Covington *et al.,* 2008; Michael *et al.,* 2003). Further elucidation of the *Arabidopsis* clock has established that the circadian clock in Arabidopsis comprises complex negative feedback loops, wherein several morning and evening clock constituents are reciprocal repressors (McClung, 2019). Within the complex feedback loops, the early loop comprises a pair of MYB-related transcription factors normally expressed in early morning, late elongated hypocotyl (LHY) and circadian associated 1 (CCA1) and the *timing of CAB2 expression1* (*TOC1*) gene, that is expressed in the evening. In the study conducted by us, the chalcone synthase gene (*chs*) (Fig. 6), a key enzyme in flavonoid biosynthesis, shows transcript abundance during the early phase of the circadian rhythm at all the MFDs, except at 50 µT, that could be due to overall suppressing effect on gene transcription by the MFD (Dhiman and Galland, 2018). The results indicate that the expression of chalcone synthase is under the control of MYB-related transcription factors. MYB (Myeloblastosis viral oncogene homolog) transcription factors (TFs) constitute one of the largest families of transcription factors in plants (Cao *et al.,* 2020). In *Arabidopdis thaliana*, MYB-TFs represent about 9% of the total transcription factors family (Riechmann *et al.,* 2000).

In addition, another set of regulator genes, pseudo-response regulator (PRR), namely, PRR9, PRR7 and PRR5, might also be responsible for controlling the transcription of chalcone synthase, as it has been observed that PRR repressors are involved in restricting the expression of CCA1 and LHY to a short duration at dawn (McClung, 2019). Similar observation was displayed by porphobilinogen synthase (Fig. 7) and phytoene dehydrogenase (Fig. 8) genes, indicating that the expression of both of these genes is also under the influence of MYB-related transcription factors and PRR

While it is pertinent to notice that the *Arabidopsis* clock is primarily based on transcriptional repression and, therefore has been termed as a "repressilator", (Pokhilko *et al.,* 2012), transcriptional activators are also shown to play a significant role in the functioning of the clock (McClung, 2019). Certain

transcriptional activators, such as, light-regulated WD1 (LWD1) and LWD2 have been reported to be employed to promoters of many *Arabidopsis* clock genes, which include PRR9, PRR5, CCA1, and TOC1 (Wu *et al.,* 2008; Wu *et al.,* 2016). There appears to be a possible involvement of two TCPs, i e., TCP20 and TCP22 by their interaction with LWD1 and LWD2 (Wu *et al.,* 2016), in regulating the expression of Chalcone synthase, Porphobilinogen synthase, and Phytoene dehydrogenase, since TCP 20 transcript shows a pre-dawn maximum (Mockler *et al.,* 2008). TCPs (Teosinte Branched1/Cycloidea/Proliferating Cell Factor) are transcription factors (TFs) which operate as plant-specific transcriptional regulators and are responsible for various functions in plant growth and development (Viola and Gonzalez 2023).

The information gathered from the kinetics of another gene, the rubisco activase (Fig. 10), shows a tendency towards being under control of circadian rhythm similar to the results shown by the *cab4* (Fig. 5). This is at least true at 0 and 50 µT, while the data at 25 and 90 µT do not conform to the required expectations of the circadian rhythm. Expression of the rubisco activase is under circadian control is already documented (Wattilon *et al.,* 1993; Perdomo *et al.,* 2021), so an abnormal behavior of rubisco activase mRNA transcripts at 25 and 90µT could possibly be due to a direct effect of MFD at these magnitudes. However, it is unclear why the rubisco activase gene shows an influence of circadian control at 0 and 50 µT and no apparent influence at 25 and 90 µT.

Conclusion

The results of the experiments performed by the authors indicate that there are quantitative differences in the expression of the analyzed genes under the influence of different magnetic flux densities i. e., at 0, 25, 50 and 90 µT. On the other hand, while maintaining these differences in the quantity of RNA produced at different magnetic flux densities, the circadian nature of the genes remains conserved. Therefore, the findings strongly indicate that despite effecting the number of transcripts of the genes studied, the variable geomagnetic field do not interfere with the circadian clock.

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Authors Contribution

Sunil Kumar Dhiman: Conception and design of the experiment, performing, collection and assembly of the data, analysis and interpretation of the data, drafting and writing of the complete article. Ashish Agnihotri: Critical revision and improvement of the article.

Conflict of Interest

Authors have no conflict of interest.

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