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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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17 **ABSTRACT**

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

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

32

33 **1. Introduction**

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

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

51 Among environmental elements, the geomagnetic field (GMF) appears to interfere with the
52 circadian rhythmicity of biological processes in animals in a blue light-dependent manner acting as a
53 clock ZT (Gegear et al., 2008, Yoshii et al., 2009). The GMF role as an external temporally
54 informative clock signal could be connected to its regular intensity variations with a fundamental
55 period of 24 hours and its different intensities at the Earth's surface (from 20 μ T at the equator to 70
56 μ T at the Poles) (Occhipinti et al., 2014). However, the GMF can also act as a stress factor on living
57 organisms because of its irregular intensity fluctuations due to external variables such as the solar

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

61 Interestingly, many circadian plant processes are known to be influenced by magnetic field (MF)
62 intensity variations as well (Galland and Pazur, 2005) and high MF values are able to synchronize
63 the clock of root cells under free running conditions (Ikeda et al., 2013). At the Earth's surface (from
64 0 to 100 μ T), flowering time, mineral nutrition (Narayana et al., 2018), gene expression and ROS
65 homeostasis (Bertea et al., 2015) seem to be differently regulated by GMF intensity (Dhiman and
66 Galland, 2018, Maffei, 2014). In particular, Arabidopsis flowering time is known to be delayed when
67 plants are grown under NNMF intensities (40 nT) and long day conditions with respect to GMF
68 control conditions (around 40 μ T) (Xu et al., 2012). Recently, we showed that under NNMF and long
69 day conditions some Arabidopsis clock genes are downregulated in flowering plants at noon (Agliassa
70 et al., 2018a, Agliassa et al., 2018b). Although these data showed the influence of NNMF on the
71 expression of clock genes with respect to GMF, the role of the GMF as a possible external cue to the
72 plant clock oscillator is still poorly studied. Moreover, Arabidopsis photoreceptor signalling is
73 differently influenced by the current GMF with respect to NNMF values, but plant magnetoreception
74 occurs even in dark conditions (Agliassa et al., 2018b), thus suggesting that the GMF could influence
75 plants' clock in the absence of light as well.

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

81 **2. Materials and methods**

82 *2.1. Plant material and growth conditions*

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

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

96 *2.2. GMF control system*

97 The GMF values (from 40 to 45 μT) were typical of the Northern hemisphere at 45°0'59" N and
98 7°36'58" E coordinate, with values ranging from 40 to 45 μT . In order to reduce the GMF to NNMF
99 values (40 nT; i.e., 1000 times less than the GMF intensity), a constant compensation was achieved
100 by using an octagonal triaxial Helmholtz coils (THC) system as detailed by Agliassa et al. (2018a).
101 The real-time measure of $B_{x,y,z}$ occurred by collecting 10 s interval data at the magnetometer probe
102 position in the middle of the THC. Data were then transformed in total B by a software (VEE, Agilent
103 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 μ L: 5 μ L 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: *ACTINI* (*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.

127 2.4. Statistical analysis

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

131 3. Results and discussion

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

140 In general, under both GMF and NNMF conditions, the switching to free running conditions under
141 CD caused the internal clock oscillator resetting to its natural period length. Therefore, the GMF
142 seems not to give any temporal signal to Arabidopsis clock under CD, thus excluding the GMF
143 influence on the internal clock period as reported in animals (Bliss and Heppner, 1976). On the other
144 hand, NNMF treatments showed that the internal clock gene amplitude was significantly ($P < 0.05$)
145 different with respect to plants exposed to local GMF conditions, regardless the light presence (Figure
146 1).

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

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

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

167 NNMF, which is known to be perceived as a stress factors by plants (Agliassa et al., 2018a, Maffei,
168 2014, Narayana et al., 2018, Rakosy-Tican et al., 2005), impacts on Arabidopsis clock gene amplitude
169 especially by reinforcing the morning loop. This observation is relevant, because transcripts with
170 high-amplitude profiles, such as *LHY*, might be expected to play a major role in controlling circadian
171 timing (Flis et al., 2015). Our results exclude a possible role of the GMF as a ZT to Arabidopsis clock
172 under CD and highlight the impact of NNMF on Arabidopsis clock gene amplitude, regardless the
173 presence of light. Dhiman and Galland (2018) have recently demonstrated that MF intensities from
174 GMF to NNMF modulate Arabidopsis seedlings gene expression under both LD and CD.
175 Furthermore, NNMF intensities can produce nonspecific biological effects on gene expression by
176 affecting RNA polymerase rotation (Binhi and Prato, 2018). Therefore, future works should evaluate

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

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

191 **Author statement**

192 CA carried out the experiments

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

194 CA and MEM wrote the article

195 **Funding information**

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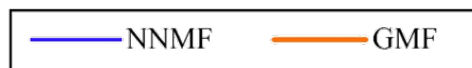
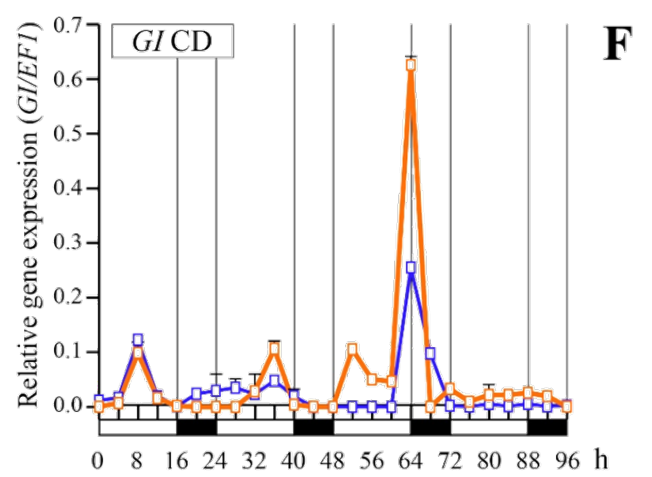
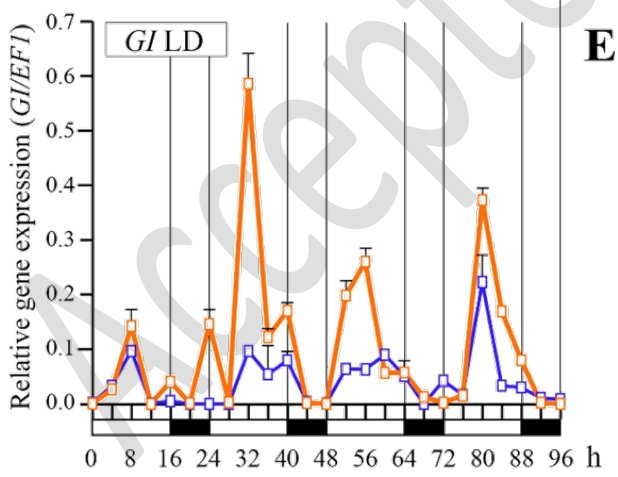
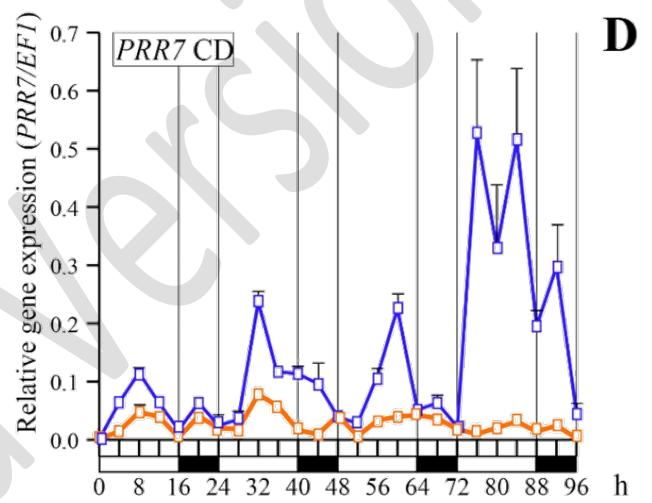
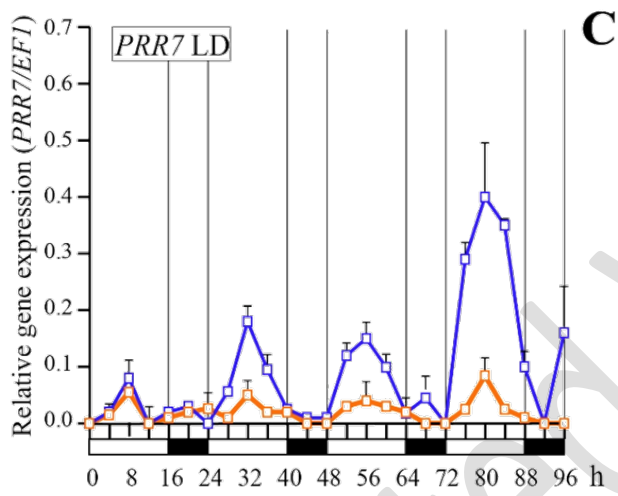
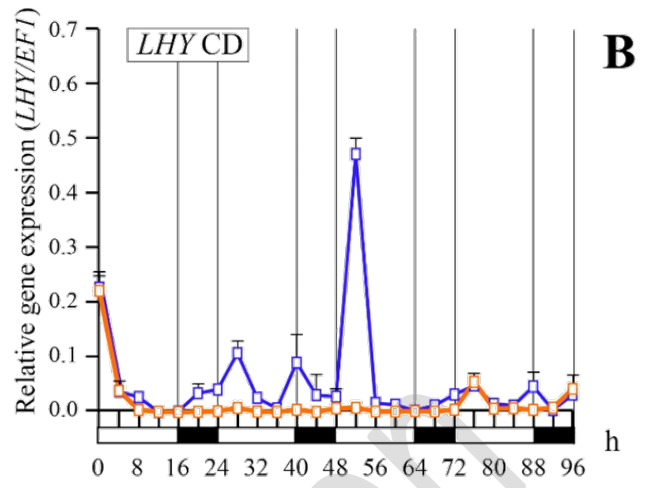
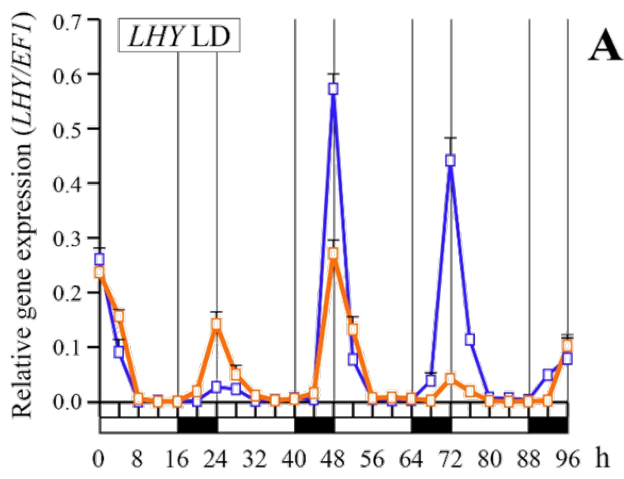
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266

267 **Legend for Figures**

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

277



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279

Supplementary Table S1: Primers used in this work

Gene code	Gene	Forward primer (5'-3')	Reverse primer (5'-3')
REFERENCE GENES			
At2g376 20	<i>ACT1</i>	TGCACTTCCACATGCTAT CC	GAGCTGGTTTTGGCTGTC TC
At5g195 10	<i>eEF1Balp</i> <i>ha2</i>	ACTTGTACCAGTTGGTTA TGGG	CTGGATGTACTCGTTGTT AGGC
At1g134 40	<i>GAPC2</i>	TCAGGAACCCTGAGGAC ATC	CGTTGACACCAACAACG AAC
At1g517 10	<i>UBP6</i>	GAAAGTGGATTACCCGCT G	CTCTAAGTTTCTGGCGAG GAG-
At1g010 60	<i>LHY</i>	GCCATTGGCTCCTAATTT CA	TGTTCCCAACTTGGCTCT CT
At5g028 10	<i>PRR7</i>	GGGCCATATGGAAGCAGTAA	CAAAGCAGCTTCCCTTTGAG
At1g227 70	<i>GI</i>	ACGCAGAGACTTCTTCTT GGAC	CAGTTCCTGGGTAGCCTT ACAC

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