Reduction of the Geomagnetic Field Delays
*Arabidopsis thaliana* Flowering Time Through Downregulation of Flowering-Related Genes

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Variations in magnetic field (MF) intensity are known to induce plant morphological and gene expression changes. In *Arabidopsis thaliana* Col-0, near-null magnetic field (NNMF, i.e., <100 nT MF) causes a delay in the transition to flowering, but the expression of genes involved in this response has been poorly studied. Here, we showed a time-course quantitative analysis of the expression of both leaf (including clock genes, photoperiod pathway, GA20ox, SVP, and vernalization pathway) and floral meristem (including GA20ox, SOC1, AGL24, LFY, AP1, FD, and FLC) genes involved in the transition to flowering in *A. thaliana* under NNMF. NNMF induced a delayed flowering time and a significant reduction of leaf area index and flowering stem length, with respect to controls under geomagnetic field. Generation experiments (F1- and F2-NNMF) showed retention of flowering delay. The quantitative expression (qPCR) of some *A. thaliana* genes expressed in leaves and floral meristem was studied during transition to flowering. In leaves and flowering meristem, NNMF caused an early downregulation of clock, photoperiod, gibberellin, and vernalization pathways and a later downregulation of TSF, AP1, and FLC. In the floral meristem, the downregulation of AP1, AGL24, FT, and FLC in early phases of floral development was accompanied by a downregulation of the gibberellin pathway. The progressive upregulation of AGL24 and AP1 was also correlated to the delayed flowering by NNMF. The flowering delay is associated with the strong downregulation of FT, FLC, and GA20ox in the floral meristem and FT, TSF, FLC, and GA20ox in leaves. Bioelectromagnetics. 39:361–374, 2018. © 2018 The Authors. Bioelectromagnetics Published by Wiley Periodicals, Inc.

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INTRODUCTION

The Earth’s magnetic field (MF), also known as the geomagnetic field (GMF), is an environmental factor affecting all organisms living on the planet, including plants. The GMF protects the Earth and its biosphere from the lethal effects of solar wind by deflecting most of its charged particles through the magnetosphere away into space [Occhipinti et al., 2014].

Since plants respond to environmental stimuli such as light and gravity with so-called phototropic and gravitropic responses, it is no wonder that the GMF is also able to influence many biological processes in plants [Maffei, 2014]. In recent years, the progress and status of research on the effect of MFs on plants has been reviewed [Phirke et al., 1996; Abe et al., 1997; Belyavskaya, 2004; Galland and Pazur, 2005; Minorsky, 2007]. The effects of both weak and
strong MFs have been thoroughly discussed, with a particular focus on the involvement of GMF reversal events on plant evolution [Maffei, 2014]. However, a detailed analysis of experiments describing the effects of MFs on plants shows a large number of conflicting reports, characterized by a dearth of plausible biophysical interaction mechanisms. Many experiments are simply unrealistic, while others lack a testable hypothesis and, ultimately, prove not to be reproducible [Harris et al., 2009].

A large number of studies on MF effects on plants have been carried out by using MF intensity higher than the GMF; however, only a limited number of studies have analyzed the effects of exposure of plant to MF with intensity lower than the GMF [Maffei, 2014]. The term “weak” or “low magnetic field” generally refers to intensities from 100 nT to 0.5 mT, whereas “super-weak,” “conditionally zero,” or “near-null magnetic field” (NNMF) refers to MFs below 100 nT [Maffei, 2014].

Investigations of NNMF effects on biological systems have attracted the attention of biologists for several reasons. Reversal of the GMF implies a period of transition that may expose living organisms to NNMF. Besides the described effects of GMF reversals and their effects on plant evolution [Occhipinti et al., 2014], interplanetary navigation will introduce humans, animals, and plants to environments where the natural MF is near 1 nT, unless artificially augmented. Therefore, the topic is of wide interest.

In Arabidopsis thaliana seedlings grown under NNMF, preliminary results showed that flowering time was found to be delayed compared with seedlings grown in normal GMF [Xu et al., 2013, 2015, 2017, 2018]. Moreover, the transcription level of a few flowering-related genes also changed [Xu et al., 2012]. Furthermore, the biomass accumulation of plants in NNMF was significantly suppressed at the time when plants were switching from vegetative growth to reproductive growth compared to that of plants grown in normal GMF. This was caused by a delay in flowering of plants in NNMF, which resulted in a significant reduction in the harvest index of plants in NNMF compared with that of control plants. Therefore, preliminary results indicate that the removal of the local GMF negatively affects the reproductive growth of A. thaliana, which thus affects the yield and harvest index [Xu et al., 2013]. Since timing of flowering is crucial to the life cycle of plants, it is not surprising that plants constantly monitor environmental signals to adjust the timing of the floral transition [Capovilla et al., 2015], but it is amazing that this is exquisitely sensitive to MF.

While the effects of day length (photoperiod) [Sanchez et al., 2011] and temperature changes [Chew et al., 2012] on flowering time have been thoroughly studied, many aspects of plant flowering delay in response to NNMF are still poorly explored. Plant flowering time is controlled by several genes, including circadian clock-associated genes [Hara et al., 2014], genes involved both in the transition from the vegetative to the reproductive phase [Gu et al., 2013] and in the precise control of flowering [Song et al., 2014], and microRNA regulation [Spanudakis and Jackson, 2014; Hong and Jackson, 2015]. Current models provide us with a basis on which to address a number of fundamental issues for a better understanding of the molecular mechanisms by which plants respond to environmental stimuli to control flowering time [Fornara et al., 2010]. Therefore, to assess the effect of NNMF on A. thaliana flowering time, we built an MF compensation apparatus, comprised of three orthogonal Helmholtz coil pairs under computer control, of dimensions sufficient for plants to grow from seed to seed. This apparatus is able to accurately reduce the normal GMF to NNMF (ca. 40 nT) (Fig. 1). This apparatus was also instrumental in our assessment of the effect of GMF reversal on A. thaliana gene expression [Bertea et al., 2015].

Previous work has shown that flowering time is delayed and the expression of a few A. thaliana genes involved in the transition to flowering is altered after exposure to NNMF [Xu et al., 2012]. However, the limited number of flowering genes analyzed in this study (COP1, CO, FT) and the lack of analysis of organ-specific gene expression do not allow for evaluation of either the interaction between genes expressed in the leaves and floral meristem or the dynamics of modulation of gene expression. In this work, we used the authoritative “SnapShot” atlas [Fornara et al., 2010] to select the key genes from all major pathways responsible for the control of flowering, expressed in the leaves and also in the floral meristem, and we significantly extended the preliminary observations of previous works [Xu et al., 2012, 2013]. For the first time, we were also able to make a comprehensive, time-course, Real-Time PCR analysis of NNMF effects.

We hypothesized that exposure of A. thaliana to NNMF would affect flowering pathways to different extents and that understanding which pathways are most sensitive to NNMF would give clues to the mechanism of plant magnetoreception.

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MATERIALS AND METHODS

Plant Material and Growth Conditions

*A. thaliana* ecotype Columbia 0 (Col-0) wild type seeds were sown in 8 cm diameter polyethylene pots with soil prepared with a mixture of peat and vermiculite (2:1). Sown pots were exposed to homogeneous irradiation from a high pressure sodium lamp source (Grolux 600W, Sylvania, Wilmington, MA) at 200 μmol m⁻² s⁻¹, at 21 °C (±1.5 °C) with a photoperiod of 14 h light and 10 h darkness. Control plants were exposed to normal Earth magnetic field (GMF), in the same laboratory and at the same time, under controlled light and temperature identical to those in the triaxial coils. Control experiments (GMF) were performed in the same lab at a distance of 8 m from the triaxial Helmholtz coils, and the measured levels of power-line frequency (50 Hz) MF associated with the triaxial coils and control GMF were similar. Treated plants were grown inside the triaxial coils under NNMF (see below in the section NNMF Generation System).

Seeds from plants growing either in the GMF control or under NNMF were harvested from brown siliques which were carefully cut at their base. In order to evaluate the generation effect, seeds were sieved to separate them from chaff and were kept in small Petri dishes (4 cm diameter) and maintained under either GMF or NNMF for 2 weeks. These seeds were then sown in pots as described above. The seeds of the first experiment (F₁ NNMF and F₁ GMF seeds) were collected and sown in pots as described above and plants were allowed to grow until full bloom. Seeds of F₁ NNMF and F₁ GMF (defined as F₂ NNMF and F₂ GMF) were then collected as above and kept under either GMF or NNMF for 2 weeks. These seeds were sown in pots as described above in order to obtain a third generation of plants experiencing either NNMF or GMF.
Leaf area index (LAI) was measured by dividing the leaf area by the pot area. Stem length was measured from the base to the tip of the flowering stem. Pictures were taken for all generations and the phenotypic behavior (leaf area index and stem length) was plotted as a function of time.

**NNMF Generation System**

The GMF (or local geomagnetic field) values were typical of the Northern hemisphere at $45^\circ$0'59" N and $7^\circ$36'58" E coordinates. Near-null MF was generated by three orthogonal Helmholtz coils (Fig. 1A) connected to three DC power supplies (model E3642A 50W, 2.5Adual range: 0-8V/5A and 0-20V/2.5A, Agilent Technologies, Santa Clara, CA) controlled from a computer via a GPIB connection. A real-time measure of the MF in the plant exposure chamber was achieved with a three-axis MF sensor (model Mag-03, Bartington Instruments, Oxford, UK) that was placed at the geometric center of the Helmholtz coils. The output data from the magnetometer were uploaded to VEE software (Agilent Technologies) to fine-tune the current applied through each of the Helmholtz coil pairs in order to maintain the MF inside the plant growth chamber at NNMF intensity. Defining the vertical axis as “y,” the GMF level at the experimental location in our lab was $B_x = 6.39 \mu T$, $B_y = 36.08 \mu T$, $B_z = 20.40 \mu T$; i.e., an MF strength ($B = (B_x^2 + B_y^2 + B_z^2)^{1/2}$) of 41.94 \mu T; by applying the following voltages $V_x = 11.36$, $V_y = 15.04$, $V_z = 13.81$ (which produced currents $I_x = 26 \ mA$, $I_y = 188 \ mA$, $I_z = 103 \ mA$), the magnetometer values were $B_x = 0.033 \mu T$, $B_y = 0.014 \mu T$, $B_z = 0.018 \mu T$ with a field strength of 40.11 nT, which is about one thousandth of the GMF strength (Fig. 1B and C). The coil diameter ($\Theta$) and separations between the Helmholtz coils (sep.) were the following: $X, \Theta = 128 \ cm, \ sep. = 55 \ cm; Y, \Theta = 150, \ sep. = 67; Z, \Theta = 135, \ sep. = 59$ (Fig. 1A). The inclination angle of B in GMF was 57.7 degrees, and in NNMF the inclination of the tiny residual field B varied between $-71.3$ and $+44.5$ degrees (see Fig. 1D). Supplementary Figure S1 shows main field inclination values on a world scale.

Sham exposure experiments were performed by keeping the field almost identical to that of the GMF but altering the direction (i.e., declination, or “North, East, or West”) of the horizontal component of the field with equal currents in the triaxial coils compared to the NNMF (see above) by altering the voltage of the coils. This sham exposure ruled out potential subtle heating or vibrational effects either from the coils themselves or from the electronics used to control the coils. Sham experiments resulted in insignificant differences between GMF and altered inclination of the GMF (data not shown). Because GMF are the natural conditions experienced by plants, we chose to use GMF as control.

Double-blind experiments were performed by applying field blinded from the personnel performing the remainder of the experiments and/or interpreting the data.

**RNA Isolation from Plants Grown Under Either NNMF or GMF**

Since the expression levels of flowering genes vary as a function of time of day, we chose to collect leaves and floral meristems from plants growing either under GMF (control) or NNMF (treatment) at noon (12:00). Samples were immediately frozen in liquid nitrogen. Fifty milligrams of either frozen leaf or frozen floral meristem material were ground in liquid nitrogen with mortar and pestle. Total RNA was isolated using Agilent Plant RNA Isolation Mini Kit (Agilent Technologies) and RNase-Free DNase set (Qiagen, Hilden, Germany). Sample quality and quantity were checked using RNA 6000 Nano kit and Agilent 2100 Bioanalyzer (Agilent Technologies), following the manufacturer’s instructions. Quantification of RNA was also confirmed spectrophotometrically, using NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA).

**Quantitative Real-Time PCR (qPCR)**

First strand cDNA synthesis was run with 1 \mu g of total RNA and random primers, using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), and following the manufacturer’s recommendations. Reactions were prepared by adding 1 \mu g total RNA, 2 \mu l of 10\times RT Buffer, 0.8 \mu l of 25 \times dNTPs mix (100 mM), 2 \mu l 10\times RT primer, 1 \mu l of Multiscribe Reverse Transcriptase, and nuclease-free sterile water to 20 \mu l. Reaction mixtures were incubated at 25 °C for 10 min, 37 °C for 2 h, and 85 °C for 5 min.

The qPCR experiments were run on an Mx3000P Real-Time System (Stratagene, La Jolla, CA) using SYBR green I with ROX as an internal loading standard. The reaction mixture was 10 \mu l, comprised of 5 \mu l of 2\times Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific), 0.5 \mu l of cDNA, and 100 nM primers (Integrated DNA Technologies, Coralville, IA). Supplementary Table S1 lists the forward and reverse primers used. Controls included non-RT controls (using total RNA without reverse transcription to monitor for genomic DNA contamination) and non-template controls (water
specifically, PCR were 10 min at 95 °C, 45 cycles of 1 s at 95 °C, 20 s at 57 °C, and 30 s at 72 °C, 1 min at 95 °C, 30 s at 55 °C. 30 s at 95 °C for At4g24540, AGAMOUS-LIKE 24 (AGL24); At1g06190, APETALAI (AP1); At2g46830, CIRCADIAN CLOCK ASSOCIATED 1 (CCA1); At5g15840, CONSTANS (CO); At4g35900, BZIP TRANSCRIPTION FACTOR FD (FD); At1g68050, FLAVIN-BINDING KELCH REPEAT F-BOX 1 (FKF1); At5g10140, FLOWERING LOCUS C (FLC); At3g00650, FRIGIDA (FRI); At1g5480, FLOWERING LOCUS T (FT); At1g78440, GIBBERELLIN 2-OXIDASE 1 (GA2ox1); At4g25420, GIBBERELLIN 20-OXIDASE1 (GA20ox1); At5g51810, GIBBERELLIN 20-OXIDASE2 (GA20ox2); At1g22770, GIGANTEA (GI); At4g20400, JMJ domain-containing histone demethylases 14 (JMJ14); At5g61850, LEAFY (LFY); At1g01060, LATE ELONGATED HYPOCOTYL (LHY); At3g10480, NAC transcription factor 50 (NAC050); At3g10490, NAC transcription factor 52 (NAC052); At1g76710, SHORT VEGETATIVE PHASE (SVP); and At1g51710, Ubiquitin (UBP6), were used to normalize the results of the qPCR. The best of the four genes was selected using Normfinder software (MOMA, Aarhus, Denmark) [Andersen et al., 2004]; the most stable gene was eEF1Balpha2. Primers used for qPCR were designed using Primer3 software (Thermo Fisher Scientific) [Rozen and Skaltsky, 2000] and are reported in Supplementary Table S1.

All amplification plots were analyzed with Mx3000P software to obtain Ct values. Relative RNA levels were calibrated and normalized with the level of eEF1Balpha2 mRNA.

qPCR data are expressed as fold change with respect to the equivalent time-point in the control.

Statistical Analyses

In general, the experiments were repeated three times (biological replicates) with at least 15 plants per each experiment. Three technical replicates were run for each biological replicate. Analysis of variance (ANOVA) and Tukey test were used to assess difference between treatments and controls. For generation experiments, at least 15 plants per experiment were used. Data were processed by Kolmogorov–Smirnov test, and Systat 10 (Systat Software, San Jose, CA) was used for univariate and multivariate tests. For all gene expression experiments, at least three samples per treatment group entered the statistical data analysis. Fold change data are expressed as mean values ± standard deviation (SD). Cluster analysis was calculated by using the Systat10 software and by using Euclidean distances with median linkage.

RESULTS

NMMF Delays Flowering and Alters Leaf Expansion and Stem Length of A. thaliana

A. thaliana seed germination in NMMF and GMF did not differ (data not shown); however, exposure of A. thaliana to NMMF and long day caused a significant delay in flowering time. NMMF-exposed plants started flowering about 4 days later with respect to control plants (GMF) and reached full bloom about 5 days later than controls (Fig. 2A, see also Supplementary Table S2 for statistical analyses). We also performed generation experiments to test whether seeds produced under NMMF were affected by further sowning in NMMF. Seeds produced under NMMF (F1-NMMF) germinated regularly but their flowering time was significantly delayed by 6 days with respect to control plants (GMF), and 1 day with respect to parent plants grown in NMMF. A second generation of seeds produced by F1-NMMF, which we indicated as F2-NMMF, did not show any significant difference in flowering time with respect to parent plants F1-NMMF (Fig. 2A, see Supplementary Table S2); however, they still maintained a delay in flowering time with respect to control plants (GMF). When F2-NMMF seeds were sown in GMF, the phenotype and flowering time were found to be the same as plants never exposed to NMMF (Fig. 2A, see Supplementary Table S2). The leaf area index was significantly (P < 0.05) lower in plants under NNMF.
than in control plants (Fig. 2B), and the same results were obtained when the floral stem was measured (Fig. 2C).

**NNMF Alters the Expression of A. thaliana Flowering-Related Genes in Leaves**

In order to dissect the effect of NNMF on the transition to flowering, we analyzed gene expression in the leaves.

In the leaves, *A. thaliana* plants under NNMF showed a significant and consistent downregulation of gene expression in early induction times (17–19 days after sowing, DAS) for *CCA1, CO, FD, FKF1, FRI, FT, GA20ox1, GA20ox2, LFY, LHY, TOC1, TSF,* and *WUS* (Table 1). *AP1, GI,* and *STM* were downregulated at later times (19–23 DAS, Table 1), whereas a significant upregulation was found for *FLC* during early floral induction, but the gene was downregulated...
TABLE 1. Time-Course Expression of Leaf Genes in *A. thaliana* Exposed to NNMF Conditions

| Genes   | 17   | 19   | 21   | 22   | 23   | 28   |
|---------|------|------|------|------|------|------|
| *API*   | -1.04 (±0.01) | -1.71 (±0.11) | -1.93 (±0.31) | -3.01 (±0.12) | -1.40 (±0.04) | 1.14 (±0.02) |
| *CCAI*  | -5.25 (±0.11) | -1.57 (±0.06) | -1.02 (±0.08) | -1.65 (±0.03) | -2.94 (±0.03) | -1.77 (±0.11) |
| *CO*    | -2.72 (±0.01) | -1.74 (±0.13) | 1.25 (±0.4) | -1.13 (±0.20) | 1.16 (±0.11) | -2.70 (±0.04) |
| *FD*    | -1.40 (±0.03) | -1.23 (±0.09) | -1.86 (±0.19) | -1.12 (±0.16) | 1.18 (±0.09) | -1.14 (±0.18) |
| *FKF1*  | -1.45 (±0.09) | -1.67 (±0.02) | -1.38 (±0.01) | -1.43 (±0.06) | -1.07 (±0.05) | -1.12 (±0.01) |
| *FLC*   | 1.98 (±0.17) | -1.12 (±0.03) | -1.10 (±0.11) | -1.75 (±0.11) | -2.22 (±0.28) | -3.81 (±0.08) |
| *FRI*   | -1.80 (±0.09) | -1.61 (±0.05) | 1.27 (±0.57) | -1.32 (±0.04) | 1.71 (±0.07) | -1.75 (±0.16) |
| *FT*    | -3.09 (±0.02) | -2.26 (±0.05) | -1.25 (±0.08) | -1.37 (±0.05) | 1.03 (±0.03) | -2.35 (±0.02) |
| *GA20ox1* | -3.11 (±0.28) | -2.43 (±0.36) | -1.24 (±0.21) | -2.29 (±0.09) | 1.22 (±0.27) | -1.20 (±0.12) |
| *GA20ox2* | -5.58 (±0.66) | -6.81 (±0.34) | 1.19 (±0.12) | 1.16 (±0.23) | 1.38 (±0.18) | -1.02 (±0.11) |
| *GI*    | 1.04 (±0.09) | 1.07 (±0.11) | 1.01 (±0.02) | -1.32 (±0.50) | -1.04 (±0.04) | 1.51 (±0.05) |
| *LFY*   | -2.07 (±0.15) | -1.71 (±0.15) | -2.15 (±0.23) | 1.15 (±0.07) | 1.56 (±0.20) | -1.66 (±0.27) |
| *LHY*   | -2.91 (±0.04) | -1.73 (±0.04) | -1.28 (±0.09) | -1.77 (±0.05) | -1.04 (±0.05) | -2.01 (±0.07) |
| *NAC050* | -1.11 (±0.15) | -1.5 (±0.23) | -1.16 (±0.15) | -1.14 (±0.06) | 1.05 (±0.09) | -1.05 (±0.12) |
| *SDG26* | -1.26 (±0.07) | -1.11 (±0.09) | -1.16 (±0.15) | -2.09 (±0.27) | -1.44 (±0.23) | 3.23 (±0.54) |
| *STM*   | -1.47 (±0.18) | -2.68 (±0.13) | 1.15 (±0.49) | -1.80 (±0.04) | 1.35 (±0.15) | -1.47 (±0.08) |
| *SVP*   | -1.08 (±0.13) | -1.01 (±0.1) | -1.31 (±0.20) | 1.32 (±0.18) | -1.15 (±0.15) | 1.41 (±0.08) |
| *TFL1*  | -1.52 (±0.18) | -1.11 (±0.16) | -1.33 (±0.13) | 1.09 (±0.05) | 2.16 (±0.05) | -1.43 (±0.13) |
| *TOC1*  | -2.16 (±0.03) | -1.54 (±0.04) | -1.39 (±0.09) | -1.13 (±0.12) | -1.23 (±0.02) | -1.13 (±0.03) |
| *TSF*   | -2.30 (±0.13) | 2.83 (±0.67) | 1.14 (±0.50) | -5.13 (±0.02) | -6.13 (±0.01) | -1.70 (±0.24) |
| *WUS*   | -3.09 (±0.10) | -2.41 (±0.05) | -1.12 (±0.33) | -1.16 (±0.06) | 1.01 (±0.10) | -1.73 (±0.01) |

Boldface numbers indicate significant (*P* < 0.05) difference between treatments and controls. Values are expressed as fold change (±SD) with respect to control plants growing in GMF conditions. See abbreviation list for gene names in Materials and Methods section.

during later stages of floral development (Table 1). SDG26 was upregulated at 22 and 28 DAS (Table 1). *A. thaliana* TFL1 and SVP gene expressions under NNMF were not significantly changed during the early floral induction period and were upregulated during early flowering (23 and 28 DAS, respectively, Table 1). Finally, exposure of *A. thaliana* to NNMF did not cause any significant regulation of NAC050 in the leaves, whereas it induced a strong downregulation of GA20ox2 in early floral induction (Table 1).

In order to analyze the pattern of expression of genes in the leaves, a cluster analysis was calculated on the data of Table 1 by using Euclidean distances with median linkage method (Fig. 3). This analysis allowed us to identify possible correlations between genes and to visualize the different patterns of gene expressions with time. We found that TSF and GA20ox2 compose two separate clusters because of their late and early downregulation, respectively, whereas CCA1 and FLC form distinct clusters because of their very early (CCA1) and late (FLC) downregulation. The remaining clusters are made by genes with either late upregulation (*GI, SDG26,* and SVP), early and late downregulation (*FRI, CO, WUS, FT, LHY*), only moderate early downregulation (*TOC1, FKF1*), or irregular regulation (all remaining genes) (Fig. 3).
Reduction of the GMF Alters the Expression of *A. thaliana* Floral Meristem Genes

In the floral meristem of *A. thaliana* plants exposed to NNMF, despite its repressing activity on flowering, *FLC* was significantly downregulated, particularly at 22 DAS (Table 2). During early times of flowering, LFY, SVP, SDG26, and, particularly, FD showed a significant upregulation, whereas AG24 was significantly downregulated in early times and upregulated during flowering (23, 28 DAS, Table 2). SOC1 regulation occurred only during late flowering, by showing a downregulation at 28 DAS (Table 2). LFY downregulation occurred only after 23 DAS. Upregulation of API occurred at 22 DAS and was followed by a significant downregulation of the gene between 23 and 28 DAS (Table 2). GA2ox1 and GA20ox1 were mildly downregulated in early phase of floral development, whereas a strong downregulation was observed for GA20ox2 during early flowering (Table 2). Both NAC050 and NAC052 were significantly downregulated only at 19 DAS, whereas JMJ14 did not show any significant regulation (Table 2).

The cluster analysis calculated on the data of Table 2 by using Euclidean distances with median linkage method (Fig. 4) showed a clear distinction between the pattern of expressions of GA20ox2 and *FLC* and all other genes. A cluster groups the expression patterns of API and GA20ox1, whereas another cluster groups genes showing early upregulation. The two *NAC* genes (NAC050 and NAC052) are grouped in a cluster because of a similar pattern of expression, whereas the pattern of expression of AG24 is separated from the other clusters because of late upregulation (Fig. 4).

**DISCUSSION**

In plants, the transition to flowering occurs after floral induction, a period separating vegetative from reproductive development. The timing of floral induction depends on environmental changes and is aimed to maximize reproductive success and seed production. In *A. thaliana* hundreds of genes have been implicated in flowering-time control [Fornara et al., 2010]. An increasing body of evidence suggests that flowering induction may be delayed by altering the MF of exposed plants. In particular, exposure of *A. thaliana* to NNMF delays flowering time [Xu et al., 2012], but the reasons why this delay occurs are far from clear. For the first time, we showed that this effect was maintained in generation experiments, when plants were constantly grown in NNMF, and normal flowering time was re-established when plants were grown in GMF. These data indicate that the effect of NNMF occurs in the growing plant, and therefore cannot be due to the conditions at the time the seed was generated. This is strongly suggestive of the presence of a plant magnetoreceptor [Occhipinti et al., 2014] that is able to interfere with the expression of genes that control flowering time [Maffei, 2014]. Furthermore, the observation that germination was not affected by MF variations suggests that in *A. thaliana* the magnetoreceptor must

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**TABLE 2. Time-Course Expression of Floral Meristem Genes in *A. thaliana* Exposed to NNMF Conditions**

| Genes     | 21 (±0.08) | 22 (±0.23) | 23 (±0.15) | 28 (±0.36) | 30 (±0.22) |
|-----------|------------|------------|------------|------------|------------|
| AGL24     | -1.06      | -3.30      | 1.96       | 1.97       | -1.37      |
| API       | -1.72      | 1.99       | -4.75      | -2.76      | 1.80       |
| FD        | 3.41       | -1.56      | 1.39       | -1.16      | -1.02      |
| FLC       | -5.00      | -14.39     | -5.54      | -3.28      | 1.75       |
| GA2ox1    | -2.72      | 1.03       | -3.54      | -3.44      | 1.19       |
| GA20ox1   | -1.83      | -1.92      | -1.39      | -1.04      | 1.03       |
| GA20ox2   | -23.87     | -53.65     | -3.11      | -47.49     | -1.25      |
| JMJ14     | 1.10       | -1.17      | 1.10       | -1.04      | 1.30       |
| NAC050    | 1.15       | -5.79      | 1.06       | -1.95      | -1.42      |
| NAC052    | -1.19      | -4.06      | -1.00      | -1.60      | -1.38      |
| SDG26     | 1.59       | -3.31      | 1.48       | -1.32      | -1.61      |
| SOC 1     | 1.44       | 1.69       | -1.15      | -2.63      | -1.04      |
| SVP       | 2.10       | -1.31      | 1.16       | -1.13      | -3.62      |

Boldface numbers indicate significant (*P* < 0.05) difference between treatments and controls.

Values are expressed as fold change (±SD) with respect to control plants growing in GMF conditions. See abbreviation list for gene names.

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be active in the developing plant (i.e., roots, shoots, or leaves).

The time-course analysis of leaves and floral meristem genes allowed for evaluation of the different patterns of expression of genes involved in flowering.

**NNMF Downregulates Expression of *A. thaliana* Circadian Clock Genes, Photoperiod, Gibberellin, and Vernalization Pathways**

The *A. thaliana* leaf circadian clock is a time-keeping mechanism that confers diurnal patterns of gene expression and has three interlocked feedback loops. The central loop has the partially redundant transcription factors *CCA1* and *LHY*, which repress transcription of *TOC1*. Although *TOC1* is genetically required for the activation of morning genes, it acts as a repressor and directly regulates the expression of *LHY* and *CCA1*. *TOC1* also forms a negative feedback loop with *GI* by repressing its expression, and *GI* in turn activates the expression of *TOC1* [Fornara et al., 2010]. In plants under NNMF, we found a significant downregulation of all genes involved in the circadian clock, particularly during the floral induction time. Therefore, we argue that this downregulation might be correlated to the NNMF-dependent delay in flowering. Flowering of *A. thaliana* is also promoted by photoperiod pathway genes that act in the leaves through a signaling cascade involving *GI* and the transcriptional regulator *CO* [Sawa et al., 2007; Sanchez et al., 2011; Song et al., 2014]. *CO* promotes flowering by initiating transcription of integrator genes *FT* and *TSF* [Hiraoka et al., 2013]. During long days (as those used for exposure under the NNMF), light promotes the interaction between *GI* and *FKF1* proteins, a family of F-box ubiquitin ligases. These interactions are known to stabilize the F-box proteins, allowing them to promote the degradation of a set of transcriptional repressors of *CO* [Fornara et al., 2010]. However, our data indicate that these interactions may also suppress *CO* expression. In *A. thaliana* under NNMF, a significant downregulation of *CO*, *FT*, *TSF*, and *FKF1* occurred in the floral induction period, whereas no significant regulation was observed for *GI* (see Fig. 3). Similar effects of NNMF on *CO* and *FT* have been observed in previous works performed in conditions similar to our experiments [Xu et al., 2012].

**GA20ox** enzyme catalyzes several steps in the biosynthesis of GA by oxidizing a number of precursors; furthermore, a reduction of this biosynthetic pathway delays flowering [Brambilla and Fornara, 2013]. NNMF induced a downregulation of *GA20ox2* immediately prior to floral induction, when usually the concentration of bioactive GA (GA₃) increases at the floral meristem [Fornara et al., 2010].

The transcription factor *LFY* plays a key role in the integration of flowering signals in parallel with *FT* to activate floral meristem identity genes [Abe et al., 2005]. Moreover, in rice *FT* forms a complex with the bZIP transcription factor *FD* and a 14-3-3 protein, triggering flowering through the activation of key floral meristem identity genes, such as *AP1* [Taoka et al., 2011]. Under NNMF, expressions of leaf *LFY*, *AP1*, and *FD* were downregulated in the floral induction period. However, *LFY* was upregulated during early flowering; therefore, we suggest that the effect of NNMF on this gene may occur in later stages of plant development. The key floral repressor *TFL1* is an *FT*-related gene, which maintains the center of the shoot apical meristem (SAM) in a vegetative state by repressing *LFY* and *AP1* [Ratcliffe et al., 1999]. The expression of *AP1* and *TFL1* is antagonistic, as *AP1* represses *TFL1*, and this is likely to be a direct effect as *AP1* directly binds *TFL1* regulatory elements [Kaufmann et al., 2010]. In *A. thaliana* leaves, *TFL1* gene expression under NNMF did not significantly change during floral induction and was upregulated during early flowering (see Fig. 3, 23 DAS).

In the leaves, *SVP* and *FLC* are known to repress the transcription of *FT* [Searle et al., 2006; Jang et al., 2009]. The vernalization pathway activates flowering by silencing *FLC* in response to prolonged exposure to low temperatures [Fornara et al., 2010]. While *SVP* showed only a late and small upregulation in plant exposed to NNMF, a significant upregulation was
found for FLC during early floral induction, whereas the gene was progressively downregulated during later stages of floral development (see Fig. 3 and Table 1). The regulator gene FRI is one of the major determinants of natural variation in flowering time. FRI encodes a protein with two coiled-coil motifs and is required to increase the FLC transcript level [Choi et al., 2011]. A slight but significant downregulation of FRI was observed in A. thaliana exposed to NNMF only in early and very late phase of floral induction (Fig. 3 and Table 1). This downregulation was associated with the progressive downregulation of FLC from early to late stages of development, with the only exception for a significant upregulation of FRI at 23 DAS.

In A. thaliana, the homeodomain gene WUS in the indeterminate shoot apical meristem is essential for maintaining the pool of stem cells, and its downregulation leads to a loss of stem cell activity [Das et al., 2009], whereas the STM gene plays an essential role in the establishment and maintenance of indeterminate development of apical and axillary meristems during all phases of plant life [Groot et al., 2005]. Exposure to NNMF downregulated both genes, although WUS showed an earlier downregulation with respect to STM. The downregulation of WUS suggests a reduction of stem cell activity and might be correlated to the flowering delay, while STM downregulation suggests a negative effect on the maintenance of vegetative growth, which may justify the observation that NNMF delays but is unable to stop the flowering of A. thaliana.

A. thaliana SDG26 is involved in the activation of flowering, as loss of function of SDG26 causes a delay in flowering [Berr et al., 2015]. In A. thaliana leaves under NNMF, no significant changes were found in SDG26 regulation before flowering, whereas a significant upregulation was observed during flowering time (Fig. 3).

The plant-specific NAC proteins form one of the largest transcription factor families in plants [Olsen et al., 2005]. Overexpression of NAC transcription factor NAC050 was found to delay A. thaliana flowering time [Ning et al., 2015]; however, despite the evident delay in flowering, exposure of A. thaliana to NNMF did not cause any significant regulation of NAC050 in the leaves.

**NNMF Regulates the Expression of GA20ox2, SVP, and FLC in A. thaliana Floral Meristem**

Floral induction is necessary to transform the shoot apical meristem from a vegetative meristem to an inflorescence meristem, which forms flowers. This morphological change is associated with dramatic changes in gene expression, including increased expression of the integrator gene SOC1, which encodes a MADS-box transcription factor [Zhao et al., 2014]. In A. thaliana floral meristems of plants under NNMF, SOC1 regulation occurred only during late flowering, by showing a downregulation at 28 DAS (see Fig. 4). FLC is also a MADS-box transcription factor that acts as a potent repressor of flowering and is responsible for much of the variation in flowering time observed in A. thaliana [Fornara et al., 2010]. FLC and SVP work together to repress the expression of SOC1 [Gregis et al., 2013]. Despite its repressing activity on flowering, FLC was significantly downregulated in floral meristems of NNMF-exposed plants, particularly at 22 DAS (Fig. 4), whereas SVP was significantly upregulated at the beginning of the flowering time and downregulated in late flowering. These data indicate that the NNMF-dependent delayed flowering time might be a consequence of SVP upregulation, more than the effect of FLC. The interaction of FT with FDR directly promotes the transcription of the MADS-box factor AP1 [Brambilla and Fornara, 2013].

A strong and significant downregulation was also observed for GA20ox2 during the early phases of floral development in NNMF. This regulation could be correlated to the upregulation of SVP, which acts either individually or in complex with FLC to repress GA20ox2 expression [Mateos et al., 2015]. On the other hand, FD and AP1 were upregulated in early phases of flower development. These results suggest that upregulation of FD at 21 DAS might compensate the strong downregulation of leaf FT by inducing AP1 upregulation at 22 DAS; however, downregulation of FD is followed by a significant downregulation of AP1. The commitment to flower is ascertained by a direct positive feedback interaction between LFY and AP1 [Valentim et al., 2015]. The transcription factor LFY is under direct control of SOC1 [Valentim et al., 2015] and is involved in the development of a determinate floral meristem [Sablowski, 2007]. As SOC1, LFY was not regulated during early flowering and was significant downregulated only after 23 DAS, and our cluster analysis confirms the pattern of expression of these two genes.

**AGL24** is one of the MADS-box genes found to promote flowering [Michaels et al., 2003]. Interestingly, AGL24 is upregulated during flowering at the same time as the downregulation of AP1, LFY, and SOC1. Upregulated levels of AGL24 expression correspond to the degree of precocious flowering, and the reduction in AGL24 expression is related to the degree of late flowering, suggesting that AGL24 is a dosage-dependent promoter of flowering [Liu et al., 2008]. Since AGL24
was significantly downregulated in NNMF-exposed *A. thaliana* in early phases of floral development and was significantly upregulated during flowering, this pattern of expression may indicate that this gene is involved in the later stages of floral development.

Gibberellin is a growth regulator that promotes flowering in *A. thaliana*. *GA20ox* and *GA3ox* can promote the production of active *GA*, whereas *GA2ox* inactivates *GA*, thus regulating its content in plants [Han and Zhu, 2011]. *GA20ox2* was downregulated under NNMF and its pattern of expression was unique, whereas the pattern of *GA2ox1* was similar to *AP1*.

In *A. thaliana*, there are 21 JmjC domain-containing histone demethylases that have been named JMJ11–JMJ31 [Lu et al., 2008] and H3K4 demethylase JMJ14 is involved in repression of the floral integrator genes *FT* and *SOC1* [Lu et al., 2010]. Recently, JMJ14 was found to be associated with *NAC* transcriptional repressors *NAC050* and *NAC052* [Ning et al., 2015]. In the floral meristem (data from Table 2), NNMF determines an early downregulation of the gibberellin pathway, *AGL24* and *AP1*, with a significant upregulation of *LFY*, *FD*, and *SVP*. In both leaves and floral meristem data, upregulation is shown in green, downregulation in light red, and no regulation in white [Fornara et al., 2010; Jaeger et al., 2013; Valentim et al., 2015].

**CONCLUSIONS**

The results of this work can be summarized in the scheme of Figure 5 and imply that

- NNMF causes a delay in the transition to flowering due to a combined regulation of leaves and floral
meristem genes. An early downregulation of clock, photoperiod, gibberellin, and vernalization pathways is accompanied by a downregulation of AP1 and GA2ox. FLC is upregulated by NNMF in early flowering induction. In the floral meristem, the strong downregulation of FT and FLC in early phases of floral development is accompanied by the downregulation of the gibberellin pathway and upregulation of FD, SVP, and the transcription factor LFY. The common downregulation of AP1 in both floral meristem and leaves is associated with the delay in flowering.

- In the floral meristem and leaves, the progressive upregulation of AGL24, AP1, GI, and SVP from early to late phase of plant development is correlated to the delay of flowering. These events are followed by the progressive reduction of gibberellin pathway downregulation. Our results indicate that NNMF do not prevent flowering, and that variations of the MF are sufficient to modulate specific genes in the early stages of flower induction that are associated with the observed delay.

- The verified delay in the transition to flowering caused by NNMF could be correlated to the observed speciation of Angiosperms after geomagnetic field reversals [Maffei, 2014; Occhipinti et al., 2014; Bertea et al., 2015], which does not exclude a hypothetical influence of GMF magnitude and polarity on plant evolution on a geological time-scale.

- Since GMF magnitude is not equal everywhere on the Earth’s surface, it is possible that changes in GMF in different places could influence plant growth and reproduction.

- However, since the results on gene expression regulation described in this work might not reflect post-translational modifications that lead to the production of proteins involved in flowering control, further proteomics studies are underway to better assess the role of NNMF on flowering control.

- Finally, experiments with one or more knock-out mutants of the genes of interest, measuring expression levels in these genotypes, will provide further insight into the nature of triggering events and signal transduction.

Having assessed the downstream events that are associated with a delay in transition to flowering caused by exposure of A. thaliana to NNMF, several questions remain unanswered: what is the magnetoreceptor molecule and what is the signaling pathway that induces the gene expression (and repression) reported here?; which point in the life-cycle of A. thaliana is most sensitive to MF perturbations?; and will crops grown in different MF have different productivity? Finally, we note that the expression changes of cryptochrome-signaling-related genes CO and FT shown in this work suggest that the effects of NNMF might be cryptochrome-related [Xu et al., 2012; Maffei, 2014; Occhipinti et al., 2014]. If a key role for cryptochrome magnetoreception were found in plants, this would make an important link to the mechanism of magnetoreception in avian navigation [Rodgers and Hore, 2009]. Experiments are underway to test this hypothesis and the results will be reported soon.

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