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Reduction of Geomagnetic Field (GMF) to Near Null Magnetic Field (NNMF) affects some *Arabidopsis thaliana* clock genes amplitude in a light independent manner

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ABSTRACT

Plant endogenous clock consists of self-sustained interlocked transcriptional/translational feedback loops whose oscillation regulates many circadian processes, including gene expression. Its free running rhythm can be entrained by external cues, which can influence all clock parameters. Among external cues, the geomagnetic field (GMF) has been demonstrated to influence plant growth and development. We evaluated the quantitative expression (qRT-PCR) of three clock genes (*LHY*, *GI* and *PRR7*) in time-course experiments under either continuous darkness (CD) or long days (LD) conditions in *Arabidopsis thaliana* seedlings exposed to GMF (~40 μT) and Near Null Magnetic Field (NNMF; ~40 nT) conditions. Under both LD and CD conditions, reduction of GMF to NNMF prompted a significant increase of the gene expression of *LHY* and *PRR7*, whereas an opposite trend was found for *GI* gene expression. Exposure of Arabidopsis to NNMF altered clock gene amplitude, regardless the presence of light, by reinforcing the morning loop. Our data are consistent with the existence of a plant magnetoreceptor that affects the Arabidopsis endogenous clock.

Keywords: *Arabidopsis thaliana*, geomagnetic field, clock genes, near null magnetic field, internal clock

1. Introduction

Circadian clocks are ubiquitous endogenous and self-sustaining timekeeping networks that are able to either continue to oscillate as free rhythms under constant environmental conditions or be entrained by variations in external cues (Harmer, 2009). By allowing organisms to anticipate and prepare for daily and seasonal changes in the surrounding environment, circadian clocks regulate the timing of various physiological and developmental processes (Muchapirei et al., 2018, Oakenfull and Davis, 2017).

In plants, the presence of the circadian clock is essential to achieve higher survival advantage and fitness despite their sessile nature (Dodd et al., 2005). Plant circadian oscillator mainly consists of interlocked transcriptional/translational feedback loops similar to mammals or insects' clocks (Harmer, 2009). Three loops have been recognized in Arabidopsis so far: the morning loop, the central loop and a group of three evening clock proteins (Inoue et al., 2017, Nohales and Kay, 2016). Changes in environmental variables impact on clock function (Muchapirei et al., 2018), sometimes by shifting its phase or giving temporally informative signals when acting as zeitberg (ZT). In particular, Light/Dark or temperature cycles reset the clock period length (Mas, 2005). Therefore, the interaction between the environment and the clock can modify the circadian rhythmicity of a wide variety of plant phenomena, such as flowering time, hypocotyl expansion, leaf movement, stomatal opening, photosynthesis and gene expression (Harmer, 2009, Muchapirei et al., 2018).

Among environmental elements, the geomagnetic field (GMF) appears to interfere with the circadian rhythmicity of biological processes in animals in a blue light-dependent manner acting as a clock ZT (Gegear et al., 2008, Yoshii et al., 2009). The GMF role as an external temporally informative clock signal could be connected to its regular intensity variations with a fundamental period of 24 hours and its different intensities at the Earth's surface (from 20 μT at the equator to 70 μT at the Poles) (Occhipinti et al., 2014). However, the GMF can also act as a stress factor on living organisms because of its irregular intensity fluctuations due to external variables such as the solar

activity and its reduction to Near Null Magnetic Field (NNMF) levels during GMF polarity reversal (Maffei, 2014). Consequently, its interaction with the clock could also give signals other than temporally informative.

Interestingly, many circadian plant processes are known to be influenced by magnetic field (MF) intensity variations as well (Galland and Pazur, 2005) and high MF values are able to synchronize the clock of root cells under free running conditions (Ikeda et al., 2013). At the Earth's surface (from 0 to 100 μT), flowering time, mineral nutrition (Narayana et al., 2018), gene expression and ROS homeostasis (Bertea et al., 2015) seem to be differently regulated by GMF intensity (Dhiman and Galland, 2018, Maffei, 2014). In particular, Arabidopsis flowering time is known to be delayed when plants are grown under NNMF intensities (40 nT) and long day conditions with respect to GMF control conditions (around 40 μT) (Xu et al., 2012). Recently, we showed that under NNMF and long day conditions some Arabidopsis clock genes are downregulated in flowering plants at noon (Agliassa et al., 2018a, Agliassa et al., 2018b). Although these data showed the influence of NNMF on the expression of clock genes with respect to GMF, the role of the GMF as a possible external cue to the plant clock oscillator is still poorly studied. Moreover, Arabidopsis photoreceptor signalling is differently influenced by the current GMF with respect to NNMF values, but plant magnetoreception occurs even in dark conditions (Agliassa et al., 2018b), thus suggesting that the GMF could influence plants' clock in the absence of light as well.

The aim of this work is to evaluate the GMF role as an external cue to Arabidopsis clock by comparing the time course expression of Arabidopsis clock genes under GMF and NNMF during either long days (LD) or continuous darkness (CD). Here we show that the Arabidopsis clock-controlled amplidudes of gene expression are changed by the shift from GMF to NNMF under light synchronized and free running rhythm in the absence of light.

2. Materials and methods

2.1. Plant material and growth conditions

Arabidopsis thaliana ecotype Col0 seeds were surface sterilized as reported elsewhere (Bertea et al., 2015) and then sown on agar plates filled with half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) without the use of sucrose. Seeds were vernalized for 48 h and then exposed vertically at 21°C under 120 μmol m^{-2} s^{-1} continuous white light (±1.5) for 24 h to induce germination and to maintain the correct geotropic position of roots and shoots. Roots were in touch with the medium whereas shoots maintained naturally a small distance from the medium while growing. Seeds were then put under 150 μmol m^{-2} s^{-1} white light (16 h light/ 8 h dark, long day, LD) for 7 days. After this period, at dawn (starting point) seedlings were either exposed at the same time to GMF (outside the triaxial coils but in the same room, as detailed by Agliassa et al. (2018a)) or NNMF where they were maintained under either LD of CD conditions. All experiments lasted 4 days.

Under both GMF and NNMF, white light was provided by a high-pressure sodium lamp source (SILVANIA, Grolux 600W, Belgium) by using a light blue spotlight film to reduce the red component of lamps. CD exposed plates were kept in paper boxes internally covered by a black cardboard.

2.2. GMF control system

The GMF values (from 40 to 45 μT) were typical of the Northern hemisphere at 45°0'59" N and 7°36'58" E coordinate, with values ranging from 40 to 45 μT. In order to reduce the GMF to NNMF values (40 nT; i.e., 1000 times less than the GMF intensity), a constant compensation was achieved by using an octagonal triaxial Helmholtz coils (THC) system as detailed by Agliassa et al. (2018a). The real-time measure of $B_{x,y,z}$ occurred by collecting 10 s interval data at the magnetometer probe position in the middle of the THC. Data were then transformed in total B by a software (VEE, Agilent Technologies) as detailed elsewhere (Bertea et al., 2015).

104 *2.3. Gene expression analysis*

105 Twenty Arabidopsis seedlings were harvested in liquid nitrogen every 4 hours since the treatment
106 started and then used for RNA extraction. Total RNA was isolated using the Agilent Plant RNA
107 Isolation Mini Kit (Agilent Technologies, Santa Clara, CA, US), following manufacturer's protocols.
108 RNA quality and quantity check and the following cDNA synthesis were performed as already
109 reported (Agliassa et al., 2018a). qPCR experiments were run on a Stratagene Mx3000P Real-Time
110 System (La Jolla, CA, USA) using SYBR green I with ROX as an internal loading standard. The
111 reaction mixture was 10 µL: 5 µL of 2X MaximaTM SYBR Green qPCR Master Mix (Fermentas
112 International, Inc, Burlington, ON, Canada), 3 ng cDNA and 300 nM primers (Integrated DNA
113 Technologies, Coralville, IA, US). Non-template controls were included. Primers were designed
114 using Primer 3.0 software (Rozen and Skaletsky, 2000) (Supplementary Table S1). Four different
115 reference genes were analysed: *ACTIN1 (ACT1*, At2g37620); *CYTOPLASMIC*
116 *GLYCERALDEHYDE3-PHOSPHATE DEHYDROGENASE (GAPC2,* At1g13440), *ELONGATION*
117 *FACTOR 1B ALPHASUBUNIT 2 (eEF1Balpha2*, At5g19510); *UBIQUITIN SPECIFIC PROTEASE*
118 *6 (UBP6*, At1g51710) and three target genes *LATE ELONGATED HYPOCOTYL (LHY*), *PSEUDO-*
119 *RESPONSIVE REGULATOR 7 (PRR7)* and *GIGANTEA (GI)*. The following qPCR conditions were
120 used: *ACT1, GAPC2, GI, LHY, PRR7, UBP6*: 10 min at 95°C, 45 cycles of 15 s at 95°C, 20 s at 57°C,
121 and 30 s at 72°C; *eEF1Balpha2*: 10 min at 95°C; 45 cycles of 15 s at 95°C, 30 s at 57°C, and 30 s at
122 72°C. All runs were followed by a melting curve analysis with the following gradient: 1 min at 95°C,
123 30 s at 55°C, 30 s at 95°C. Primer efficiencies and all amplification plots were analysed as previously
124 described (Agliassa et al., 2018a). Relative RNA levels were normalized to the transcripts of
125 *eEF1Balpha2*, which was selected as the most stable reference gene by using the Normfinder
126 software.

2.4. Statistical analysis

Analysis of variance (ANOVA) and the Tukey test were used to assess difference between treatments and controls (three biological replicates each one) by using the Systat10 software. Fold change data are expressed as mean values ± standard deviation (SD).

3. Results and discussion

The GMF has been considered as a possible entrainer to animals' endogenous clock under blue light exposure (Yoshii et al., 2009); however, plant magnetoreception occurs in dark conditions as well (Agliassa et al., 2018b). In this work, we evaluated whether the GMF was able to provide signals to Arabidopsis clock oscillator by reducing the GMF to NNMF conditions. The expression of genes belonging to the morning and the evening clock loop (*LHY*, *PRR7* and *GI*) was monitored every 4 h in a time-course experiment under LD and CD conditions. We found that the rhythmic expression of Arabidopsis clock genes was different under GMF with respect to NNMF not only under LD, but also under free rhythm running conditions in CD.

In general, under both GMF and NNMF conditions, the switching to free running conditions under CD caused the internal clock oscillator resetting to its natural period length. Therefore, the GMF seems not to give any temporal signal to Arabidopsis clock under CD, thus excluding the GMF influence on the internal clock period as reported in animals (Bliss and Heppner, 1976). On the other hand, NNMF treatments showed that the internal clock gene amplitude was significantly ($P < 0.05$) different with respect to plants exposed to local GMF conditions, regardless the light presence (Figure 1).

Under both LD and CD conditions, reduction of GMF to NNMF prompted a significant increase of the gene expression of *LHY* (Figs. 1A, 1B) and *PRR7* (Figs. 1C, 1D). Even though exposure to CD is known to reduce the amplitude of the clock rhythm (Salome et al., 2008), this was not observed for *LHY* (Fig. 1B) and *PRR7* (Fig. 1D) under NNMF. Apart from light, some other variables are known

to interfere with the *LHY* clock gene amplitude oscillation, including chlorophyll, sugar and starch contents, together with some stress factors (Adams and Carre, 2011). In particular, copper deficiency increases the amplitude of the oscillatory expression of *LHY* (Perea-Garcia et al., 2016), as observed under our NNMF conditions. Our data also support a general interconnected crosstalk of clock transcriptional loops under NNMF. Under LD conditions, NNMF downregulates Arabidopsis *CCA1, LHY* and *TOC1* transcript levels 6 h after dawn, at the time when PRR factors are known to be approximately at their expression peak time (Agliassa et al., 2018a). PRR factors act as repressors of *CCA1* and *LHY* expression, whereas CCA1 and LHY promote *PRR7* transcription (Oakenfull and Davis, 2017). Therefore, we hypothesize that NNMF might also enhance CCA1 and LHY protein level. Ongoing research aims in understanding whether the MF variations are also able to affect clock translational loops by monitoring the protein clock level with time.

An opposite trend with respect to *LHY* and *PRR7* was found in *GI* time-course expression, because the reduction of GMF to NNMF always decreased the gene expression both under both LD (Fig. 1E) and CD (Fig. 1F). Interestingly, *GI* expression is known to be repressed by LHY and vice versa (De Caluwe et al., 2017), thus suggesting that clock protein level might be also regulated by GMF intensity.

NNMF, which is known to be perceived as a stress factors by plants (Agliassa et al., 2018a, Maffei, 2014, Narayana et al., 2018, Rakosy-Tican et al., 2005), impacts on Arabidopsis clock gene amplitude especially by reinforcing the morning loop. This observation is relevant, because transcripts with high-amplitude profiles, such as *LHY*, might be expected to play a major role in controlling circadian timing (Flis et al., 2015). Our results exclude a possible role of the GMF as a ZT to Arabidopsis clock under CD and highlight the impact of NNMF on Arabidopsis clock gene amplitude, regardless the presence of light. Dhiman and Galland (2018) have recently demonstrated that MF intensities from GMF to NNMF modulate Arabidopsis seedlings gene expression under both LD and CD. Furthermore, NNMF intensities can produce nonspecific biological effects on gene expression by affecting RNA polymerase rotation (Binhi and Prato, 2018). Therefore, future works should evaluate

whether a range of MF intensities (from GMF values to NNMF values) differently affect Arabidopsis clock oscillation by monitoring the related circadian plant processes. The relative MF-independence may represent a novel property of the plant clock. Our data are in line with the classical view that the effect of external cues, like temperature, may affect differently the clock, with period length and phase being largely independent of the prevailing steady-state cue (Rensing and Ruoff, 2002).

Considering that Arabidopsis clock oscillation is known to be different in both its amplitude and period dependently from the plant organ (Bordage et al., 2016), studies aimed in discriminating the GMF role as a cue to the internal clock of different plant organs will provide new insights on magnetoreception. The amplitude changes here reported did not greatly affect the period length or the phase, indicating that the clock was resistant to the actual amounts of clock mRNA. We know, however, that after prolonged periods of NNMF plants respond with a delayed flowering time (Agliassa et al., 2018a), suggesting that clock gene amplitude more than gene expression shifting might be related to magnetoreception. Finally, the use of clock mutant lines will be instrumental to investigate whether Arabidopsis response to GMF is mainly dependent on the clock.

Author statement

CA carried out the experiments

CA and MEM conceived of the study and participated in its design and coordination

CA and MEM wrote the article

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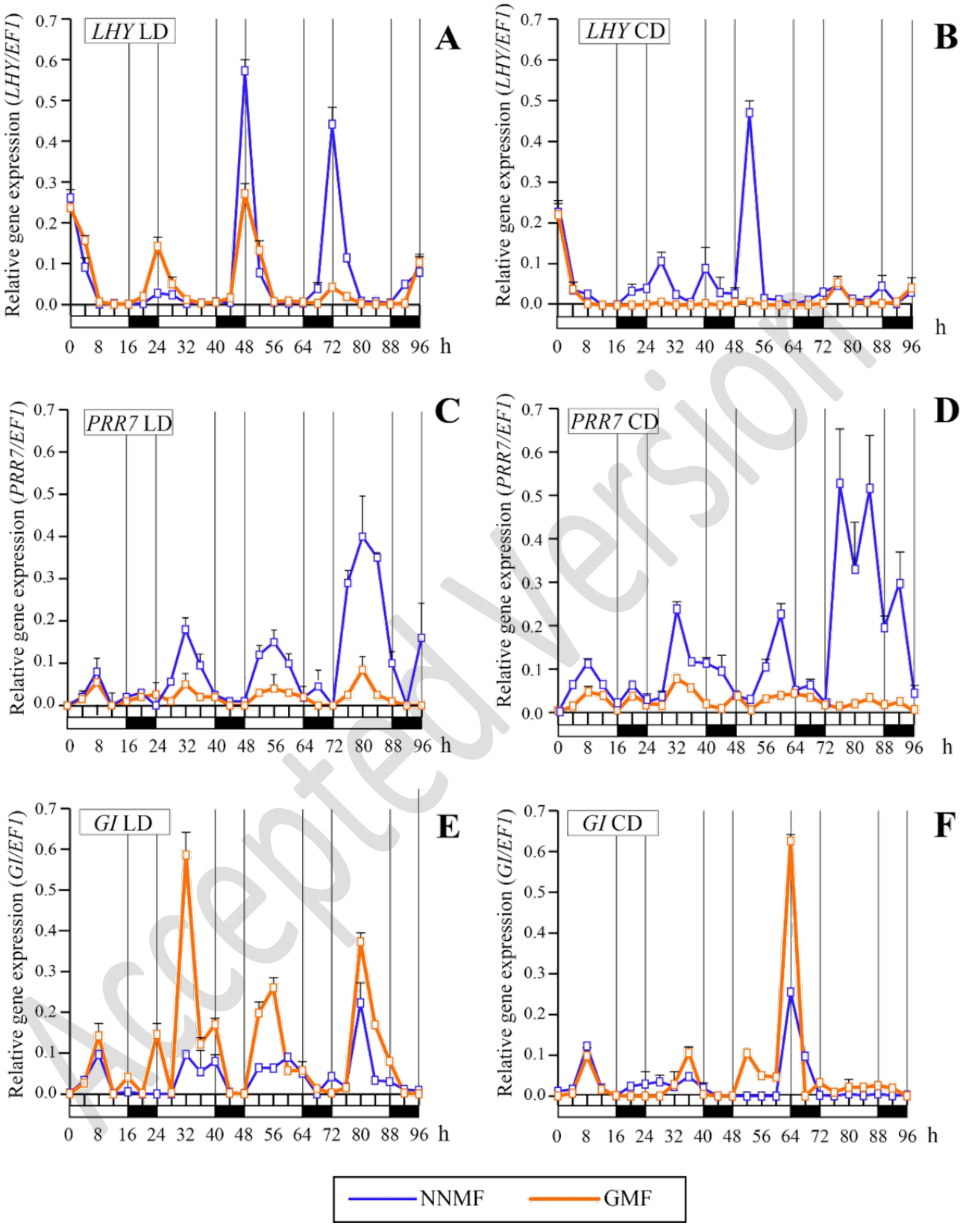
Legend for Figures

Figure 1. Time course *LHY*, *PRR7* and *GI* relative expression in *Arabidopsis thaliana* grown under geomagnetic field (GMF) and Near Null Magnetic Field (NNMF) in long day conditions (LD) and in continuous darkness (CD). *LHY* under LD (**A**) and CD (**B**) conditions as well as *PRR7* under LD (**C**) and CD (**D**) conditions always show increases gene expressions under NNMF, with respect to GMF. *GI* under LD (**E**) and CD (**F**) conditions always show a reduced gene expression under NNMF, when compared to GMF. Data are expressed as the ratio between the given gene and *eEF1Balpha2* (*EF1*) gene expression. Metric bars indicate standard deviation of three experiments. In LD plots, white boxes indicate the light phase, whereas black boxes indicate the dark phase, whereas in CD plots, the white and dark boxes indicate the subjective day and night, respectively.



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280 **Supplementary Table S1: Primers used in this work**

Gene code	Gene	Forward primer (5'-3')	Reverse primer (5'-3')
REFERENCE GENES			
At2g37620	*ACT1*	TGCACTTCCACATGCTATCC	GAGCTGGTTTTGGCTGTCTC
At5g19510	*eEF1Balpha2*	ACTTGTACCAGTTGGTTATGGG	CTGGATGTACTCGTTGTTAGGC
At1g13440	*GAPC2*	TCAGGAACCCTGAGGACATC	CGTTGACACCAACAACGAAC
At1g51710	*UBP6*	GAAAGTGGATTACCCGCTG	CTCTAAGTTTCTGGCGAGGAG-
At1g01060	*LHY*	GCCATTGGCTCCTAATTTCA	TGTTCCCAACTTGGCTCTCT
At5g02810	*PRR7*	GGGCCATATGGAAGCAGTAA	CAAAGCAGCTTCCCTTTGAG
At1g22770	*GI*	ACGCAGAGACTTCTTCTTGGAC	CAGTTCCTGGGTAGCCTTACAC

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